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Tina Schiavello  
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# **GENETIC AND NON-GENETIC FACTORS INVOLVED IN MODIFYING THE CLINICAL SEVERITY OF AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE**

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**A Thesis Submitted for the Award of Doctor of Philosophy in Human Biology  
at the Faculty of Computing, Health and Science, Edith Cowan University**

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**Date of submission:** 18<sup>th</sup> June 2002

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## ABSTRACT



Polycystic kidney

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# ABSTRACT

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Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common Mendelian disorders, affecting approximately 1 in 1000 individuals. The disease is recognised as a systemic disorder, which expresses a complex phenotype between and within families. Mutations in at least two genes (*PKD1* and *PKD2*) result in ADPKD, however, additional genetic and non-genetic factors are expected to contribute to the observed phenotypic variance.

While several factors including locus and allelic heterogeneity, trans-heterozygous and somatic mutations, modifying genes, sex, hypertension and environmental determinants may influence disease progression, the factors, which modify the disease remain largely unknown. Reasons for this include difficulties in genotype-phenotype correlations due to a high proportion of private mutations (*i.e.* confined to individual families), inter-population differences in terms of biological and environmental variables, ascertainment, small sample numbers and differences in study design.

This thesis was designed to investigate some major plausible factors that may modify ADPKD disease severity. In addition, this is the first study in ADPKD to quantify the genetic contribution of the variable phenotype. The study included a total of 322 affected individuals drawn from three separate geographical locations: Western Australia, Bulgaria and Poland and represented a large sample size in comparison to previous studies. The ADPKD phenotype was graded according to kidney function, hypertension and ultrasonographic findings. Kidney function was assessed by serum creatinine (SCr) values and two renal outcomes were measured: chronic and end-stage renal failure. Chronic renal failure was defined as a SCr of 150  $\mu\text{mol/L}$  and ESRF defined as the age at which an individual required dialysis or kidney transplantation. Ultrasound findings were based on kidney length, cyst size and number and the presence of extrarenal cysts. The effect of inter-population differences, locus heterogeneity, sex, and genetic variation within the renin-angiotensin system (RAS) were investigated. This included analysis of an insertion/deletion (I/D) polymorphism in the angiotensin converting enzyme (ACE) gene, a dinucleotide repeat within the

angiotensinogen (AGT) gene and a tetranucleotide repeat flanking the renin gene. The relationship between recognised environmental risk factors (smoking, alcohol consumption, physical activity and lipidaemia) and the ADPKD phenotype was also examined.

The results excluded the influence of inter-population differences. A similar age at onset and progression to renal failure was observed between the three countries. In terms of locus heterogeneity, no significant difference in age at onset or renal disease progression could be found between *PKD1* and *PKD2* individuals. However, after adjusting for the effect of sex, *PKD2* females were found to reach renal failure at a later age than *PKD2* males and *PKD1* male and females. No association between genetic variants within the RAS (ACE I/D polymorphism, angiotensinogen and renin) and renal failure was observed, except that the ACE I/D polymorphism was found to be associated with an increase in left ventricular mass when adjusted for hypertension. However, subtle relationships between smoking and renal disease progression, and smoking, lipidaemia and cystogenesis, were observed. The summary of the results is shown at the end of the abstract.

Based on these findings and the fact that the ADPKD phenotype can be regarded as a complex trait, principles and methods traditionally used in the study of complex traits were employed to estimate the overall contribution of genetic versus environmental factors.

Multivariate component modelling was undertaken to compare the correlations for age at CRF and slope of decline of kidney function between related individuals, and estimate the 'narrow' sense heritability. The results revealed a higher correlation coefficient between sibs and cousins compared to parent-offspring and uncle/aunt-niece/nephew relationships in terms of age at CRF and the slope of decline of kidney function. Moreover, the estimated heritability was <5%. The results indicated that apart from the PKD mutations, environmental factors common to sibs and cousins were important determinants of disease severity, but non-shared factors were equally important since differences in the age at CRF and the slope of decline of kidney function still existed between sibs and parent offspring pairs. It is possible that

environmental factors are important in providing the second mutation (second hit) in the remaining wild-type copy of the PKD gene, helping to explain (at least in part) the different rates of progression of disease in ADPKD patients.

Based on a large sample size, the results from this thesis provide substantial evidence for the contribution of environmental factors on phenotypic variance. These findings have significant implications for the direction of future research in the study of modifying factors. Clinically, the findings further support the need to encourage ADPKD patients to cease smoking and lower cholesterol and triglycerides by dietary and pharmaceutical intervention to slow the progression of renal disease in ADPKD.

**Summary Table: Factors examined in this thesis contributing to phenotypic variation in ADPKD**

<b>Factor</b>	<b>Specific factor</b>	<b>Contribution to CRF</b>	<b>Contribution to ESRF</b>
Inter-population differences	Country	0	0
Locus heterogeneity	PKD1:PKD2	0	0
Gender	Female	0	-
Modifying genes	ACE, AGT, Renin	0	0
Environment	Smoking	+	+
	Lipids	0	+
General genetic contributions	Heritability	<5%	0%

*0 no effect/difference; - negative effect; + positive effect*

# DECLARATION

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I certify that this thesis does not, to the best of my knowledge and belief:

- (i) incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;
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Signature.....*J. Schiavello*.....

Date.....*30 - 3 - 04*.....

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# ABBREVIATIONS

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<b>ACE</b>	Angiotensin converting enzyme
<b>ADPKD</b>	Autosomal dominant polycystic kidney disease
<b>AGT</b>	Angiotensinogen
<b>AngI</b>	Angiotensin I
<b>AngII</b>	Angiotensin II
<b>ANOVA</b>	Analysis of variance
<b>ANCOVA</b>	Analysis of covariance
<b>AT1R</b>	Angiotensin II type 1 receptor
<b>bFGF</b>	Basic fibroblast growth factor
<b>BMI</b>	Body mass index
<b>BP</b>	Blood pressure
<b>Ccr</b>	Creatinine clearance
<b>CHD</b>	Coronary heart disease
<b>Cr</b>	Creatinine
<b>CRF</b>	Chronic renal failure
<b>DBP</b>	Diastolic blood pressure
<b>DD</b>	Deletion/deletion
<b>DNA</b>	Deoxyribonucleic acid
<b>DN</b>	Diabetic nephropathy
<b>DR</b>	Diabetic retinopathy
<b>DZ</b>	Dizygotic (twins)
<b>ECM</b>	Extracellular matrix
<b>EGFR</b>	Epidermal growth factor receptor
<b>ESRF</b>	End stage renal failure
<b>GBM</b>	Glomerular basement membrane
<b>GFR</b>	Glomerular filtration rate
<b>HbA</b>	Haemoglobin A
<b>HBP</b>	High blood pressure
<b>HDL</b>	High density lipoprotein
<b>HSPG</b>	Heparin sulfate proteoglycan

<b>HWE</b>	Hardy-Weinberg Equilibrium
<b>ID</b>	Insertion/deletion
<b>IDDM</b>	Insulin dependent diabetes mellitus
<b>IgA</b>	Immunoglobulin A
<b>II</b>	Insertion/insertion
<b>QTL</b>	Quantitative trait loci
<b>LDL</b>	Low density lipoprotein
<b>LRA</b>	Logistic Regression Analysis
<b>LRR</b>	Leucine rich repeat
<b>LVH</b>	Left ventricular hypertrophy
<b>LVM</b>	Left ventricular mass
<b>MA</b>	Microalbuminuria
<b>MI</b>	Myocardial infarction
<b>MRA</b>	Multiple regression analysis
<b>MZ</b>	Monozygotic (twins)
<b>NIDDM</b>	Non-insulin dependent diabetes mellitus
<b>NRF</b>	Normal renal function
<b>OR</b>	Odds ratio
<b>p</b>	Significance level (probability)
<b>PAI-1</b>	Plasminogen activator inhibitor-1
<b>PC1</b>	Polycystin 1
<b>PC2</b>	Polycystin 2
<b>PKD1</b>	Polycystic kidney disease 1
<b>PKD2</b>	Polycystic kidney disease 2
<b>PCR</b>	Polymerase chain reaction
<b>PRA</b>	Plasma renin activity
<b>RAS</b>	Renin angiotensin system
<b>REJ</b>	Receptor for egg jelly
<b>RF</b>	Renal failure
<b>RRT</b>	Renal replacement therapy
<b>sACE</b>	Serum angiotensin converting enzyme
<b>SBP</b>	Systemic blood pressure
<b>SCr</b>	Serum creatinine

<b>SD</b>	Standard deviation
<b>SE</b>	Standard Error
<b>TGF-<math>\beta_1</math></b>	Transforming growth factor $\beta_1$
<b>UAE</b>	Urinary albumin excretion
<b>UPE</b>	Urinary protein excretion
<b>UTI</b>	Urinary tract infection
<b>WA</b>	Western Australia

# TERMS AND DEFINITIONS

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***Allele:*** alternative forms of a gene or a marker.

***Anticipation:*** Apparent earlier occurrence and more severe expression of a disorder in succeeding generations.

***Autosomal:*** pertaining to the 22 non-sex chromosomes.

***CentiMorgan (cM):*** a unit of genetic distance equivalent to a 1% probability of recombination during meiosis. One centiMorgan is equivalent, on average, to a physical distance of approximately 1 megabase in the human genome.

***Compound heterozygote:*** genotype in which two different mutant alleles are present at the same locus on homologous chromosomes.

***Confounder:*** an additional factor that might be responsible for an observed relationship between exposure and outcome. Confounding variables are associated with the exposure in question, and are independently associated with disease risk but are not on the direct causal pathway of the outcome of interest.

***Degrees of relatedness:*** closeness of the genetic connection between two people, based on the proportion of shared genes. First-degree relatives share one-half of their genes: parents, siblings, offspring. Second-degree relatives share one-fourth of their genes: grandparents, aunts, uncles, nieces, nephews, grandchildren. Third-degree relatives share one-eighth of their genes: great-grandparents, great-aunts and great-uncles, first cousins.

***Dependent variable:*** a variable whose values are estimated or predicted by a regression equation.

***EF-hand motif:*** is a calcium binding domain in a protein (for example, calmodulin). It is a helix-turn-helix motif, with the EF referring to the numbering of the helices from the N terminus of the protein. For example, the first alpha helix at the N terminal end of a protein is called Helix A, the second Helix B etc. The calcium ion binds in the turn or “loop” between Helices E and F.

***Epidemiology:*** the study of the occurrence, distribution and causation of the disease in humankind.

***Epistasis:*** a type of interaction between genes at different loci on a chromosome in which one is able to mask or suppress the expression of the other. The epistatic effect, which is nonallelic and therefore the opposite of the dominance relationship, may be caused by the presence of homozygous recessives at one gene pair, as occurs in the Bombay phenotype, or by the presence of a dominant allele that counteracts the expression of another dominant gene.

***Genetic heterogeneity:*** a characteristic of complex diseases whereby similar phenotypes may result from different allelic combinations.

***Hardy-Weinberg law:*** the simple relationship between gene frequencies and genotype frequencies that is found in a population under certain conditions.

***Hereditary:*** genetically transmitted to offspring from parents.

***Imprinting:*** the differential expression of genetic material depending on whether it has been inherited through the mother or through the father.

***Independent variable:*** the explanatory or predictor variable in a study.

***Index case:*** defined as the first case of a disease.

***Intercept of a line:*** the point on the vertical axis of a scatter plot where the line crosses this axis.

**Intron:** noncoding DNA which separates neighbouring exons in a gene. During gene expression introns, like exons, are transcribed into RNA but the transcribed intron sequences are subsequently removed by RNA splicing and are not present in mRNA.

**Linkage:** the situation where two syntenic loci are inherited together. More specifically, two loci are said to be linked if they are close enough to each other on a chromosome such that recombination during meiosis is uncommon enough for their co-segregation to be detectable within families. Linkage analysis relies on statistical methods, and seeks to estimate the recombination fraction (?) between two or more loci in identifiable meiotic events.

**Linkage disequilibrium:** the increased frequency of haplotypes within a population due to co-inheritance of linked alleles.

**Microsatellite DNA:** small array (often less than 0.1 kb) of tandem repeats of a very simple sequence, often between 1 and 4 bp.

**Modifier gene:** a gene whose expression can influence a phenotype resulting from mutation at another locus.

**Mosaic:** a genetic mosaic is an individual who has two or more genetically different cell lines derived from a single zygote.

**Odds ratio (OR):** the ratio of the odds of a dichotomous outcome (eg disease affection) among exposed individuals relative to that among unexposed individuals.

**Phenocopy:** is defined as those individuals who develop a disease as a result of environmental or random causes (*i.e* no inheritance or predisposing alleles).

**Phenotype:** the entire physical, biochemical and physiological makeup of an individual; any qualitative or quantitative characteristic of an individual.

**Polymorphism:** classically a gene with one or more allele, each allele having a frequency of at least 5% in a given population, was said to be 'polymorphic'. In recent usage, 'polymorphism' describes a phenotypically neutral (usually non-coding) sequence variation in a gene.

**Probe:** a DNA or RNA fragment which has been labeled in some way, and used in a molecular hybridization assay to identify DNA or RNA sequences which are closely related to it in sequence.

**Recombination:** crossing over of chromosomes during meiosis.

**Relative risk (RR):** measure of disease-exposure relationship. Estimates the magnitude of an association between exposure and disease and indicates the likelihood of disease in exposed individuals compared with those who are not exposed. RR is the incidence of disease in the exposed group over the corresponding incidence in the non-exposed group.

**Slope of a line:** a number that indicates whether a line rises or falls and how steeply the line rises or falls.

**Synten:** the property of occurring on the same chromosome.

**Two-hit hypothesis:** Knudson's theory that hereditary cancers require two successive mutations to affect a single cell.



**CHAPTER ONE: GENERAL INTRODUCTION AND  
LITERATURE REVIEW**

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# CHAPTER ONE: AN OVERVIEW

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## 1.1 GENERAL INTRODUCTION

### 1.1.1 *What is ADPKD?*

This thesis is concerned with genetic factors as well as environmental determinants that may play a role in modifying the clinical severity of ADPKD.

The literature review begins with a general description of ADPKD, including the prevalence, age of onset, clinical features and genetics of the disease. Subsequently, the pathogenesis of ADPKD is discussed, beginning with an overview of the normal kidney, followed by a description of the pathophysiology and genetic mechanisms of cyst formation. The final part of the review discusses the clinical picture of ADPKD, by addressing factors that may contribute to the observed inter-and intra-familial variation. Because of the scope and diversity of this thesis, this review is intended only as a general overview of the field. More specific reviews pertaining directly to each aspect of the study are provided at the beginning of each chapter.

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic disorders, with a frequency of 1 in 1000 in the general population (Dalgaard, 1957). The disease is recognised as a systemic disorder and is marked by extensive phenotypic heterogeneity between and within families, suggesting that apart from the PKD genes, additional factors may play a role in its aetiology and pathogenesis. Recent experimentation from various laboratories has shown that an acquired somatic mutation in the originally normal parental allele, as well as the existence of other modifying loci, may account for the observed inter- and intra-familial variability of the disease. The most prominent feature of this disease is the focal formation of cysts within the kidney and liver, which increase in size and number over the lifetime of an individual. The process of nephrogenesis (formation of the nephron; the functional unit of the kidney) is disrupted, leading to cyst formation and other clinical manifestations. The development and progressive enlargement of fluid-filled cysts in the kidney may be one of the processes associated with fibrosis of the normal renal tissue, resulting in kidney failure in approximately 50% of patients by the age of 60 years.

## **1.2 GENERAL DESCRIPTION OF ADPKD**

### **1.2.1 *Prevalence and Age at Onset***

There is wide variation in both the gene frequency and clinical expression of ADPKD. The gene frequency in Wales is estimated to be 1 in 2459 (Davies *et al.*, 1991), while in Denmark, Hungary and Bulgaria it is estimated to be 1 in 1000 (Papp, 1987 [cited in Tauszik *et al.*, 1989]; Forrai *et al.*, 1989; Todorov and Nacheva, 1991; Davies *et al.*, 1991). A higher gene frequency of 2.5 in 1000 has been reported in Northern European individuals in the USA (Iglesias *et al.*, 1983), and in a Gypsy community residing in South Western Hungary. In the Gypsy community, the gene frequency is 23 fold the national average (Tauszik *et al.*, 1989). In addition to gene frequency, the prevalence of clinically expressed ADPKD differs between populations. In the Japanese population a prevalence of 1 in 4000 is observed (Higashihara *et al.*, 1992), whilst in the USA (Simon and Thompson, 1955) and Seychelles (Yersin *et al.*, 1997) a higher prevalence of 1 in 2438 and 1 in 544 respectively is seen. Differences in the gene frequency and the prevalence of clinically expressed ADPKD may be explained by the fact that many patients remain asymptomatic and undiagnosed throughout life, as well as by differences in diagnostic criteria, geographical variations or true population differences.

The clinical onset of ADPKD is typically in the third to fifth decade of life. Earlier manifestations of ADPKD are well known (Kaplan *et al.*, 1977), and some cases with prenatal onset have been reported (Zerres *et al.*, 1982). It is estimated that approximately 2% of gene carriers present with severe manifestation in childhood (Sedman *et al.*, 1987) and several studies have reported a high recurrence risk to siblings for early manifestation of ADPKD (Zerres *et al.*, 1985, 1993; Kaariainen, 1987).

## **1.3 ADPKD AND KIDNEY FUNCTION**

### **1.3.1 *Gross Structure of the Kidney***

The kidney is a complex organ, which performs several important functions, including formation of urine, water and salt metabolism, acid-base balance and hormonal regulation of blood pressure, bone metabolism and red cell formation (Irion, 2000). Each kidney is composed of approximately 1 million units called nephrons (renal tubules), which are long coiled tubules located in the cortex and medulla that produce

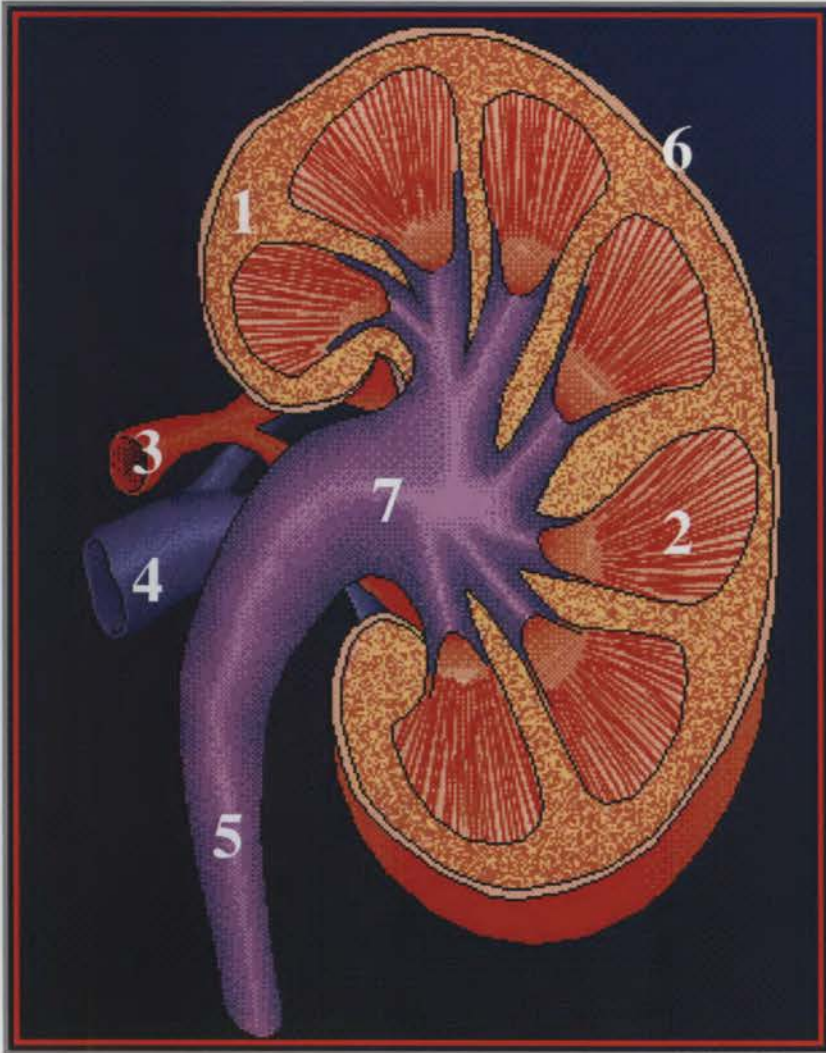
urine and drain into the renal pelvis. A schematic representation of a normal kidney is shown in Figure 1.1.

The nephron is the functional unit of the kidney that has several important functional segments (Figure 1.2). Each nephron is completely surrounded by its own continuous basement membrane, which is a non-cellular layer of tissue or extracellular matrix (ECM) that secures the overlaying layers of stratified epithelium and is associated with epithelia, endothelia, muscle, fat and peripheral nerve. The most proximal part of the nephron is the glomerulus, which is an invagination of an original epithelial vesicle or sac known as Bowman's capsule and is a ball-shaped collection of capillaries and mesenchyme. The primary function of the glomerulus is the production of glomerular filtrate, which is a selective process. The glomerular basement membrane (GBM) is the critical component of the ultrafiltration barrier that lies between the plasma and the urinary space. It is an unusually thick basement membrane bordered on its vascular face by fenestrated endothelial cells and on its urinary space face by the interdigitated foot processes of visceral epithelial cells (podocytes). Like all basement membranes, the GBM contains four major classes of molecules: collagen IV, laminin, entactin/nidogen and heparin sulfate proteoglycan (HSPG). Recent work has shown that there are complex laminin and collagen IV isoform switches in the developing GBM, and that failure of some of these switches to occur may lead to renal insufficiency (Irion, 2000).

Once it passes the GBM, the glomerular filtrate flows into the proximal tubule whose main function is the reabsorption of solutes such as sodium, potassium, phosphate, glucose and water. This tubule loops backward towards the glomerulus to become the distal tubule, which eventually carries urine towards the collecting tubules and the calyces (Irion, 2000).

### **1.3.2      *Nephronal Function***

The nephron has three basic functions: filtration, secretion and reabsorption. Approximately 20% of the plasma that enters the kidneys is filtered into the renal tubules via the glomeruli. In addition, the tubular cells themselves can transport substances from the interstitial space outside the tubules into the tubules, providing a second pathway for solutes to enter the tubule: this process is called secretion. The



**Figure 1.1:** The normal kidney. 1. renal cortex; 2. renal medulla; 3. renal artery; 4. renal vein; 5. ureter; 6. renal capsule; 7. renal pelvis  
*Source: <http://www.nhpress.com/pkd/kidney/parts.html>*



**Figure 1.2:** The Structure of the Nephron.

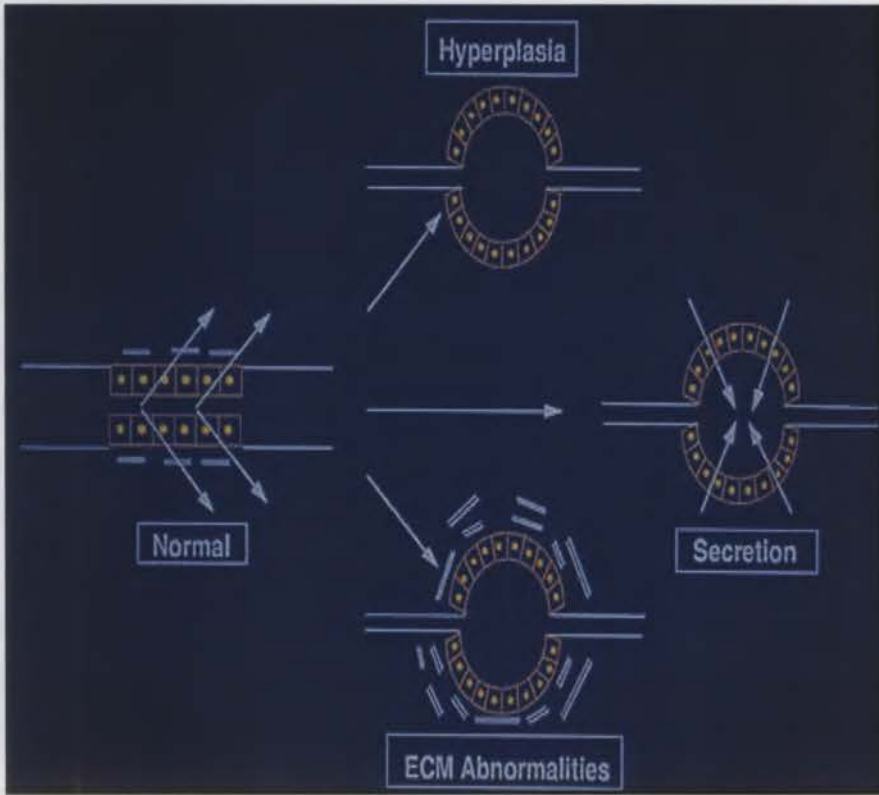
Source: <http://www.nhpress.com/pkd/kidney/howwork.html>

process of reabsorption is the transport of solute from the inside of the tubule into the interstitial space surrounding the tubules. Blood vessels called peritubular capillaries transport to the interstitial space surrounding the renal tubules substances to be secreted by the tubules, and take reabsorbed solute back to the circulation (Irion, 2000).

### **1.3.3      *Pathophysiology of Cystogenesis in ADPKD***

Renal cysts are thought to initiate as small dilations in renal tubules, which then expand into fluid-filled cavities of relatively large size (Calvet, 1993). The development of cysts is a slow, progressive process which seems to depend on the proliferation of tubular epithelial cells, increased production of abnormal extracellular matrix (ECM) and tubular basement membrane, and abnormal fluid transport towards the lumen (Grantham *et al.*, 1987; Grantham, 1996). A schematic representation of these processes is shown in Figure 1.3. Thus, cystic epithelial cells undergo a transition in their phenotypic state, yet retain many basic epithelial characteristics (Grantham *et al.*, 1987). Cyst enlargement may cause disruption of the normal renal parenchyma, which interferes with glomerular filtration and vascular blood flow, ultimately causing renal failure in some patients (Calvet, 1993).

Morphological studies suggest that as some cysts enlarge, they may lose their tubular connection and become isolated from the glomerulus, hence requiring a net influx of fluid (tubular secretion) to expand further and to prevent the collapse of the cyst wall (Grantham *et al.*, 1987; Murcia *et al.*, 1999). The fluid composition of cystic cells ranges between low and high sodium concentrations (Grantham, 1996). These differences have been interpreted as reflecting different sites of origin of the cysts within the nephron. It has been proposed that fluid secretion in ADPKD may result from several mechanisms. The first mechanism may involve activation of the adenylate cyclase signal transduction pathway by forskolin or cAMP (Sullivan and Grantham, 1996; Murcia *et al.*, 1999). The second mechanism may involve mislocalization of the Na,K-ATPase (Wilson *et al.*, 1991; Murcia *et al.*, 1999), which is an enzyme responsible for pumping sodium out of renal tubule cells and establishing sodium gradients which drive numerous transport processes related to reabsorption (Wilson, 1991). The third mechanism includes chloride secretion through the cystic fibrosis transmembrane regulator (CFTR) (Sullivan *et al.*, 1998; Murcia *et al.*, 1999), which has



**Figure 1.3:** The Processes of Cystogenesis. A normal kidney can become cystic because the cells grow too quickly (hyperplasia), because fluid is secreted into the space as opposed to absorbed across it, or because of abnormalities in the extracellular matrix (ECM). Source: HDCN Highlights of the 9th Annual Conference on PKD. <http://www.hdcn.com.symp/98pkd/avner/avner2.htm>



recently has been implicated in the pathway of fluid secretion in ADPKD cystic epithelia (Sullivan *et al.*, 1998; Murcia *et al.*, 1999). CFTR is a cAMP-dependent chloride channel that is required for chloride secretion and fluid production in airway epithelium (Jiang *et al.*, 1993; Murcia *et al.*, 1999).

Alterations in the composition of the ECM are suggested by the abnormal appearance of the basement membrane synthesised by cystic renal epithelial cells in culture (Wilson *et al.*, 1992). Matrix abnormalities are not unique to ADPKD but also occur in a variety of experimental models, suggesting a central role for matrix alterations in cystogenesis (Ojeda *et al.*, 1990; Liu *et al.*, 1992; Calvet, 1993). The most significant and obvious ECM abnormality is a thickening of the basement membrane, which was first noted in a rat model (Darmady *et al.*, 1970) and was then documented in pathological specimens of human ADPKD kidneys (Cuppage *et al.*, 1980) and later confirmed in biopsy specimens from kidneys of young affected ADPKD patients (Milutinovic and Agodoa, 1983). A relationship between cyst induction and basement membrane thickening has been found in chemically induced cyst models, such as the 2-amino-4, 5-diphenyl thiazole hydrochloride (DPT) treated rat (Kanwar and Carone, 1984; Carone *et al.*, 1989) and in the corticosteroid treated rabbit (Ojeda *et al.*, 1990). In both these models progressive thickening, splitting and lamination of the basement membranes occurred in tandem with the development of cystic formation, raising the possibility that BM abnormalities are possibly pathogenetically related to cystogenesis (Carone *et al.*, 1989).

Degradation of the ECM is mediated by several proteases, including matrix metalloproteinases (MMPs) and cysteine proteinases (Wilson *et al.*, 1996). MMPs are in turn regulated by secreted proteins known as tissue inhibitors of metalloproteinases (TIMPs). Alterations in MMPs, TIMPs, and cysteine protease activities have been correlated with abnormal cell migration in murine models of ADPKD (Murcia *et al.*, 1999). The array of systemic abnormalities in ADPKD (namely diverticular disease, herniae and the production of abnormal vascular tissue), are compatible with a defect in the composition of the ECM (Gabow, 1993). It could contribute to the formation of aneurysms and cardiac abnormalities, and could also influence vascular reactivity, thus playing an important part in the development of hypertension.

Evidence of increased epithelial cell proliferation includes the expression of proliferating cell nuclear antigen within cells lining cysts (Nadasdy *et al.*, 1995), an absolute increase in the number of cells (Grantham *et al.*, 1987), and expression of proto-oncogenes (*c-myc*, *c-fos*, *c-jun*, *c-ha-ras* and *c-ki-ras*) in humans and several mouse models of PKD (Cowley *et al.*, 1991; Klingel *et al.*, 1992). It is not yet clear whether these changes play an early role or reflect secondary changes in the microenvironment of cystic lesions. Studies on murine models suggest that early expression of proto-oncogenes may be sufficient to induce hyperplasia and predispose tubules to cystic disease (Trudel *et al.*, 1998). The proliferative process of tubular epithelial cells can be modulated by endocrine, paracrine, and autocrine factors including the epidermal growth factor (Munemura *et al.*, 1994; Du and Wilson, 1995), cyst activating factor (Grantham, 1996), and the Ras protein (Raguram *et al.*, 1996; Wang and Strandgaard, 1997).

Apoptosis, or programmed cell death, of normal tissue may also be involved in renal cyst formation (Woo, 1995; Winyard *et al.*, 1996). Apoptosis uses molecular pathways to ablate individual cells in response to external stimuli or developmental genetic programs (Savill, 1994). During kidney development, high levels of apoptosis are localised to the nephrogenic zone and the developing medullary papilla (Coles *et al.*, 1993).

Proliferating epithelial cells of renal cysts appear morphologically less differentiated than noncystic tubular epithelium. Differences associated with the cystic phenotype include persistent expression of clusterin, vimentin, and Pax-2, as well as polarity abnormalities, including E-cadherin, cathepsins B and H, gelatinase A, TIMP's, and apical epidermal growth factor receptor (EGFR) expression (Grantham, 1996). Initiation and maintenance of polarisation depend on cell-cell and cell-matrix adhesion. In the absence of cellular adhesion, epithelial cells demonstrate few characteristics of polarised cells. Polarised organisation of proteins in renal epithelium is required for the function of these cells. The spatial arrangement and unique protein composition of apical and basolateral domains facilitate cellular processes including differentiation, proliferation, cell migration and morphogenesis. The selective or limited polarization defects in ADPKD suggest that specific developmental pathways of epithelial polarity

and its maintenance are affected in the disease. The adhesion receptor proteins (cadherin and integrin) mediate cell-cell interactions and are required for the establishment and maintenance of epithelial polarity. Cadherin and integrin adhesions act as nucleation sites for the localised assembly of specialised cytoskeletal and signaling networks at the point of cell contacts (Murcia *et al.*, 1999).

The pathologic processes which are the primary result of the gene defect are not known. However, studies in transgenic mice suggest a role for proto-oncogenes in the development of renal cystic disease (Trudel *et al.*, 1991). The occurrence of renal cysts in multiple congenital, genetic and non-genetic disorders, including tuberous sclerosis (Bernstein *et al.*, 1986), von-Hippel-Lindau syndrome (Christoferson *et al.*, 1961; Maher *et al.*, 1990), autosomal recessive polycystic kidney disease (McDonald and Avner, 1991) and acquired cystic kidney disease of longstanding renal failure (Ishikawa, 1991) suggests that renal cysts represent a final common pathway for many abnormalities (Gabow, 1993). Further advances in knowledge about ADPKD will provide a better understanding of the disease process and regulation of cell growth and differentiation, epithelial fluid secretion and ECM abnormalities (Gabow, 1993), and help separate cause and effect.

## **1.4 SYMPTOMS AND CLINICAL MANIFESTATIONS OF ADPKD**

The renal complications of ADPKD largely arise as a consequence of structural deformities. Of these, the most common complications include renal failure, hypertension, chronic pain (in the back, flank and abdomen), bleeds from cysts (producing haematuria or acute back pain), urinary tract infection (UTI) and kidney stones. Many studies have examined the clinical spectrum of ADPKD, however, differences in the reported frequency of symptoms and clinical findings are observed between studies (see Table 1.1).

### **1.4.1 *Kidney Failure***

The most feared and detrimental outcome of ADPKD is the development of end-stage renal failure (ESRF). The cause of renal failure is probably multi-factorial, including the

**Table 1.1: Symptoms and Clinical Findings Reported in Different Studies of ADPKD**

Author	Ethnicity	Sample	HBP	Back/ flank pain	UTI	Renal calculi	Haematuri a	Extrarenal cysts	Mean age at CRF	Mean age at ESRF
Rall & Odel, 1949	European American	* 207 ADPKD cases * 26 necropsy cases	73% (initial exam)	28% (initial exam)	17% (initial exam)	Not recorded	11.5% (initial exam)	<b>Necropsy data:</b> * Hepatic cysts (33%) * Pancreatic cysts (9%) * Splenic cysts (2%)	Not recorded	Not recorded
Ward <i>et al.</i> 1967	European American	* 53 ADPKD cases	62%	Not recorded	11%	50%	47%	* Hepatic cysts (73%) * Ovarian cysts (13%) * Pancreatic cysts (13%) * Splenic cysts (7%)	Not recorded	Not recorded
Higgins, 1952	European American	* 94 ADPKD cases	73%	33%	Not recorded	Not recorded	41%	Not recorded	Not recorded	Not recorded
Simon & Thompson, 1955	European American	* 366 ADPKD cases	55% (initial exam), 65% (all)	45% (initial exam); 73% (all)	21% (initial exam), 38% (all)	Not recorded	31.4% (initial exam), 38% (all)	Not recorded	Not recorded	Not recorded
Dalgaard, 1957	Danish	* 284 cases and their families	52%	Not recorded	Not recorded	18%	39%	Not recorded	Not recorded	53 years?
Segal <i>et al.</i> 1977	European American	* 100 ADPKD cases	Not recorded	Not recorded	Not recorded	20%	Not recorded	Not recorded	Not recorded	Not recorded
DeBono and Evans (1977)	British	* 65 ADPKD cases	55% (initial exam)	43% (all)	27% (all)	Not recorded	58% (all)	Not recorded	Not recorded	Approx. 50 years
Iglesias <i>et al.</i> 1983	Northern European	* 56 patients: 34 symptomatic; 6 family screening; 16 autopsy	83% (all),	79% (all); 94% (Sx), 17% (FS), 69% (Au)	34% (all), 44% (Sx), 17% (FS), 19% (Au)	Not recorded	<b>Gross &amp; Micro:</b> 29%, 38% (all); 44%, 38% (Sx); 0%, 0% (FS); 6%, 50% (Au)	<b>Autopsy :</b> * Hepatic cysts (55%) * Pancreatic cysts (10%) * Splenic cysts (5%)	Not recorded	Not recorded
Milutinovic <i>et al.</i> 1984	European American	* 140 ADPKD cases	13% (initial exam); 52% (all)	20% (initial exam); 41% (all)	24% (initial exam); 46% (all)	0.7% (initial exam); 11% (all)	14% (initial exam); 39% (all)	Not recorded	Not recorded	Not recorded

Churchill <i>et al.</i> 1984	European Canadian	* 140 ADPKD cases	40% not in renal failure	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded
Delaney <i>et al.</i> 1985	European American	* 53 symptomatic patients	21% (initial exam); 64% (all)	Not recorded	Not recorded	34%	19% (initial exam); 64% (all)	* Hepatic cysts (44%) * Pancreatic cysts (9%)	Not recorded	50 years
Zeier <i>et al.</i> 1988	German	* 158 ADPKD cases	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	38.5 yrs	Not recorded
Davies <i>et al.</i> 1991	Welsh	* 303 ADPKD cases	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	53.5 yrs
Parfrey <i>et al.</i> 1990a	European - Canadian	* 152 ADPKD cases	31%	Not recorded	22%	15%	9% (20-39yrs); >60% (>40 yrs)	Not recorded	Not recorded	59.3 yrs
Gabow <i>et al.</i> 1992b	American	* 580 ADPKD cases	70%	Not recorded	* 44%	Not recorded	43%	* Hepatic cysts 49%	Not recorded	Not recorded
Roscoe <i>et al.</i> 1993	European Canadian	* 80 pre-dialysis patients * 320 ESRF ADPKD patients * 4129 other disease	Not recorded	Not recorded	Not recorded	10%	Not recorded	* Hepatic cysts (46%) * Ovarian (13%) * Pancreatic (0%)	Not recorded	54.4 in pre-dialysis patients
Choukroun <i>et al.</i> 1995	French Caucasians	* 157 patients: (A) 109 (ESRF); (B) 48 (CRF)	* (A) 97%; (B) 100%	Not recorded	Not recorded	Not recorded	Not recorded	* (A) Hepatic cysts (74%)	Not recorded	52.7 yrs
Higashihara <i>et al.</i> 1992	Japanese	* 316 ADPKD cases; 72 with ESRF	64% (all)	* 42% (all) * 52% (ESRF)	Not recorded	* 18% (all) * 7% (ESRF)	<b>Gross:</b> * 39% (all) * 48% (ESRF)	* Hepatic cysts (54%) * Pancreatic cysts (7%) <b>ESRF:</b> * Hepatic cysts (67%)	Not recorded	52 yrs
Higashihara <i>et al.</i> 1998	Japanese	* 1082 ADPKD cases (without dialysis)	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded	53.4 yrs

*HBP* – high blood pressure; *UTI* – urinary tract infection; *CRF* – chronic renal failure; *ESRF* – end-stage renal failure; *Sx* – symptomatic; *FS* – family screening; *Au* – autopsy

pressure effect of expanding cysts; intrarenal vascular sclerosis from systemic hypertension; and interstitial fibrosis.

The development of cysts leads to enlarged kidneys, which on gross examination may appear to be completely replaced by cysts (Figure 1.4). The renal cysts vary in size and appearance from a few millimetres to many centimetres and from clear to cloudy to chocolate-coloured, suggesting previous haemorrhage (Gabow, 1993).

Serum creatinine (SCr) is the most widely used surrogate indicator of glomerular filtration rate (GFR). Increasing SCr levels imply a decrease in GFR as a measure of overall kidney function. Unlike glomerulonephritis and type 1 diabetes mellitus, where renal function declines in a linear fashion from onset, ADPKD patients may show a preservation of renal function for many years, followed by a phase of rapid, almost linear, deterioration (Franz and Reubi, 1983).

Several studies have reported average age at ESRF (see Table 1.1), which ranges between 50 and 59 years (Dalgaard, 1957; Delaney *et al.*, 1985; Parfrey *et al.*, 1990a; Davies *et al.*, 1991; Higashihara *et al.*, 1992; Roscoe *et al.*, 1993; Choukroun *et al.*, 1995; Higashihara *et al.*, 1998). The only study to report average age at CRF (defined as a SCr >1.4 mg/dl [93.5 µmol/L]), found to be 39 years, was that of Zeier *et al.* (1988).

#### **1.4.2      *Hypertension***

Hypertension (discussed briefly here and in more detail in the literature review in Chapter Five) is a very common and detrimental clinical manifestation of ADPKD, which may develop as an early symptom or occur later in the course of the disease. Hypertension affects approximately 30% of children (Gabow, 1993; Zeier *et al.*, 1993), and as many as 60% of adults before the onset of renal insufficiency and more than 80% of patients with ESRF (Milutinovic *et al.*, 1984).

Several studies have examined the development and frequency of hypertension between patients stratified by sex, mode of ADPKD diagnosis, severity of renal impairment and at autopsy. As shown in Table 1.2, the frequency of hypertension reported by several



**Figure 1.4:** Picture of a polycystic kidney.

Source: <http://www.medlib.med.utah.edu/WebPath/RENAHTML/RENALIDX.html>

**Table 1.2 Studies Examining the Development and Frequency of Hypertension in ADPKD**

Study	Sample Size	Definition of HBP	Stage of ADPKD progression				
			At ADPKD diagnosis	Any time of study	CRF	ESRF	Autopsy
Schacht (1931)	74 ADPKD cases	SBP:>140 mmHg or DBP:>90 mmHg	Not examined	73%	Not examined	Not examined	75%
Rall and Odel (1948)	207 ADPKD cases & 46 with necropsy data	SBP:>140 mmHg or DBP:>90 mmHg	17% (presenting complaint)	73%	Not examined	Not examined	Not examined
Ward <i>et al.</i> (1967)	53 ADPKD cases	Not recorded	62%	Not examined	Not examined	Not examined	Not examined
Higgins (1952)	94 ADPKD cases	Not recorded	Not examined	73%	Not examined	Not examined	Not examined
Simon and Thompson (1955)	366 ADPKD cases & 35 autopsy cases	Not recorded	55%	65%	Not examined	Not examined	Not examined
Mitcheson <i>et al.</i> (1977)	78 ADPKD cases	SBP:>150 mmHg or DBP:>100 mmHg	24%	Not examined	Not examined	Not examined	Not examined
DeBono and Evans (1977)	65 ADPKD cases	DBP: >100mmHg	55%	Not examined	Not examined	Not examined	Not examined
Iglesias <i>et al.</i> (1983)	56 ADPKD cases	DBP:>90 mmHg or DBP:>100 mmHg	83%	>90 (88%) >100 (55%)	Not examined	Not examined	DBP:>90 mmHg (69%) DBP:>100 mmHg (31%)
Milutinovic <i>et al.</i> (1984)	140 ADPKD cases	SBP:≥150 mmHg or DBP:≥90 mmHg	13%	52%	Not examined	82%	Not examined
Delaney <i>et al.</i> (1985)	53 ADPKD cases	SBP:>150 mmHg or DBP:>100 mmHg	21%	64%	Not examined	Not examined	Not examined
Parfrey <i>et al.</i> (1990a)	152 ADPKD cases	SBP:>95 <sup>th</sup> percentile DBP:>95 <sup>th</sup> percentile for age and sex	Not examined	25% children & young adults; & 62% adults	Not examined	Not examined	Not examined
Higashihara <i>et al.</i> (1992)	316 ADPKD cases	SBP:>150 mmHg or DBP:>90 mmHg	Not examined	64%	Not examined	Not examined	Not examined
Choukroun <i>et al.</i> (1995)	157 ADPKD cases: 109 with ESRF & 48 with CRF	SBP:>160 mmHg or DBP:>95 mmHg	Not examined	97%	Not examined	100%	Not examined

*HBP* – high blood pressure; *CRF* – chronic renal failure; *ESRF* – end-stage renal failure; *SBP* – systolic blood pressure; *DBP* – diastolic blood pressure; *mmHg* – millimetres mercury



studies varies, and may be attributed to differences in the definition of hypertension, methods of blood pressure measurement and stage of ADPKD.

The frequency of hypertension as the initial presenting symptom at the time of ADPKD diagnosis ranges between 13 and 88% (Rall and Odel, 1948; Simon and Thompson, 1955; Ward *et al.*, 1967; De Bono and Evans, 1977; Mitcheson *et al.*, 1977; Iglesias *et al.*, 1983; Milutinovic *et al.*, 1984; Delaney *et al.*, 1985). At any one time of the study the reported frequency of hypertension ranges between 52 and 97% (Schacht, 1931; Rall and Odel, 1948; Higgins, 1952; Simon and Thompson, 1955; Iglesias *et al.*, 1983; Milutinovic *et al.*, 1984; Delaney *et al.*, 1985; Parfrey *et al.*, 1990a; Higashihara *et al.*, 1992; Choukroun *et al.*, 1995).

Two studies reported the frequency of hypertension in patients with ESRF (Milutinovic *et al.*, 1984; Choukroun *et al.*, 1995), while two studies reported the frequency in patients diagnosed with ADPKD at autopsy (Schacht, 1931; Iglesias *et al.*, 1983). Another study compared systolic (SBP) and diastolic (DBP) blood pressure between three groups, based on renal function: group 1 - SCr  $\leq 1.5$  mg/dl, group 2 -  $>1.5$  mg/dl and group 3 - dialysis. Blood pressure was found to be higher in group 2 than groups 1 and 3 (Higashihara *et al.*, 1992).

A renal mechanism for the development of hypertension seems likely. Growth of cysts may lead to hypertension through several mechanisms. The first mechanism involves the renin-angiotensin system (RAS), which has been repeatedly incriminated in the pathophysiology of hypertension in ADPKD, due to its involvement in physiological renal sodium handling and extracellular volume regulation (Florijn *et al.*, 1992). In addition, evidence of RAS activation has been demonstrated as an early feature in the course of ADPKD (Chapman *et al.*, 1990; Harrap *et al.*, 1991). The RAS may be activated by displacement and narrowing of the renal arterioles leading to focal intrarenal hypoperfusion and ischaemia. Compression of normal renal tissue attenuates the blood vessels and alters the blood supply to the kidney, which may stimulate the RAS.

The second mechanism by which the growth of cysts may lead to hypertension involves increased release of endothelin (a potent vasoconstrictor released by endothelial cells)

into the stretched and narrow arterioles around expanding cysts (Barendregt *et al.*, 1995). The rise in plasma endothelin in ADPKD patients may be associated with increased endothelial shear stress or focal ischaemia in the kidneys, or by differences in the production or clearance of endothelin elsewhere in the body (Barendregt *et al.*, 1995). A deficiency or defective release of endothelial-derived relaxing factors such as nitric oxide has been observed in some patients with hypertension (Baylis and Vallance, 1996) and end-stage renal disease patients (Schmidt *et al.*, 1995; Wang and Strandgaard, 1997).

The third mechanism for hypertension caused by cyst growth may also involve an increase in afferent nerve activity from the kidneys leading to a generalised increase in sympathetic nerve activity (Iversen *et al.*, 1996). Enhancement of local sympathetic nerve activity may occur in ADPKD by attenuation of blood vessels around the cysts (Iversen *et al.*, 1996).

The effects of hypertension are not localised to the kidney but also have a profound effect on the cardiovascular system. Hypertension contributes to an increase in the left ventricular mass (LVM) and other cardiovascular complications (Chapman and Gabow, 1997). Apart from renal failure, the primary cause of death in ADPKD patients is cardiovascular disease. This is discussed in section 1.4.7.

### **1.4.3 Back/flank pain**

Back/flank pain, often described as a dull or nagging ache, is caused by the pressure produced by the swelling of the kidneys, the rupture of a cyst or blood vessel, concomitant renal calculi or musculo-skeletal pain caused by the weight of the kidneys. Many patients with persistent pain are at significant risk for long-term disability, depression and analgesic abuse (Wilson and Guay-Woodford, 1999). However, with the utilization of a variety of traditional and available pain management techniques, pain can be managed in most of these patients (Bajwa *et al.*, 2001).

Back/flank pain is experienced by approximately 60% of patients, however variation in its frequency at the time of initial examination and during follow-up has been observed between studies (see Table 1.1). The reported frequencies range between 20 and 45% at

the time of initial examination (Rall and Odel, 1948; Simon and Thompson, 1955; Milutinovic *et al.*, 1984; Higashihara *et al.*, 1992) and 33-79% at follow-up (Higgins, 1952; Simon and Thompson, 1955; De Bono and Evans, 1977; Iglesias *et al.*, 1983; Milutinovic *et al.*, 1984; Higashihara *et al.*, 1992).

Only one study compared the frequency of back/flank pain according to the reason for examination (Iglesias *et al.*, 1984). In that study, 94% of patients initially examined because of symptoms, were found to suffer from back/flank pain, followed by 69% reported by autopsy records and 17% in patients examined because of family history of ADPKD.

#### **1.4.4 Haematuria**

Haematuria is defined by the presence of blood in the urine and may present as microscopic or gross haematuria (Simon and Thompson, 1955). It is a common renal manifestation associated with pain occurring as a result of a cyst bleed. It may be precipitated by trauma or excessive physical exertion (Simon and Thompson, 1955), or may occur spontaneously.

The frequency of haematuria from cyst rupture is reported to correlate with the size of the kidneys; the incidence of haematuria increasing dramatically when one or both of the kidneys is larger than 15 cm (Gabow *et al.*, 1992a). In addition, hypertensive subjects are more likely to have gross haematuria than normotensive subjects (Johnson and Gabow, 1997).

The reported frequency of haematuria varies considerably between studies (see Table 1.1). At the time of initial ADPKD investigation, it ranges from 11 to 31% (Rall and Odel, 1948; Higgins, 1952; Simon and Thompson, 1955; Dalgaard, 1957; Ward *et al.*, 1967; Iglesias *et al.*, 1983; Milutinovic *et al.*, 1984; Delaney *et al.*, 1985; Parfrey *et al.*, 1990a; Higashihara *et al.*, 1992), whereas during follow-up the reported frequency is between 9 and 60% (Higgins, 1952; Simon and Thompson, 1955; Dalgaard, 1957; Ward *et al.*, 1967; De Bono and Evans, 1977; Iglesias *et al.*, 1983; Milutinovic *et al.*, 1984; Delaney *et al.*, 1985; Parfrey *et al.*, 1990a; Higashihara *et al.*, 1992). This wide variation may be explained by differences in diagnostic procedures used to examine for

the presence of haematuria, for example urinalysis versus urine microscopy versus macroscopic, as well as presentation to urology versus nephrology clinics.

#### **1.4.5            *Urinary Tract Infection (UTI)***

Approximately 50% of ADPKD patients suffer at least one UTI episode and these are more prevalent in women than in men (Sklar *et al.*, 1987; Bajwa *et al.*, 2001)(as applies to UTI in the general population). Cyst infection represents a serious complication and is due either to lower urinary tract infections or, rarely, to hematogenous spread. Most infections are caused by Gram-negative organisms (Schwenger and Zeier, 1999), and are often resistant to therapy.

The incidence of UTI reported at the time of initial examination is found to range between 17 and 24% (Rall and Odel, 1948; Simon and Thompson, 1955; Milutinovic *et al.*, 1984; Delaney *et al.*, 1985). The occurrence of UTI at any time during follow-up ranges between 11% and 61% (Simon and Thompson, 1955; Ward *et al.*, 1967; De Bono and Evans, 1977; Iglesias *et al.*, 1983; Milutinovic *et al.*, 1984; Delaney *et al.*, 1985; Gabow *et al.*, 1992b). Iglesias also reported a frequency of 44% in patients initially examined because of symptoms, 17% because of family history and 19% at autopsy. The reported frequencies are shown in Table 1.1.

#### **1.4.6            *Kidney Stones (Renal Calculi)***

Kidney stones are a less common complication of ADPKD. The pathogenesis of stone formation is not well understood in ADPKD, however the majority contain calcium or urate (derived from uric acid) (Schwenger and Zeier, 1999).

The frequency of renal calculi varies between studies (see Table 1.1). As early as 1967, Ward *et al.* reported a prevalence of 62%, whereas other studies have reported a lower frequency ranging between 10 and 34% (Dalgaard, 1957; Segal *et al.*, 1977; Milutinovic *et al.*, 1984; Delaney *et al.*, 1985; Parfrey *et al.*, 1990a; Higashihara *et al.*, 1992; Roscoe *et al.*, 1993). Milutinovic *et al.* (1984) examined the frequency of renal calculi at initial investigation and reported only 0.7%, which increased to 11% throughout the course. Higashihara *et al.* (1992) compared the frequency of renal calculi by sex and between patients with normal and impaired renal function. In their study, the frequency of renal

calculi was more common in men (21%) than in women (13%) and more common in patients not on dialysis (21%) than in those on dialysis (7%).

#### **1.4.7 Extrarenal Manifestations**

As previously mentioned, ADPKD is a systemic disorder consistent with a defect in extracellular matrix integrity (see section 1.3.3).

##### *Extrarenal cysts*

Liver involvement is the most frequent extrarenal cystic manifestation in ADPKD. Liver cysts are responsible for most hepatic complications, but other liver changes may occasionally be encountered, including congenital hepatic fibrosis and segmental dilation of the biliary tract. Liver cysts are focal in nature and seem to develop later than renal cysts and increase in frequency with advancing age. Although liver cysts are very rare for people younger than 20 years, their prevalence increases from 20% in the third to 70% in the seventh decade of life (Chauveau *et al.*, 2000). Women are more prone to liver cystic involvement. Cysts are not only recognised earlier but are also more numerous and larger in females than in males. Previous use of estrogens has been identified as a risk factor for development of liver cysts (Sherstha *et al.*, 1997). Although most patients with ADPKD report no liver symptoms, some experience chronic manifestations related to progressive enlargement in size of the polycystic liver (Chauveau *et al.*, 2000), and occasionally require hepatectomy and/or liver transplantation.

Pancreatic cysts as well as splenic and ovarian cysts are also found to occur in about 10% of ADPKD patients, but are generally clinically insignificant (Gabow, 1993).

A list of studies that have examined for the presence of extrarenal cysts is presented in Table 1.1. The frequency of hepatic cysts ranged between 33 and 74% (Rall and Odel, 1948; Ward *et al.*, 1967; Iglesias *et al.*, 1983; Delaney *et al.*, 1985; Higashihara *et al.*, 1992; Gabow *et al.*, 1992b; Roscoe *et al.*, 1993; Choukroun *et al.*, 1995). Six studies examined the presence of pancreatic cysts. In five of these studies, the frequency of pancreatic cysts ranged between 7 and 9% (Rall and Odel, 1948; Ward *et al.*, 1967; Iglesias *et al.*, 1983; Delaney *et al.*, 1985; Higashihara *et al.*, 1992), whereas no

pancreatic cysts were detected in the remaining study (Roscoe *et al.*, 1993). The presence of splenic cysts was investigated by three groups, who reported a frequency of 2-7% (Rall and Odel, 1948; Ward *et al.*, 1967; Iglesias *et al.*, 1983). Only two studies examined the presence of ovarian cysts and reported an incidence of 13% (Ward *et al.*, 1967; Roscoe *et al.*, 1993).

#### *Intracranial Aneurysms (ICA)*

The most devastating extrarenal manifestation is a ruptured intracranial aneurysm (ICA). The estimated prevalence of aneurysms in ADPKD patients with a positive family history is 26%. By comparison, ICA occurs in approximately 2% of the general population (van Dijk *et al.*, 1995). The risk for rupture of an ICA associated with ADPKD has not been well defined, but epidemiologic data suggest that a significant fraction of ICAs never rupture (Pirson and Chauveau, 1996). There may be family clustering of ruptured ICA, and a rupture of an ICA in ADPKD seems to occur at a younger age and with smaller aneurysms than in the general population (Fick and Gabow, 1994).

#### *Cardiovascular Abnormalities*

Cardiac valvular abnormalities are the main non-cystic extrarenal manifestation of ADPKD (Leier *et al.*, 1984; Hossack *et al.*, 1988). In particular, mitral valve prolapse and mitral and aortic regurgitation have been documented in patients with ADPKD (Hossack, 1988). The prevalence of these abnormalities ranges from 0% to 30% (Perrone, 1997). In a large prospective study, 26% of ADPKD individuals were found to have mitral valve prolapse as compared to 2% in a control population (Hossack *et al.*, 1988).

Another cardiovascular abnormality is left ventricular hypertrophy (LVH), which may be a non-specific reflection of hypertension target organ damage, rather than related to ADPKD *per se*. Nonetheless, LVH has been reported as a major risk factor for mortality in uraemic patients undergoing dialysis (Parfrey *et al.*, 1990b). LVH is reported to occur in approximately half the hypertensive patients with ADPKD (Chapman *et al.*, 1997b), while an increased left ventricular mass index has also been found in the early stages of the disease (Zeier *et al.*, 1993; Saggar-Malik *et al.*, 1994).

## *Herniae*

The development of herniae occurs in approximately 10% of all ADPKD patients. There are two types of herniae: 1) inguinal, which develop in the groin between the lower part of the torso and the leg and; 2) umbilical, which involve the navel. Both probably develop because of the increased intra-abdominal pressure produced by the size of the cysts (Isselbacher *et al.*, 1994), but an intrinsic weakness of the abdominal wall cannot be excluded.

## *Diverticulitis*

Colonic diverticulae occur with a prevalence between 30 to 80% (Gabow, 1993). Scheff *et al.* (1980) reported colonic diverticulae at a frequency of 82% in 12 ADPKD patients who were receiving dialysis, as compared with a frequency of 32% among individuals receiving dialysis because of other renal diseases and 38% in age-matched control subjects who were not receiving dialysis. Once again, this may be associated with intrinsic weakness of the colonic wall (Isselbacher *et al.*, 1994).

# **1.5 THE GENETICS OF ADPKD**

## **1.5.1 *Inheritance of ADPKD***

As its name suggests, ADPKD is transmitted in an autosomal dominant mode, meaning that every offspring of an affected parent has a 50% risk of inheriting the disease gene (Dalgaard, 1957). Similar to other genetic disorders, *de novo* mutations occur, but they appear to account for 10% or fewer of all cases (Gabow, 1993).

## **1.5.2 *Genetic Heterogeneity***

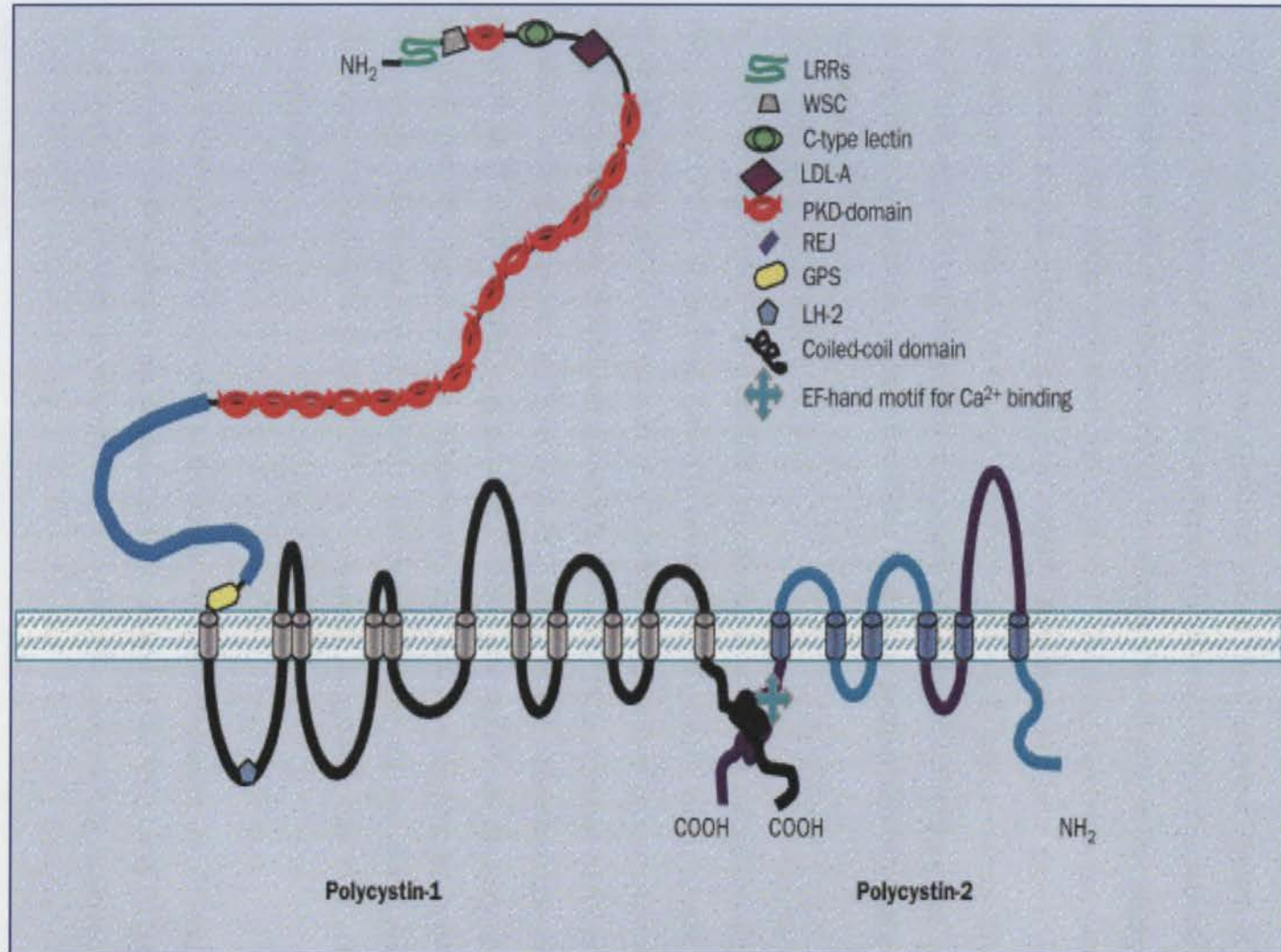
At least three different genes could be involved in the development of ADPKD. The major breakthrough in the genetics of ADPKD was the localisation of a gene for ADPKD to the short arm of chromosome 16 using genetic linkage techniques (Reeders, 1985). Approximately 85% of all Caucasian ADPKD families demonstrate linkage to this gene, designated *PKD1* (Peters and Sandkuijl, 1992). Another major finding was the subsequent discovery of a family whose ADPKD was not linked to chromosome 16 (Kimberling *et al.*, 1988). This second ADPKD gene, *PKD2*, was mapped to 4q (Kimberling *et al.*, 1993; Peters *et al.*, 1993), and other affected families linked to *PKD2* have subsequently been identified (Romeo *et al.*, 1988; Kimberling *et al.*, 1993;

Peters *et al.*, 1993; San Millan *et al.*, 1995). Further evidence of genetic heterogeneity has been reported by various groups (Bogdanova *et al.*, 1995; Daoust *et al.*, 1995; de Almeida *et al.*, 1995) suggesting the existence of a third ADPKD gene.

The *PKD1* gene was cloned in 1994 (The European Polycystic Kidney Disease Consortium, 1994; Hughes *et al.*, 1995; The American PKD1 Consortium, 1995; The International Polycystic Kidney Disease Consortium, 1995) and its protein product named Polycystin 1 (PC1). PC1 is predicted to be a very large membrane-associated glycoprotein, containing 4302 amino acids, with a molecular weight of 460 kDa. A picture of the predicted protein structure is shown in Figure 1.5. PC1 consists of a long extracellular N-terminal portion, 11 transmembrane domains and a shorter intracellular C-terminal portion. The extracellular portion consists of two leucine-rich repeats (LRR's), encoded by exons 2 and 3, which are very close to the 5' terminus and are flanked by two cysteine-rich domains (Hughes *et al.*, 1995). The combination of LRR's and cysteine-rich domains suggests that PC1 interacts with other proteins and that it may be involved in cell recognition, binding to components of the ECM, or signal transduction (The American PKD Consortium, 1995).

The next functional domain is a C-type lectin domain, predictive of protein-carbohydrate interactions. The function of this domain is to bind to specific carbohydrates in the presence of calcium ions, also associated with cell adhesion and recognition. The extracellular portion also consists of fifteen copies of an 80-90 amino acid repeat, which form an immunoglobulin-like (Ig-like) domain. A study of the structure of Ig-like PKD domains (Bycroft *et al.*, 1999) suggests that this region forms a  $\beta$ -sandwich fold and may play a role in ligand-binding. More recent evidence (Ibraghimov-Beskrovnya *et al.*, 2000) indicates that the Ig-like repeat region plays a particular role in mediating intercellular adhesion, likely to involve E-cadherin and several catenin subtypes (Huan and van Adelsberg, 1999). A region extending over 1000 amino acids, that follows the PKD domain, is homologous to the receptor for egg jelly (REJ) of the sea urchin. REJ, a 210-kDa sperm membrane glycoprotein has been shown to function as an ion channel regulator and is believed to trigger the sea urchin sperm acrosome reaction (Moy *et al.*, 1996; Hughes *et al.*, 1999).





**Figure 1.5:** The predicted structure of the PKD1 and PKD2 proteins and their interaction forming the polycystin complex.

Source: Peters and Breuning 2001 358:1439-44

The intracellular C-terminal portion of PC1 contains several phosphorylation sites, that have been demonstrated to trigger the activation of activator protein-1 (AP-1), a transcription factor involved in cell proliferation, differentiation and apoptosis (Arnould *et al.*, 1998). G proteins may initiate this signal transduction pathway. A G-protein activation domain, which has been identified within the C-terminal domain of PKD1, and *in vitro*, binds to and activates G proteins (Parnell *et al.*, 1998). The intracellular C-terminal also stabilises  $\beta$ -catenin, which is a multifunctional protein that engages in at least three intracellular regulatory pathways: cell adhesion, transcriptional regulation, and tubular morphogenesis (Barth *et al.*, 1997). Recent studies have shown that PC1 participates in complexes containing E-cadherin and  $\alpha$ -,  $\beta$ - and  $\gamma$ -catenin (Huan and van Adelsberg, 1999) which provide a structural link between cell-cell surface adhesion and the actin cytoskeleton. The intracellular C-terminal portion also contains a coiled-coil domain.

In 1996, the *PKD2* gene was identified (Mochizuki *et al.*, 1996) and its protein named Polycystin 2 (PC2). It has been shown to encode a 5.4 kb mRNA within approximately 68 kb of genomic DNA (Mochizuki *et al.*, 1996). The predicted protein of 968 amino acids is approximately 25% identical and 50% similar to PC1. A picture of the predicted protein structure is shown in Figure 1.5. PC2 contains six transmembrane domains, intracellular C and N termini, and putative EF hand and coiled-coil domains in the C-terminal portion, suggestive of calcium binding and protein-protein interactions, respectively (Mochizuki *et al.*, 1996). The transmembrane regions share significant homology with voltage-activated  $\text{Ca}^{2+}/\text{Na}^{2+}$  channels, suggesting that PC2 may be a channel protein.

So far, *PKD1* and *PKD2* are used to designate the genes. However, PKD1 and PKD2 are also used to describe the phenotype of families linked to the *PKD1* and *PKD2* genes. Since these terms are used interchangeably, referral to the genes will be italicised.

#### *Novel PKD2-like genes*

At least two members of the *PKD2*-like (*PKDL*) gene family, *PKD2L* and *PKD2L2*, have been identified. *PKD2L* is localised to chromosome 10q24-25 (Nomura *et al.*, 1998; Wu *et al.*, 1998), and has a tissue-specific expression pattern, as well as

alternative transcripts (2.4, 2.7 and 3kb) in different tissues (Wu *et al.*, 1998). Polycystin-L, the gene product of *PKD2L*, contains 805 amino acids and shows approximately 50% identity and approximately 70% similarity to *PKD2*. In the carboxyl-terminus of this gene product, an EF-hand motif, similar to that seen in *PKD2*, and a coiled-coil domain have been predicted (Veldhuisen *et al.*, 1999). Polycystin-2 and polycystin-L have structural similarity with cation channel subunits (Nomura *et al.*, 1998; Wu *et al.*, 1998). It has been suggested that both polycystin-2 and polycystin-L may act as cation channels to regulate calcium-mediated signal transduction.

The second member of the *PKDL* family, *PKD2L2*, lies in an approximately 51kb genomic region on chromosome 5q31. This gene has a 2.2kb transcript and encodes a 613 amino acid protein with 6 to 8 predicted transmembrane domains (Veldhuisen *et al.*, 1999). The gene product polycystin-2L2 exhibits approximately 48% identity and 67% similarity with both *PKD2* and *PKD2L*. However, unlike *PKD2L*, no putative EF-hand or coiled-coil domain has been predicted in its carboxyl-terminal portion (Veldhuisen *et al.*, 1999). The function of this novel protein therefore remains unknown.

### 1.5.3 *The Polycystin Complex*

Individuals linked to either *PKD1* or *PKD2* share common clinical features, which together with the commonality of cyst formation, suggests that PC1 and PC2 may interact and participate in a common cystogenic pathway (Mochizuki *et al.*, 1996). The physiological role of this pathway may be to maintain planar polarity and cell contact-dependent growth control among epithelial cells. Data from *in vitro* protein interactions and heterologous expression systems suggest that PC1 does indeed form heterodimers with PC2 (Figure 1.5) (Qian *et al.*, 1997a; Tsiokas *et al.*, 1997). Other components of the same complex are Tg737 (a putative polycystin-1 ligand), actin (which mediates attachments to the cytoskeleton),  $\alpha$ - and  $\beta$ -catenins, cadherins and the epidermal growth factor receptor. According to this model, it is speculated that signals from other cells or the extracellular milieu are sensed by PC1 and passed on to PC2, causing calcium channel activity and leading to downstream regulation of other activities at gene expression level (Murcia *et al.*, 1999). Disruption of the polycystin complex may alter the ability of renal epithelial cells to mature fully. This may result in abnormal cellular proliferation, altered amounts of otherwise normal electrolyte transport proteins

(resulting in excessive secretion of solute and fluid into the cysts) and complex changes in the extracellular matrix (Murcia *et al.*, 1999).

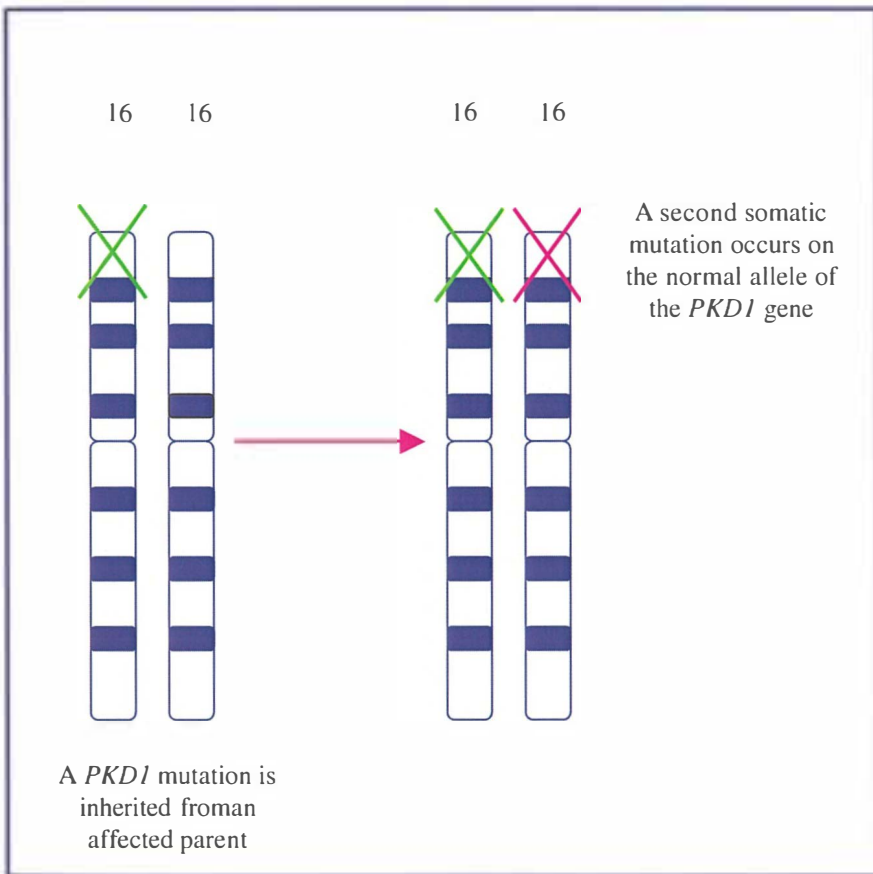
## 1.6 THE PATHOGENESIS OF ADPKD

### 1.6.1 *The Genetic Mechanisms of Focal Cyst Formation*

A striking characteristic of ADPKD is that, although all nucleated cells carry the mutant gene, only a small fraction of nephrons (*i.e.* <5%) become cystic (Osathanondh and Potter, 1964; Baert, 1978; Harris *et al.*, 1995; Koptides *et al.*, 1998, 1999; Qian *et al.*, 1997a). The “two hit” model of germline and superimposed somatic mutation is used to explain this paradox. Somatic mutations are frequent and inevitable, but are often of no consequence. Human mutation rates are typically  $10^{-5}$  to  $10^{-7}$  per gene per generation, and our bodies contain about  $10^{14}$  cells. The minority, which do cause problems, usually do so by fostering uncontrolled cell growth (Strachan and Read, 1996). Knudson (1971) was the first to propose the ‘two-hit’ model for explaining the origin of retinoblastoma. In inherited cancer syndromes caused by germline mutations in tumor-suppressor genes such as the retinoblastoma 1 (*RBI*) gene, tumors only occur through the chance event of additional somatic mutations (one or more) in susceptible cells (Knudson, 1985).

It has been suggested that, at the organism level, ADPKD is inherited as a dominant trait, but at the cellular level it is recessive. In this model, an inherited mutation predisposes an individual to the disease by the deleterious alteration of the first PKD allele. The germline mutation supplies the rate-limiting “first hit” at the locus. Analysis of individual cysts has revealed that independent “second hits” occur randomly in the normal *PKDI* allele in individual renal epithelial cells (Qian *et al.*, 1996; Brasier and Henske, 1997). A schematic representation of the “two hit” mechanism is shown in Figure 1.6.

The hypothesis that a second hit may be required for cyst formation and disease development is supported by work on *PKDI*, which demonstrated loss of heterozygosity (LOH) and somatic mutations in DNA from renal cystic epithelial and liver cells, in addition to the inherited germline mutation. Loss of heterozygosity is defined as the loss of alleles on one chromosome detected by assaying for markers for which an individual is constitutionally heterozygous. In all cases, the wild type *PKDI* allele was lost (Qian



**Figure 1.6:** Schematic Representation of the “Two Hit” Model for Cystogenesis in ADPKD. Modified from Pei *et al.* (2001) *Am J Hum Genet* 68:355-363

*et al.*, 1996; Brasier and Henske, 1997; Koptides *et al.*, 1998) or had mutated (Watnick *et al.*, 1998). Based on the similarity in phenotypes, it is assumed that the same hypothesis may hold true for *PKD2*. Using a murine model, Wu *et al.* (1998) showed that cyst formation is the result of somatic inactivation of both *Pkd2* alleles. This hypothesis was further supported by Koptides *et al.* (1999) who directly showed for the first time the presence of somatic inactivating mutations in the inherited normal allele of *PKD2*, in the cells of kidney cysts of an ADPKD patient.

Qian *et al.* (1996) analysed polymorphic markers located within the *PKD1* gene in isolated renal epithelial cells derived from polycystic kidneys. Clonality of the renal cystic epithelial cells was found in 82% of the samples studied. The data were consistent with loss of heterozygosity, caused by a deletion of the normal *PKD1* allele, or with a somatic mutation arising from errors in transcription-coupled repair. All the above data support the suggestion that cyst formation requires a ‘second hit’ in the inherited normal copy of the *PKD1* locus (Qian *et al.*, 1996). The requirement for a second mutation event would account for the relative infrequency of cyst formation by nephrons (Brasier and Henske, 1997).

The focal formation of liver cysts suggests a similar mechanism. Like renal cysts, inactivating somatic mutations have also been found in *PKD1* and *PKD2* liver cyst epithelia, supporting the “two-hit” model as a unifying mechanism of cystogenesis (Watnick *et al.*, 1998; Pei *et al.*, 1999). Heterozygous mice with targeted mutations in *Pkd1* (Lu *et al.*, 1999) or *Pkd2* (Wu *et al.*, 1998) develop liver cysts that are indistinguishable from those found in ADPKD patients. In contrast, homozygous mice with a deletion of exon 34 of *Pkd1* (*del34* previously named *Pkd1<sup>-</sup>*) that die *in utero* or early in the postnatal period have a normal biliary system and no liver cysts. This suggests that *Pkd1* and *Pkd2* are required in the maintenance but not in the formation of biliary ducts (Chauveau *et al.*, 2000).

## **1.7 PHENOTYPIC HETEROGENEITY: INTER- AND INTRA-FAMILIAL VARIATION**

As mutations have been identified for a series of diseases, it has become clear that the correlation between genotype and phenotype is often incomplete. What has emerged is

the recognition that, for many diseases, only a subset of all mutations reliably predict phenotype. Consequently, many genetic diseases exhibit significant phenotypic variation between families (inter-familial variation) and also between affected individuals within the same family (intra-familial variation). Additional, independently inherited, genetic variation and/or environmental factors may be involved in modifying the clinical severity of such disorders.

ADPKD is one of the examples of considerable phenotypic variation between and within families. The age at onset of terminal renal failure and the rate of progression are known to vary between affected individuals. Whereas some individuals develop ESRF within five years after the onset of symptoms, others may develop ESRF ten years later or even not at all. While this variability can be explained partly by locus and mutation heterogeneity, differences within families suggests that progression may be influenced by other factors, acting independently or in combination with each other.

Phenotypic variability in ADPKD, as well as in other disorders, greatly increases the burden for affected families. This variability confounds both genetic counselling and clinical management and impairs the ability to understand the pathogenesis of the disease. From the point of view of potential therapy, the most important question raised is: Why does one affected patient fail to develop symptoms by age 70 years whereas his/her grandchild may need dialysis by age 35 years? Several studies have examined the extent of inter- and intra-familial variation in ADPKD (Dalgaard, 1957; Ravine *et al.*, 1992; Hateboer *et al.*, 1999a), however there is no general agreement about intra-familial variation (Dalgaard, 1957; Milutinovic *et al.*, 1992; Torra *et al.*, 1995). Differences in sample size, ascertainment of cases and methodology may in part account for the discordant findings, suggesting that further analysis is required to investigate the extent of intra-familial variation and factors that may be involved in modulating the course of the disease.

### **1.7.1      *Factors That May Influence Phenotypic Variation in ADPKD***

There are many factors that may influence phenotypic variation. A list of the factors and examples are presented in Table 1.3, and discussed in the following sub-sections.

**Table 1.3: Factors Potentially Leading to Phenotypic Variation in ADPKD and Other Inherited Disorders**

Factor	Example
Locus heterogeneity	* <i>PKD1</i> , <i>PKD2</i>
Allelic heterogeneity	* <i>PKD1</i> mutations associated with early disease onset * CFTR mutations associated with severe pancreatic insufficiency
Somatic mutations	* Cystic epithelial cells revealing germline and somatic mutations in the <i>PKD1</i> and <i>PKD2</i> genes
Compound trans-heterozygote mutation	* Individuals with mutations in both <i>PKD1</i> and <i>PKD2</i> alleles
Modifying genes	* ACE I/D polymorphism in ADPKD * AGT and blood pressure in humans * MOP1 and MOP2 in mouse PKD * <i>Nek1</i> gene in mouse PKD
Genomic imprinting	* Maternal inheritance – more severe phenotype in ADPKD
Environmental factors	* Smoking and $\alpha$ -1 ATD deficiency * Diet in PKU * Dietary protein in ADPKD * Smoking and ADPKD
Other factors	* Potential effect of female sex * Parental hypertension * Populations (racial ethnicity, affluence, health care systems etc)

**1.7.1.1 Locus Heterogeneity**

Locus/genetic heterogeneity (described briefly here and discussed in detail in the literature review in Chapter Four) is defined as mutations in any one of several genes that may result in identical phenotypes, such as when the genes are required for a common biochemical pathway or cellular structure (Lander and Schork, 1994). Examples of such conditions include hereditary nonpolyposis colon cancer (Fishel *et al.*, 1993, 1994) and early onset Alzheimer’s disease (St. George-Hyslop *et al.*, 1990). Two disorders displaying a high level of locus heterogeneity are retinitis pigmentosa, which can result from mutations in any of at least 32 different loci (<http://www.sph.uth.tmc.edu/RetNet>) and Zellweger syndrome resulting from mutations in any of 13 loci (Brul *et al.*, 1988).



In ADPKD, the different genetic forms generally share the same main clinical features. However, it has been shown that patients with PKD1 present with a more severe phenotype than PKD2 patients, in regard to age at onset, frequency of complications and development of ESRF (Parfrey *et al.*, 1990a; Gabow *et al.*, 1992b; Ravine *et al.*, 1992; Roscoe *et al.*, 1993; Wright *et al.*, 1993; Coto *et al.*, 1995). These findings have been reinforced in a large multi-centre study by Hateboer *et al.* (1999b) that compared the clinical outcome (*i.e.* survival to ESRF or death) between a large number of patients linked to *PKD1* or *PKD2*, and normal controls. Controls were spouses of affected individuals and unaffected siblings (normal renal imaging and linkage results showed their genetic risk was <1%). Results from the study showed that patients linked to *PKD1* were diagnosed at a younger age and had a higher prevalence of hypertension. The study also found that the median age at ESRF in PKD1 patients was 53 years, compared to 69 years for PKD2 and 78 years for controls (Hateboer *et al.*, 1999b). This is further discussed in Chapter Four.

#### 1.7.1.2 Allelic Heterogeneity

Allelic heterogeneity is defined as multiple disease-causing mutations at a single genetic locus (Lander and Schork, 1994). In many cases, phenotypic variation has been correlated with allelic heterogeneity. The type and position of mutations within a gene may be used to predict the severity of the disease. An example of this is cystic fibrosis (CF), where individuals exhibit a wide variety of clinical symptoms. The correlation between genotype and phenotype has been investigated for a number of specific mutations in the cystic fibrosis transmembrane regulator (CFTR) gene. In some mutations, such as the 3 bp deletion that causes the omission of a phenylalanine residue at codon 508 (*i.e.*  $\Delta F508$ ), which is present in 70% of all cases of CF (Kerem *et al.*, 1989, 1990), the CFTR protein is produced, but does not associate with the plasma membrane. As a result, regulation of chloride ion transport is absent and clinical symptoms are severe (Sheppard *et al.*, 1993). However, the relationship between genotype and phenotype has proved to be difficult. While the pancreatic status and the susceptibility to *Pseudomonas aeruginosa* colonization seem genetically determined, pulmonary involvement is more variable (The Cystic Fibrosis Genotype-Phenotype Consortium, 1993). In the case of the  $\Delta F508$  mutation, the clinical manifestations in patients homozygous for this mutation have been extensively studied (Santis *et al.*,

1990; Kerem *et al.*, 1990; Campbell *et al.*, 1991). Individuals generally have pancreatic insufficiency of early onset with markedly elevated sweat chloride concentrations, but the pulmonary manifestations are widely variable.

In ADPKD, the correlation between genotype and phenotype is also not straightforward. This is due to the large number of private mutations (*i.e.* confined to individual families) that make genotype-phenotype correlations difficult to assess and the fact that the number of patients with the same mutation is minute (Krawczak and Cooper, 1997; Afzal *et al.*, 2000; Aguiari *et al.*, 2000). The Q4041X mutation originally reported by Turco *et al.* (1995) is the most common *PKD1* mutation which has been detected in only a handful of patients world-wide (Turco *et al.*, 1995; Daniells *et al.*, 1998; Torra *et al.*, 1998; Bogdanova *et al.*, 2000). Hence, the analysis of such correlations has to be limited to comparing the general effects of the nature of mutations (truncating versus missense) and localisation in specific domains (McCluskey *et al.*, 2002).

The first mutation detected in ADPKD was a combined deletion of the *PKD1* and tuberous sclerosis 2 (*TSC2*) genes that lie close together, tail-to-tail, on the short arm of chromosome 16. It has been found that deletions eliminating part or all of both genes were associated with a variant of TSC often characterised by a severe PKD in childhood (Brook-Carter *et al.*, 1994). Watnick *et al.* (1999) provided further evidence regarding the relationship between genotype and phenotype in 10 *PKD1* families presenting very early onset of ADPKD and aneurysms. Their study reported 7 mutations located in exon 15 of the *PKD1* gene and 3 of these mutations were identical 2 bp deletions, suggesting that the particular genotype may be correlated with the severity of ADPKD phenotypes (Watnick *et al.*, 1999). A similar relationship between genotype and phenotype is also observed in *PKD2* (Hateboer *et al.*, 2000). Twenty-two *PKD2* mutations (exon 1-13) were identified in 24 unrelated families. A score based on the clinical severity was calculated for each individual and patients were grouped according to the location of their mutation. Although this study did show that the location of the mutation shared by family members influences phenotype, the involvement of additional factors is evidenced by the fact that two families (1 Spanish and 1 Dutch) with identical *PKD2* mutations displayed significantly different phenotypes (Hateboer *et al.*, 2000).

#### 1.7.1.3 Germline and Somatic Mutations

As previously discussed in section 1.6.1, a second hit mutation might be required and necessary for cyst formation and disease development. Indeed, mutation detection in cystic epithelial cells revealed germ-line as well as somatic mutations present in both the *PKD1* and *PKD2* genes (Qian *et al.*, 1996; Brasier and Henske, 1997; Koptides *et al.*, 1998, 1999). Since one mutated allele is already present in every cell throughout the kidney, the inactivation of the second allele would result in the alteration of PC1 and PC2 activity, respectively, in a subset of tubular epithelial cells (Qian *et al.*, 1996; Brasier and Henske, 1997; Koptides *et al.*, 1998). Consequently, the combined effect of a germline mutation and the nature and number of somatic mutations may result in a more severe ADPKD phenotype. It can be speculated that the occurrence of extrarenal manifestations including hepatic cysts, cerebral and aortic aneurysms and cardiac valvular disease might arise by this mechanism (Qian and Germino, 1997).

#### 1.7.1.4 Compound Trans-Heterozygous Mutations

Trans-heterozygosity is the inheritance of two mutations by the same individual. In ADPKD, compound trans-heterozygosity may result by the inheritance of two mutant alleles (for example, bilineal inheritance of both a *PKD1* and a *PKD2* mutation).

The first documentation of bilineal disease in ADPKD, arising from independently segregating *PKD1* and *PKD2* mutations in the same family, was reported by Pei *et al.* (2001). Of 28 affected individuals, 12 were found to have a *PKD2* mutation and 15 a *PKD1* mutation, while 2 affected individuals were compound trans-heterozygotes with mutations involving both genes. The 2 affected individuals with trans-heterozygous ADPKD mutations had a more severe clinical phenotype compared to ADPKD individuals who had either mutation alone. One mechanism that may explain the increased disease severity in the two individuals with trans-heterozygous mutations involves a “threshold” effect. This results from the interactions of two mutant proteins that are components of a multi-molecular complex or pathway. Therefore it is plausible that the interactions of the mutant proteins arising from the trans-heterozygous *PKD1* and *PKD2* mutations could lead to a reduction of the functional signalling complex below a “threshold”, which would predispose more cells to a cystic phenotype (Pei *et al.*, 2001).

Wu *et al.* (2000) generated a compound heterozygote mouse line with a targeted mutation in *Pkd1* and another in *Pkd2*. Their study showed that the compound heterozygote, with one mutant allele in both *Pkd1* and *Pkd2*, has a more severe cystic phenotype in the kidney than age-matched heterozygous *Pkd1* or *Pkd2* alone.

#### 1.7.1.5 Modifying Genes

Interaction between (mutations in) different genes may also cause phenotypic diversity. This type of variability in disease phenotype may be caused by modifier genes, which have been implicated in some disorders (Romeo and McKusick, 1994). For example, in a mouse model of familial polyposis coli, a modifying locus has been identified (*Mom-1*) (Dietrich *et al.*, 1993), and the risk of ovarian cancer in human subjects with *BRCA1* mutations appears to be modified by allelic variation at the *H-RAS* locus (Phelan *et al.*, 1996). Furthermore, in a large study of affected relative pairs with neurofibromatosis type 1, there was significant evidence for modifier effects on the number of café au lait spots and neurofibromas (Easton *et al.*, 1993).

In view of the importance of hypertension in ADPKD, genes involved in the pathogenesis and regulation of hypertension may modify the ADPKD phenotype. It has been reported that the coexistence of primary hypertension and ADPKD within families is associated with earlier age at onset of ESRF (Geberth *et al.*, 1995b). Moreover, the association between hypertension and renal disease progression suggests a role for hypertension in ADPKD (see section 1.4.2).

Hypertension is regarded as a complex trait (Ward, 1990) involving several different loci (Hilbert *et al.*, 1991). A large number of candidate loci are being studied and include components within the renin-angiotensin system (RAS), namely the angiotensin converting enzyme (ACE), angiotensinogen (AGT) and the renin genes. In humans, some studies have reported an association between an insertion/deletion (I/D) polymorphism in the *ACE* gene and renal disease progression in ADPKD (Baboolal *et al.*, 1997; Perez-Oller *et al.*, 1999). The effect of variation in the RAS genes is discussed extensively in Chapter Five.

The spontaneously hypertensive rat and the stroke-prone spontaneously hypertensive rat are useful models for the study of human hypertension. Hilbert *et al.* (1991) reported both autosomal and sex-linked genes in the development of hypertension. Linkage studies in crosses between the stroke-prone spontaneously hypertensive rat and the normotensive control strain Wistar-Kyoto have led to the localization of two genes, *BP/SP-1* and *BP/SP-2*, that contribute significantly to blood pressure variation in the F<sub>2</sub> population. *BP/SP-1* and *BP/SP-2* have been assigned to rat chromosomes 10 and X respectively. Comparison of the human and rat genetic maps indicates that *BP/SP-1* could reside on human chromosome 17q in a region that also contains the *ACE* gene (Hilbert *et al.*, 1991).

Experimental models of PKD have identified modifying genes not involving the RAS. Two modifier loci that strongly modulate the progression of the polycystic kidney phenotype in *pcy/pcy* mice have been observed (Woo *et al.*, 1997). The first locus (MOP1) maps to mouse chromosome 4 in a region that shows conserved synteny with the human chromosome region 9p22-q32. The second locus (MOP2) maps to mouse chromosome 16 in a region that shows conserved synteny with the human 3q13-28. MOP1 accounted for 36.7% of the phenotypic variance in 68 *pcy/pcy* with mild PKD, while MOP2 accounted for 46.8% of the phenotypic variance in 46 *pcy/pcy* with severe PKD (Woo *et al.*, 1997).

Mutations in the *Nek1* gene, a NIMA (never in mitosis A)-related kinase, were identified from a mouse model for PKD (Upadha *et al.*, 2000). *Nek1* has been previously cloned and characterised from a mouse erythroleukemia cDNA library. The *Nek1* gene encodes a 774 amino acid protein kinase that contains an N-terminal domain with homology to the catalytic domain of NIMA, a protein that controls the initiation of mitosis in *Aspergillus nidulans* (Letwin *et al.*, 1992). Two mutations, *kat* and *kat*<sup>2J</sup> were found to be responsible for the PKD phenotype. The mutation in *kat* was a partial deletion in the *Nek1* gene, whereas the mutation in *kat*<sup>2J</sup> was a single-base insertion that caused a frameshift and a premature termination codon. This finding revealed that a kinase-related gene, *Nek1*, was able to contribute to the cystic phenotype of the kidney and implies that *Nek1* may act as an additional signalling pathway to regulate renal epithelial proliferation and cystogenesis.

Mouse models of human disorders are potentially powerful tools for understanding heritable contributions to complex genetic traits. The availability of mutations on inbred genetic backgrounds, along with the development of extensive molecular and computational resources for genome-wide analysis, makes these systems ideal for genetic analysis (Kuida and Beier, 2000). The mapping of modifier genes and their eventual identification will help uncover factors that can delay disease progression and subsequently be used to design suitable modes of therapy for various forms of human PKD (Upadhyaya *et al.*, 1999).

#### 1.7.1.6 Genomic Imprinting

Genomic imprinting (*i.e.* differential expression of genetic material depending on whether it has been inherited from the mother or father) was clearly recognised by the mule breeders in Asia Minor more than 3000 years ago (Savory, 1970), however, formal demonstration of genomic imprinting was not achieved until 1991 when the selective maternal expression of *Igf2r*, the paternal expression of *Igf2* and the maternal expression of *H19* in mice were reported (Barlow *et al.*, 1991; Bartolomei *et al.*, 1991; DeChiara *et al.*, 1991). Since then, numerous genes have been shown to be subject to genomic imprinting. We now recognize several phenotypes in humans, mice and other animals, which show a pattern of inheritance consistent with the involvement of imprinted genes. Some examples of human imprinted genes include: *p73*, a putative tumour suppressor gene involved in neuroblastoma (Caron *et al.*, 1993; Kaghad *et al.*, 1997); *KvLQT1* (Lee *et al.*, 1997), *IPL* (Qian, *et al.*, 1997b) and *IMPT1* (Dao *et al.*, 1998) in the imprinted cluster of genes on 11p15; *necdin* (*NDN*) (MacDonald and Wevrick, 1997; Jay *et al.*, 1997; Watrin *et al.*, 1997) and *UBE3A* (Rougeulle *et al.*, 1997; Vu *et al.*, 1997) in the Prader-Willi/Angelman disease locus on 15q; and genes on the X chromosome (Chu *et al.*, 1994; Skuse *et al.*, 1997) which influence the phenotype of Turner syndrome. The study of murine genes also continues to provide a source of novel imprinted genes including that for neuronatin on chromosome 2 (Kagitani *et al.*, 1997; Kikyo *et al.*, 1997); *Grb10*, a candidate for growth retardation on chromosome 11 (Miyoshi *et al.*, 1998); the serotonin receptor 2a gene on chromosome 14 (Kato *et al.*, 1998) and *Impact* on chromosome 18 (Hagiwara *et al.*, 1997).

In ADPKD, some studies have observed a parent-of-origin effect on disease severity, consistent with genetic imprinting, however no conclusive evidence (for example by direct detection of parent-of-origin-specific transcription from a gene) has been documented. Bear *et al.* (1992) described a parent-of-origin effect on disease progression in 10 Newfoundland families with PKD1. They found that age at onset of ESRF was significantly earlier in persons inheriting the disease from their mothers than from their fathers (50.5 vs 64.8 years,  $p = 0.004$ ). Fick *et al.* (1994) and Zerres *et al.* (1993) revealed a statistically significant predominance of affected mothers transmitting the mutated gene in early manifesting ADPKD. Fick *et al.* (1994) also showed that in 65% of the 52 parent-offspring pairs with ESRD anticipation, the transmitting parent was the mother. Of 79 children (64 index patients and 15 affected sibs) with early onset ADPKD from 64 families, 41 mothers and 23 fathers had ADPKD. A significant deviation from the assumption of an equal sex ratio among the affected parents was found in this study (Zerres *et al.*, 1993). Conversely, Geberth *et al.* (1995a) showed a trend for later age at renal death in offspring with maternal transmission (Geberth *et al.*, 1995a). The above observations of a parent-of-origin effect in ADPKD disease severity suggest that genomic imprinting may play a role in disease progression. However, the mechanism of imprinting is complex and can vary between tissues and also during development. Therefore, further studies and conclusive evidence is warranted to suggest a role in ADPKD.

#### 1.7.1.7 Environmental Factors

Environmental factors should also be considered when discussing modifiers of disease severity. An example of this is  $\alpha$ -1 antitrypsin deficiency ( $\alpha$ -1 ATD), which is the most common genetic cause of emphysema yet identified (Cox, 1989). Although  $\alpha$ -1 ATD may cause emphysema regardless of the environment, the course of lung disease is strongly dependent on exposure to cigarette smoke (Larsson, 1978; Brantly *et al.*, 1988). Another major environmental modifying factor to be considered is diet. In some diseases, such as phenylketonuria (PKU) or galactokinase deficiency, the exclusion of certain foods containing phenylalanine or galactokinase from the diet prevents the onset of clinical symptoms (Rohr *et al.*, 1987; Hanley *et al.*, 1996).

In experimental models of ADPKD, numerous studies support the concept that dietary protein restriction and control of blood pressure delay progression of renal disease (Brenner, 1983; Klahr *et al.*, 1983; Keane *et al.*, 1989). Early studies in humans have also suggested that a restriction of dietary protein is beneficial, especially in patients with advanced renal disease (Mitch *et al.*, 1984; Rosman *et al.*, 1984; Hunsicker, 1989). Some of these studies were however inconclusive because of deficiencies in their design, for example changes in renal function were assessed only by measurements of serum creatinine, which may in itself be affected by diet. A subsequent major study where ADPKD patients formed the majority of subjects, the Modification of Diet in Renal Disease Study, was unable to show a clinically significant beneficial effect of dietary protein restriction (Klahr, 1996).

Additional environmental factors that may act as modifiers of disease severity in ADPKD include smoking and alcohol. Several studies have reported an association between smoking and heavy alcohol consumption and renal disease progression in diabetic populations as well as in ADPKD (Orth *et al.*, 1997,1998; Perneger *et al.*, 1999). The relationship between environmental factors and ADPKD is further discussed in Chapter Six.

#### **1.7.1.8 Other Factors**

Several studies have reported that renal disease progression is less aggressive in women than men (Dalgaard, 1957; Gretz *et al.*, 1989; Gabow *et al.*, 1992b). In men, the occurrence of UTI appears to worsen the course of renal disease (Gabow *et al.*, 1992b).

In regard to the relationship between hypertension and ADPKD disease progression, the presence of hypertension in the non-affected parent may influence disease progression in ADPKD individuals. Geberth *et al.* (1995b) showed that the coexistence of ADPKD and genetic predisposition to primary hypertension is associated with an earlier onset of terminal renal failure in families with ADPKD.

The occurrence of hepatic cysts and the effect of three or more pregnancies have been shown to be independently associated with worse renal function in women (Gabow *et al.*, 1992b). The incidence of UTI has been shown to be associated with poorer renal



function in men, and early age at diagnosis, gross haematuria and increased renal size (expressed as mean renal volume) with poorer renal function in both males and females (Gabow *et al.*, 1992b).

#### **1.7.1.9 Inter-population differences**

Additional factors that may influence disease outcome but have not been addressed to date, include inter-population differences. Inter-population differences can be classified as biological (for example PKD locus and mutation heterogeneity, as well as differences in genetic background) and environmental variables (socioeconomic position, health care, diet and lifestyle) and differences in ascertainment. Some of these factors are significant in determining patterns of population health. Inter-population differences will be discussed in Chapter Three, which aims to compare ADPKD clinical findings between individuals from three diverse ethnic backgrounds.

## **1.8 GENETIC COUNSELLING AND DIAGNOSTIC TESTING**

In ADPKD, there are two main types of diagnostic testing. These include ultrasound and genetic diagnosis.

### **1.8.1 Ultrasound Diagnosis**

Imaging studies including ultrasound, computer tomography (CT) and magnetic resonance imaging (MRI) are the mainstay for clinical diagnosis. However, ultrasound is the most widely used because it is safe, relatively inexpensive, non-invasive and provides reproducible results. A positive ADPKD diagnosis by ultrasound is definitive, whereas a negative finding must be interpreted in the context of the patient's age. Ultrasonographic criteria for PKD1 have been established as the presence of two cysts, either unilateral or bilateral, in individuals younger than 30 years, two cysts in each kidney in individuals aged between 30 and 59 years, and at least four cysts in each kidney in individuals aged above 60 years (Ravine *et al.*, 1994). Ultrasound evidence of a single renal cyst in a child at-risk due to family history is strongly suggestive of ADPKD. In non-PKD1 families, similar diagnostic guidelines have also been established (Bear *et al.*, 1992; Demetriou *et al.*, 2000). The general consensus is that over the age of 30, a negative, good quality ultrasound, not fulfilling the diagnostic

criteria of at least two renal cysts bilaterally, is 100% reliable in excluding PKD2 (Demetriou *et al.*, 2000).

### **1.8.2            *Genetic Diagnosis***

Since the discovery of loci involved in ADPKD, genetic linkage analysis has provided an alternative approach to diagnosis as well as a tool for investigators to compare the phenotype between the different genetic forms (Kimberling *et al.*, 1991). This method uses an array of DNA polymorphisms for known chromosomes, which are tested in multigenerational families who have several individuals with the characteristic phenotype. If a particular polymorphic allele is transmitted from parent to child in association with the ADPKD phenotype, this suggests that the gene of interest must be inherited with that DNA polymorphism and hence is located on the same chromosome (Kimberling *et al.*, 1991).

The availability of this method of diagnosis has raised the question of whether at-risk individuals should be tested by this method. Essentially, genetic linkage analysis should only be performed as part of the overall care and in combination with sound genetic counselling. Linkage analysis may be particularly helpful in defining the genetic status of young unaffected relatives of ADPKD patients, who are potential transplant donors, and for individuals whose family planning would be strongly influenced by such knowledge (Gabow, 1990).

Although genetic linkage analysis is available, there is limited information regarding patients' attitudes about its use. Sujansky *et al.* (1990) revealed that 97% of at-risk subjects stated that they would use the test to define their own status. Similarly, 88% said they would use the test to define the status of their offspring, but only 4% of affected individuals stated that they would abort a fetus with the ADPKD genotype (Sujansky *et al.*, 1990).

### **1.8.3            *Implications of Diagnostic Testing***

As reported in the literature (Kimberling *et al.*, 1991), diagnostic testing has important implications especially in asymptomatic patients. The first point concerns the advisability of presymptomatic diagnosis. In families with a strong family history of

ICA, it is advisable for an individual to be tested for the presence of ADPKD. It is also advisable for an ADPKD relative (50% risk) who has the potential of being a transplant donor to be tested for ADPKD. Secondly, presymptomatic diagnosis provides an opportunity for an affected individual to carefully monitor and treat high blood pressure and other modifiable clinical manifestations associated with ADPKD. However, presymptomatic diagnosis using genetic linkage methods may have psychological implications on family members and may also introduce health insurance issues.

## **1.9 SUMMARY OF THE LITERATURE REVIEW**

The literature review has provided a descriptive picture of the clinical and genetic aspects of ADPKD, and factors that may modify the phenotype. Despite the progress in ADPKD research, the factors that are involved in modifying ADPKD progression remain unclear. Therefore the central aim of this thesis was to investigate some plausible genetic and non-genetic factors that may influence the ADPKD phenotype. The specific research aims are detailed below.

- 1) Patients from three geographical locations were recruited to increase the power of the study and used to examine differences in ADPKD severity between countries;
- 2) A large joint clinical/genetic/environmental database was constructed with the purpose of grading the phenotype based on a number of clinical parameters including extent of renal involvement and hypertension;
- 3) Possible modifiers of phenotype severity were investigated by:
  - i) performing genetic linkage analysis and comparing the clinical severity between the different genetic forms of ADPKD;
  - ii) analysing genetic variants within the renin-angiotensin system (RAS) including the angiotensin converting enzyme (ACE) insertion/deletion (I/D) polymorphism;
  - iii) examining the influence of environmental factors including smoking, alcohol, dietary lipids and physical activity on the ADPKD phenotype.

- 4) The overall importance of genetic factors was assessed in a study of affected sib, parent-offspring and cousin pairs using analysis of variance and multivariate component modelling.

To address and achieve the multiple aims of this thesis, several methods were employed and performed by various individuals with specific expertise. The subsequent chapter (Chapter Two) describes the recruitment phase of the project and the methodologies employed. Chapters Three, Four, Five and Six investigate some of the plausible genetic and non-genetic factors as possible modifiers of ADPKD severity. The study on the overall assessment of genetic and environmental factors to the ADPKD phenotype is examined in Chapter Seven and a discussion on the overall findings from this thesis is presented in Chapter Eight.

# CHAPTER TWO: GENERAL METHODS

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## CHAPTER TWO: GENERAL METHODS

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This chapter describes the general epidemiological, molecular, data management and statistical methods used in this thesis.

### 2.1 RESEARCH DESIGN

A critical element in any study is to target a specific population(s) to be investigated. In order to investigate the epidemiological factors involved in a disease, a population-based sample is generally required. By contrast, genetic studies generally require highly selected families, enriched for certain phenotypic traits, in order to reduce the effect of heterogeneity and phenocopies, and to provide sufficient sample sizes to detect genotype-phenotype associations.

In this study, both epidemiological and genetic aspects of ADPKD were examined. As progression of ADPKD is closely associated with hypertension and renal impairment, data on cardiovascular mortality and risk factors will also be discussed throughout the course of this thesis.

### 2.2 SOURCES OF DATA

The index populations studied came from three geographic locations: Western Australia, Bulgaria and Poland (Figures 2.1, 2.3, 2.4). The age range of the population was 10-86 years. The criteria for inclusion in the study for index cases and relatives were based on a clinical diagnosis of ADPKD by ultrasonography (described in section 2.3.1).

#### 2.2.1 *The Australian Sample*

Geographically, Australia is the world's sixth largest country and has a total area of 7 682 292 km<sup>2</sup>. The overall population in 1999 was 18 338 000 people (roughly 0.3% of the world's population). In this study, the families were recruited from Western Australia (WA) which has a land area of approximately 2.5 million km<sup>2</sup> (33% of the area of Australia) and a population of approximately 1.5 million (Australian Bureau of Statistics - [www.abs.gov.au](http://www.abs.gov.au)). The population of WA is concentrated in the Perth



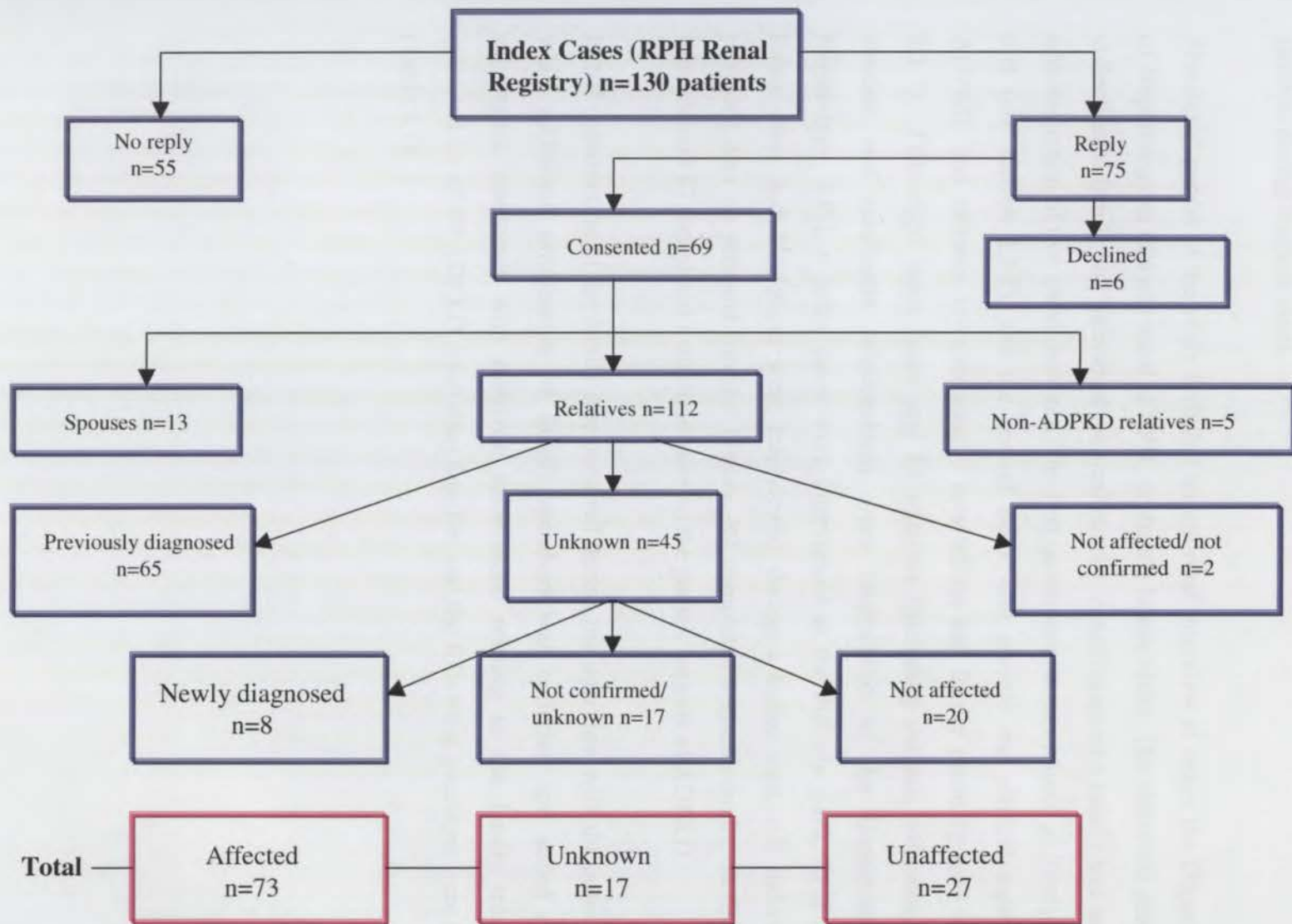
metropolitan area (population approximately 1.3 million). A map of Australia is shown in Figure 2.1.

The overall life expectancy for men in Australia is 77 and for women 82 years. According to recent mortality data reported by the Global Cardiovascular Infobase (<http://www.americanheart.org/statistics/biostats/bioin.htm>), cardiovascular disease (CVD) represents approximately 43% of all deaths in Australia. Mortality due to CVD can be broken down into ischaemic heart disease (IHD) (56%), cerebrovascular diseases (23%), hypertensive diseases (2%), rheumatic heart disease (1%) and other CVD (18%).

A total of 69 Western Australian ADPKD families (126 affected individuals) from diverse ethnic backgrounds were represented in this study. Forty-seven families (69%) were of British descent, while the remaining 22 families included 6 Asian (9%), 2 Macedonian (3%), 2 Polish (3%), 2 German (3%), 1 Spanish (1%), 1 Italian (1%), 1 Irish (1%) and 7 (10%) where the ethnic origin was not known.

Recruitment began with an invitation package consisting of an invitation letter, an information sheet about ADPKD and the study, and a consent form (see Appendices 1, 2 and 3). The invitation packages were sent to 130 index patients (defined as the first case of a disease) selected through the Royal Perth Hospital (RPH) Renal Registry (see Figure 2.2). A total of 75 (58%) individuals replied: 50 by telephone and 25 by mail, and 69 index cases and their families participated in the study. In 3 cases, the spouse of a deceased index case responded by phone. A further 3 cases replied asking for further information but decided to refrain from taking part in the study. Of the remaining 55 packages, 10 individuals were lost to follow-up, 6 packages were returned by post because of incorrect addresses and the remaining 39 did not respond. No information was obtained on the reasons why all the patients failed to participate, however, the extensive involvement of the study examinations may have deterred families from participating. However, it must be pointed out that data on the 75 individuals who responded versus the 55 who didn't are representative of the 130 individuals invited to participate in the study. As demonstrated throughout this thesis, the affected individuals represented a range of ADPKD clinical severity with and without known family histories of ADPKD. Similarly, the families who did not participate also comprised a





**Figure 2.2:** The Recruitment Process and ADPKD Status of Australian Index Cases and Relatives Participating in the ADPKD Research Project

range of clinical severity and with some families also demonstrating a known family history of ADPKD as determined by hospital records and contact with some of the families during hospital visits.

The initial period of the study included an informal interview at either the Department of Nephrology or dialysis ward at RPH, or during house visits. The interview involved collection of family and individual medical history, construction of a family tree and the administration of two questionnaires. The first questionnaire was related to lifestyle and diet (see section 2.3.7), and the second dealt with genetic and clinical aspects of ADPKD, and attitudes towards genetic counselling and family planning (see section 2.3.7). Fifty-eight index cases and 83 relatives (including parents, offspring, sibs, cousins and aunt/uncle relationships) were interviewed at the Department of Nephrology, RPH, 6 index cases were interviewed at the dialysis ward, RPH and 5 index cases and 2 relatives were interviewed during a house visit. All individuals provided data on personal medical history, 89 completed a questionnaire on diet and lifestyle and 87 completed a questionnaire on the genetic aspects of ADPKD.

Initial construction of the family tree occurred during the interview with the index case, while additional information on family members was collected and added during subsequent interviews with relatives. Information relating to the family tree was collected on all 69 ADPKD Australian families and the following questions were asked (Table 2.1).

**Table 2.1: Questions Relating to the Collection of Family Tree Data**

PKD status	Question	
<b>Affected Individual</b>	1)	What age or year was ADPKD diagnosed?
	2)	Has individual reached CRF or ESRF or required a transplant?
	3)	Does individual suffer from HBP? If yes, when was HBP diagnosed, is individual on treatment and when was treatment commenced?
	4)	Does the individual suffer from diabetes?
	5)	Has the individual suffered from a brain stroke? If yes, age and number of strokes and does brain stroke coincide with ADPKD?
	6)	Has the individual had cancer? If yes, where and when?
	7)	Does the individual suffer from any other conditions or complications ?
<b>Unaffected/Unknown Status</b>	8)	Has individual been tested by ultrasound for ADPKD? If yes, when? and what age was last ultrasound performed?
	9)	Questions 2-7 repeated
<b>Deceased</b>	10)	Questions 1-7 repeated
	11)	What was the cause of death?
	12)	At what age or year did he/she die?

During the interview, individuals were asked if they would contribute further to the study by undergoing various clinical examinations, and permission was also obtained to collect information from general practitioners and other specialists. The following clinical investigations were performed: physical examination, biochemical analysis, ultrasound examination/review, blood pressure assessment and echocardiography. These are discussed in Section 2.3.

### **2.2.2 The Bulgarian Sample**

The second sample of ADPKD individuals involved in this study originated from Bulgaria, which is located in Eastern Europe (see Figure 2.3). The population in 1995 was 8,774,000 million people. Approximately 71% of the population live in urban areas. According to the Global Cardiovascular Infobase (<http://cvdinfobase.ic.gc.ca/>), the overall life expectancy in Bulgaria is 68 for males and 75 years for females. In contrast to Australia (43%), the mortality data show that cardiovascular disease contributes to 62% of all deaths in Bulgaria. This consists of IHD (33%), cerebrovascular disease (33%), hypertensive heart diseases (5%), chronic rheumatic heart diseases (0.5%) and other CVD diseases (29%).

A total of 32 Bulgarian ADPKD families (128 affected individuals) were recruited and gave informed consent. The recruitment process and assessment of the Bulgarian



**Figure 2.3:** Map of Bulgaria. This figure shows the two cities (Pleven and Plovdiv) where the Bulgarian index patients were ascertained.  
Source: <http://www.nationalgeographic.com/xpeditions/atlas/>

ADPKD population was carried out by a team from the cities of Plovdiv and Pleven which included: Dr Nadja Bogdanova, Dr Vassil Todorov and Dr Dimitar Dimitrakov.

Data and DNA samples from this population were made available to the author for further analysis. Clinical information including serum creatinine (SCr) measurements, blood pressure, ultrasound data and clinical presentation is discussed in section 2.3.

### **2.2.3            *The Polish Sample***

The third sample of ADPKD individuals originated from Poland, which is also located in Eastern Europe (see Figure 2.4) and has a population of approximately 38,388,000 million people (1995). The overall life expectancy for Polish individuals is similar to that seen in Bulgaria, being 68 years for males and 77 for females. Half of the overall deaths are caused by CVD and consist of the following: IHD (20%), cerebrovascular diseases (15%), hypertensive diseases (4%), rheumatic heart diseases (1%) and other CVD (60%).

A total of 16 Polish ADPKD families (70 affected individuals) were recruited and gave informed consent. The recruitment and assessment of the Polish ADPKD population were carried out by a team from the city of Krakow, which included: Dr Piotr Jasik, Prof. Wladyslaw Sulowicz, Dr Andrzej Krasniak and Dr Tomasz Stompor.

Data and DNA samples from this population were made available to the author for further analysis. Clinical information including SCr measurements, blood pressure, ultrasound data and clinical presentation is discussed in section 2.3.

## **2.3            CLINICAL INVESTIGATIONS**

A number of clinical and laboratory parameters were assessed in an attempt to grade the ADPKD phenotype. These are outlined below and in subsequent chapters.

### **2.3.1            *Ultrasound Investigations***

Ultrasound investigations were performed or reviewed to establish the diagnosis of ADPKD and to assess the extent of renal involvement.



**Figure 2.4:** Map of Poland. *This figure shows the city of Krakow where the Polish index patients were ascertained.*

Source: <http://www.nationalgeographic.com/xpeditions/atlas/>

### **2.3.1.1 Ultrasonographic diagnosis**

Eligibility of subjects for this study was based on standard age-stratified ultrasonographic criteria (Ravine *et al.*, 1994). The majority of scans were performed and interpreted by a single radiologist at each of the representing cities (WA, Bulgaria and Poland). It must be pointed out that no standard protocol (*i.e.* ultrasonographic protocol) for the assessment of the kidneys and presence of extra-renal cysts was used across the three representing centres. Therefore, any significant differences in kidney size, number and size of extra-renal cysts may be the product of differences in assessment.

Patients were divided into two groups: 1) index cases and 2) relatives. Group two was further divided into the following sub-groups based on ADPKD status, as defined by age adjusted ultrasonographic criteria: a) previously diagnosed; b) newly diagnosed; c) not confirmed or unknown and d) not affected. The “not confirmed” category included subjects whose scan was negative, however because of the age of the individual, a positive diagnosis could not be excluded. These individuals were informed about the age-related penetrance of the condition and were advised to repeat the ultrasound every couple of years or undertake gene testing, which could provide a definitive diagnosis of ADPKD.

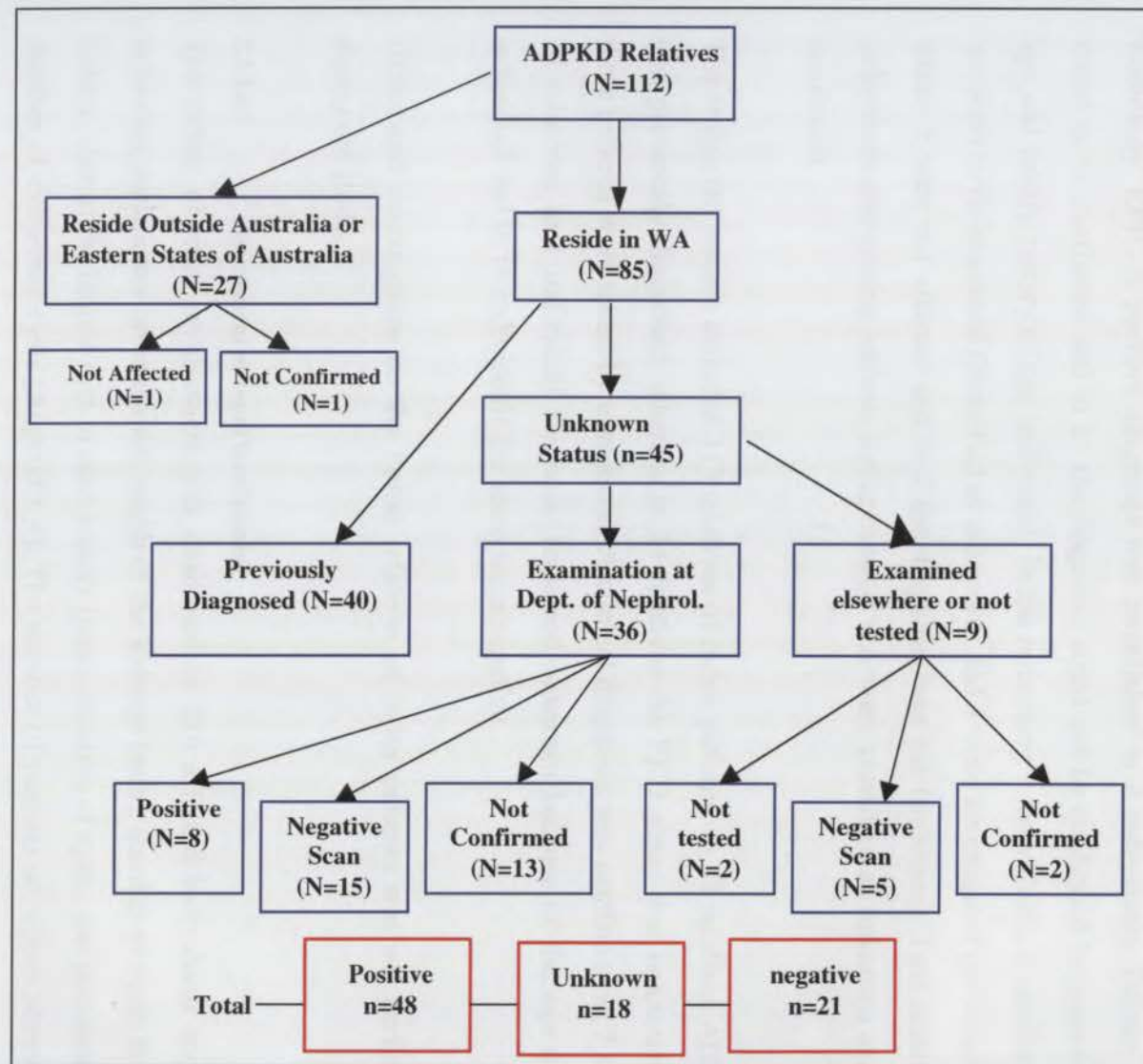
### **2.3.1.2 Index cases**

This group consisted of individuals who were initially designated as the index case (*i.e.* the first member of the family diagnosed with ADPKD) and identified through the renal registries at one of the representing centres. This included 69 from Australia, 32 from Bulgaria and 16 from Poland.

### **2.3.1.3 ADPKD relatives**

In the Australian sample, 112 ADPKD relatives (*i.e.* 50% pre-test probability) were further classified into the following sub-groups based on ADPKD status: a) previously diagnosed as having ADPKD; b) newly diagnosed with ADPKD; c) not confirmed or unknown and: d) not affected (Figure 2.5). The number of previously diagnosed Australian relatives was 65, newly diagnosed 8, not confirmed 16, unknown 2 and not affected 21.





**Figure 2.5:** Ultrasound Examination and Results in Previously Undiagnosed Australian ADPKD Relatives



Eighty-five of the 112 ADPKD relatives (76%) resided in WA and 27 (24%) lived in England, South Africa, USA and the East Coast of Australia. The diagnosis of ADPKD was previously established in 40 of the 85 WA residents (47%) and in 25 of the 27 (93%) residing outside WA. In the other 45 WA subjects and 2 outside WA, the diagnosis was either not confirmed or unknown. An ultrasound examination was performed on 36 of these individuals by Dr Steve Melsom at the Department of Radiology, RPH. A positive diagnosis was established in 8 individuals. Fifteen were found to be unaffected and in 13 the diagnosis could not be confirmed because of the age and family history of the individual. In the remaining 9 individuals, 2 subjects did not want to be examined (classified as unknown) and 7 were investigated previously, of whom 5 were not affected and in 2 the diagnosis was not confirmed. Two remaining individuals comprising patients living outside WA were classified as unknown and not confirmed.

The Bulgarian sample included 113 relatives of index patients. In 89 relatives ADPKD was previously diagnosed, whereas in 24 relatives the PKD status was unknown and these patients were tested by ultrasound. A clinical diagnosis was confirmed in 7 of the 24 relatives, in 1 individual diagnosis remained unknown because of the age of the individual and in 16 individuals the scans were negative.

Ultrasound investigation on the Polish relatives of index patients was not performed during the study.

**2.3.1.4            Assessment of renal involvement**

The extent of renal involvement was examined in 216 affected individuals and the following parameters investigated: length of the kidneys (mm), number of cysts in each kidney, size of the largest cyst in each kidney (mm), location of cysts, and presence and number of extrarenal cysts (Appendix 4). The number of patients on whom ultrasound information was available is shown in Table 2.2.

**Table 2.2: The Number of Patients with Available Ultrasound Information**

<b>Ultrasound parameters examined</b>	<b>Australia (N=126)</b>	<b>Bulgaria (N=128)</b>	<b>Poland (N=70)</b>
Length R kidney	76	44	69
Length of L kidney	76	44	69
number of cysts R	67	44	69
number of cysts L	67	44	69
Size of large cyst R	69	27	69
Size of large cyst L	69	27	69
Size of small cyst R	10	Not examined	Not examined
Size of small cyst L	10	Not examined	Not examined
<b>Location of cysts:</b>			
R kidney	80	36	59
L kidney	80	36	59
<b>Extrarenal cysts:</b>			
<b>Liver:</b>			
Number of liver cysts	95	52	69
<b>Pancreas:</b>			
Number of pancreatic cysts	95	52	69
<b>Spleen:</b>			
Number of splenic cysts	95	52	69
<b>Ovaries:</b>			
Number of ovarian cysts	95	52	69
Nephrectomy	95	52	69

### **2.3.2 Biochemical Investigations**

Urine samples and peripheral whole blood were collected from patients residing in WA. The following biochemical tests were performed at the Department of Biochemistry, RPH: spot urine (sodium, potassium, protein and creatinine), urea, electrolyte and bicarbonate (UEB), total serum cholesterol (Chol), total serum triglycerides, aldosterone and plasma renin activity (PRA). In addition, analysis of angiotensin converting enzyme

(ACE) activity was performed at the Department of Pathology, Sir Charles Gairdner Hospital. In this study, only serum creatinine (SCr), total serum cholesterol, total serum triglycerides and ACE activity levels were used for analysis. The biochemical techniques are described below. Additional data on these variables were collected via hospital records at RPH and from general practitioners.

1) SCr was measured in 126 Australian subjects. The reaction involved creatinine with alkaline picrate to form a red chromophore (rate of production is proportional to the concentration of creatinine in the sample), with a normal reference range between 50-120  $\mu\text{mol/L}$ . The investigations were performed at the Department of Biochemistry, Royal Perth Hospital, UWA.

2) Total serum cholesterol was measured in 59 Australian subjects. Serum cholesterol was detected by the enzymatic (cholesterol esterase/cholesterol oxidase) method, with a reference range  $<5.5 \text{ mmol/L}$  (covariance 2.0% at 3.6 mmol/L and 1.7% at 7.4 mmol/L). The reference range is in accordance with the National Heart Foundation guidelines (<http://www.heartfoundation.com.au>). The investigations were performed at the Department of Biochemistry, Royal Perth Hospital, UWA.

3) Serum triglycerides were measured in 37 Australian subjects. Total serum triglycerides was detected by the enzymatic (lipase/glycerol kinase) method, with a reference range  $<1.8 \text{ mmol/L}$  (covariance 2.0% at 3.6 mmol/L and 2.5% at 1.2 mmol/L). The reference range is in accordance with the National Heart Foundation guidelines (<http://www.heartfoundation.com.au>). The investigations were performed at the Department of Biochemistry, Royal Perth Hospital, UWA.

4) Plasma angiotensin converting enzyme activity was measured in 92 Australian patients. The investigations were performed at the Department of Pathology, Sir Charles Gairdner Hospital, UWA. Plasma ACE activity was determined using the kinetic method of Buttery and Stuart (1993), with a reference range of 23-100 U/L (covariance 3.2% at 125 U/L and 8.1% at 31 U/L).

Total serum cholesterol and triglyceride measurements were available on 54 Polish patients and were subsequently used to investigate the relationship between environmental determinants and the ADPKD phenotype (see Chapter Six).

**2.3.3            *Kidney Function***

Creatinine measurements in plasma and urine have traditionally been employed to assess chronic renal failure (CRF) (the initial stage of renal impairment) and end-stage renal failure (ESRF), as well as the rate of progression to renal failure (Walser *et al.*, 1988). The most important clinical outcome in ADPKD is the development of ESRF and the assessment of severity is based on this parameter and factors that may accelerate the progression. Therefore a crucial element in this study was to collect serial SCr measurements to examine both CRF and ESRF. CRF was defined as SCr value of 150 µmol/L and ESRF was defined as the point at which a person required dialysis or renal transplant. An arbitrary SCr value of 800 µmol/L was chosen as a reliable indicator of ESRF if the above information was not available. The limitations of defining CRF as SCr values of 150 µmol/L were discussed with several Consultant Nephrologists from various teaching hospitals in Western Australia. A cut-off point of SCr 150 µmol/L (rather than a GFR of <90 [=mild CRF] or <60 [=moderate] mls/min) was set because calculations of GFR were impossible for many individuals included in the study, due to missing height and weight data. Furthermore, a SCr concentration of 150 µmol/L is clearly elevated, thus eliminating the chance of any false positive diagnoses of CRF, however potentially missing some early CRF patients (especially the elderly, females and individuals with low muscle mass). In addition, because of the wide range in age (10 – 86 years) and therefore in the stage (in regard to renal function) of ADPKD, few values were available for some individuals. For statistical purposes, the age at CRF was estimated by extrapolation methods to increase the power of the study (see section 2.7).

The number of available serial SCr measurements is outlined in Table 2.3.

**Table 2.3: The Number of Individuals with Serum Creatinine Measurements by Country**

SCr values	Australia (N=126)	Bulgaria (N=128)	Poland (N=70)
1 value	22	7	9
≥2 values	104	121	61

2.3.4                    **Blood Pressure (BP) Assessment**

2.3.4.1                **Serial blood pressure measurements**

Blood pressure (BP) is a major factor risk factor in the progression of kidney disease. Information on BP values was available on 118 Australian, 76 Bulgarian and 70 Polish ADPKD individuals (Table 2.4). Hypertension was defined according to the World Health Organisation criteria (Chalmers *et al.*, 1999) as  $\geq 140$  mmHg systolic or  $\geq 90$  mmHg diastolic blood pressure. Information on antihypertensive medication was also collected and adjusted for in statistical analyses.

**Table 2.4: The Number of Individuals with Blood Pressure Data and on Antihypertensive Medication by Country**

<b>Blood pressure measurements</b>	<b>Australia (N=118)</b>	<b>Bulgaria (N=76)</b>	<b>Poland (N=70)</b>
Number with hypertension	86 (73%)	67 (88%)	43 (61%)
ACE inhibitor treatment	54 (63%)	Not known	41 (95%)

Age at onset of hypertension was defined as the age at which a patient had a blood pressure  $>140$  or  $90$  mmHg (systolic and diastolic respectively) or the age at which anti-hypertensive treatment was commenced as indicated by a clinician on the hospital records or in the clinical protocol.

2.3.5                    **Echocardiography**

Echocardiography could be performed on 86 Western Australian affected individuals to detect an increase in left ventricular mass (LVM) suggestive of left ventricular hypertrophy, as an indication of hypertensive target organ damage. Only 73 patients with adequate LVM data were included in the analysis.

Echocardiography involves three methods. A combination of M-mode and two dimensional echocardiography used to measure left ventricular size, wall thickness and function, and Doppler echocardiography used to detect blood flow velocity and turbulence. All echocardiograms were performed by the Department of Cardiology, RPH.

### 2.3.6 *Physical Examination*

Physical examination was performed on 57 WA ADPKD subjects at the Department of Nephrology, RPH. A standard study protocol (see Appendix A4) collected information on clinic blood pressure, palpable organomegaly (liver, spleen and kidneys), oedema, heart, bone deformities and herniae (see Table 2.5). Clinic blood pressure was recorded by sphygmomanometer, in a relaxed seated position, using Korotkoff phase IV for diastolic pressure. The numbers in Table 2.5 vary and indicate that in some individuals the parameter was not examined by the clinician.

**Table 2.5: Parameters Examined during Medical Examination in 57 WA ADPKD Subjects**

Physical parameters examined and number of ADPKD individuals with adequate information								
Clinic BP	Liver	Spleen	Right Kidney	Left Kidney	Oedema	Heart	Hernia	Bone
N=55	N=49	N=55	N=53	N=52	N=55	N=55	N=55	N=55

### 2.3.7 *Questionnaire-Based Parameters*

Two questionnaires: 1) diet and lifestyle (Appendix 5) and 2) genetic information (Appendix 6), were administered to individuals from Australia and Poland only. Ascertainment of the Bulgarian families and the collection of samples were performed by Dr Nadja Bogdanova (who resides in Germany), Dr Vassil Todorov and Dr Dimitar Dimitrakov prior to the Australian study. Consequently, due to time and distance the questionnaires were not administered to the Bulgarian sample. Information on diet and lifestyle factors collected with the first questionnaire, was used to investigate the relationship between recognised environmental determinants (*i.e.* smoking, alcohol, lipids and physical activity) and renal disease progression in ADPKD. The second questionnaire aimed to assess individual understanding of the genetic nature of ADPKD and attitudes towards genetic counselling and family planning options.

Eighty-nine affected Australian and 67 affected Polish patients completed the diet and lifestyle questionnaire and 87 affected Australian and 67 affected Polish individuals completed the genetic questionnaire (see Table 2.6). The results of the diet and lifestyle questionnaire are discussed in Chapter Six. Information collected from the genetic questionnaire is not discussed in this thesis.

**Table 2.6: Administration of Questionnaires**

<b>Diet and lifestyle questionnaire</b>	<b>Number of participating individuals</b>	
	<b>Australia</b>	<b>Poland</b>
Number of affected individuals	89	67
Number of unaffected relatives	0	0
<b>Genetic questionnaire</b>		
Number of affected individuals	87	67
Number of unaffected relatives	12	0
Number of patients referred for genetic counselling	1	0

### **2.3.7.1 Lifestyle questionnaire**

The questionnaire collected four categories of information: (1) smoking history, (2) alcohol consumption; (3) physical activity; and (4) dietary lipid intake.

#### *Questions related to physical activity*

Questions 1-7 related to the type of exercise and amount of physical activity (hours/week). Activity was classified by energy requirements, expressed in terms of metabolic equivalent tasks (METs). The MET, or ratio of working metabolic rate/resting metabolic rate, is a convenient method of expressing energy expenditure that is independent of body size or sex. The MET values were based on a compendium by Ainsworth *et al.* (1993). The questionnaire used in this study did not allow for recall of activity pace (*i.e* speed; rate of progression), therefore the general (average moderate intensity) MET value was chosen for each activity based on the Ainsworth *et al.* (1993) compendium. Different types of physical activity were recorded and coded accordingly (Appendix A.6). We multiplied the intensity of each activity (defined in metabolic equivalents [MET]) by duration. If an individual left blank one or more activities, we assumed the duration to be zero. The product of each activity was summed to give a total activity score in MET\*hours per week. Subjects were classified into three activity groups based on MET values and hours of exercise per week. The three groups were: 1) moderate-vigorous; 2) light-moderate and 3) low physical activity.

#### *Questions related to smoking*

Questions 8-14 included detailed information about the type of tobacco consumption (cigarettes, cigars, pipe) and the amount of tobacco consumed. The age at commencement and the duration of smoking were also recorded using categorical variables, which did not allow an assessment of changes in smoking habits over time to permit accurate calculation of pack years (PY). The categories for age at commencement of smoking included: 1) <16 years; 2) 16-30 years; 3) 31-50 years; 4) >51 years; and 5) not applicable. For duration of smoking, the categories included 1) <1 year; 2) 1-5 years; 3) 5-10 years; 4) 10-20 years; 5) >20 years; 6) not applicable.

A cigarette smoker was defined as a person who was regularly smoking one or more cigarettes per day at the time of the interview. A former smoker was considered an individual who quit smoking before the interview and a non-smoker was considered a person who had never smoked one or more cigarettes per day.

To examine the relationship between smoking and ADPKD phenotype severity, the following three variables were used: (1) non-smokers and smokers; (2) duration of smoking: non-smoker, ≤20 years, >20 years; (3) daily number of cigarettes: non-smoker, ≤20, >20 cigarettes per day (in past and current smokers).

#### *Questions related to alcohol consumption*

Three questions related to alcohol consumption, which collected two categories of information: (1) frequency of beer, wine and spirits and intake (2) maximum consumption of alcohol during any three-month period. The amount of alcohol consumed was measured in mls of alcohol per week and calculated using the following formula:

$$\text{ethanol (mls)} = \% \text{ content}/100 \times \text{volume (mls)}$$

The average concentration of ethanol for beer (3.1%), wine (11%) and spirits (41%) were used in the calculations. The common volume measures for beer, wine and spirits are shown in Table 2.7.



**Table 2.7: Common Volume Measures**

Alcohol type	Alcohol measure	Volume (mls)
Beer	bottle	750
	can	375
	glass	200
	jug	1140
	midi	285-300
	poni	100-140
	pot	600
	schooner	375
	stubby	375
White wine	glass	85-110
	bottle	750
	flagon	2000
Red wine	glass (small)	85
	glass (large)	110-150
Fortified wines	small glass	85
Spirits and liquors	nip or small glass	30
Ciders	Stubby	375

Patients were divided into four alcohol groups: (1) non-drinker (<15 mls per week), (2) light drinker ( $\geq 15$ -70mls per week for females;  $\geq 15$ -210mls for males per week), (3) moderate drinker (70-140mls per week for females; 210-280mls per week for males) and (4) heavy drinker (>140mls per week for females; >280mls per week for males) (see Table 2.8). However, for statistical purposes, groups 2 and 3 were pooled.

**Table 2.8: Classification of the Four Alcohol Consumption Groups Used in This Study**

Drinker/group	mls ETOH p/week	Sex allowance
Group 1 - non-drinker	<15 mls	F and M
Group 2 - light	$\geq 15$ -70mls	F
	$\geq 15$ -210 mls	M
Group 3 - moderate	70-140 mls	F
	210-280 mls	M
Group 4 - heavy	>140 mls	F
	>280 mls	M

*1 glass beer = 6.2 ml/EtOH; 1 glass wine = 9.35 ml/EtOH; 1 glass spirit = 12.3 ml/EtOH*

### 2.3.7.2 The dietary questionnaire

The dietary questionnaire comprised 8 questions related to lipid intake (saturated fat, monounsaturated fat, polyunsaturated fat). The aim of the questionnaire was designed to examine the relationship between dietary lipid intake and ADPKD disease severity. Each question in the dietary and lifestyle questionnaire was given a score. For example,

a score of 2 indicated a high level of saturated fat and a score of 0 indicated no saturated fat intake. The scores for each question were summed to give a dietary index score reflecting the total amount of fat consumed in the diet. Subsequently, an arbitrary value or cut-off score, based on low to medium (<4) or medium to high (>4) intake of fat, was used to categorise patients according to the total level of saturated fat in their diet. This value was based on discussion with a dietician at the Department of Nutrition, Health and Biomedical Sciences at Edith Cowan University. The scores (or values) for each question are shown in Appendix 5.

### **2.3.8            *Other Sources of Retrieval of Clinical Data***

In order to collect as much clinical data as possible, two additional methods were employed for the Australian sample. These included information collected from GP's and hospital records. For the Polish and Bulgarian samples, clinical data were collected from hospital records by the clinicians who had taken care of the patients.

#### **2.3.8.1            Clinicians**

Seventy letters were written and mailed to clinicians requesting specific information on both index cases and affected relatives (refer to Appendix 7).

#### **2.3.8.2            Hospital records**

Additional clinical information was retrieved on 69 index cases and 30 affected ADPKD relatives through the hospital records at RPH.

## **2.4            ETHICAL CONSIDERATIONS**

All procedures were safe and routinely used in the participating hospitals. Medically qualified personnel took all blood samples and performed clinical examinations. Informed personal or parental consent was obtained for all subjects, and all individuals were advised that they could withdraw from the study at any time without prejudice to further care. Subjects were advised to contact their local doctor or renal physician for individualised assessment of the clinical significance of any findings. Psychological counselling or genetic counselling was available through Royal Perth Hospital and Princess Margaret Hospital for any subjects wishing further assistance.

The author maintained confidentiality of named data in accordance with the Edith Cowan University (ECU) and Royal Perth Hospital (RPH) Ethics Committees guidelines. All paper records were kept under lock and key, computer named records were coded and kept separate from associated medical records. The data will be kept secure for 5 years post-study completion.

The data collection activities in this thesis were approved by the ECU and RPH Ethics Committees.

## 2.5 LABORATORY METHODS

This section relates to laboratory methods used to extract DNA from blood samples and blood spots and the molecular genetic analysis techniques.

A total of 160 blood samples were collected at the Department of Nephrology, RPH for DNA extractions (see Table 2.9). Because of geographical barriers, 29 blood kits were sent to Australian individuals residing outside WA (see Appendix 8). The blood kits were sent to individuals for the purpose of genetic linkage analysis and to investigate the effects of modifying genes. This method involved the collection of several drops of blood on filter paper using a lancet. DNA was extracted using the phenol-chloroform method described in section 2.5.1.2.

**Table 2.9: The Number of Blood Samples Collected in the Australian Sample for DNA extractions**

Blood samples Collected at the Department of Biochemistry, RPH, WA	Index patients	ADPKD relatives	Spouses and non-ADPKD relatives	Total
EDTA (for DNA extraction):	69	75	16	160
Blood spots	0	27	2	29

### 2.5.1 Preparation of DNA

#### 2.5.1.1 Salt precipitation extraction of genomic DNA

The salt precipitation method was used to isolate DNA from peripheral blood within 24 hours of collection using standard techniques (Miller *et al.*, 1988). Approximately 21mL of peripheral blood was collected in 7mL Vacutainers containing the anti-coagulant ethylenediaminetetraacetic acid (EDTA) and transferred into a 50ml Falcon Corning

tube. Red cell lysis buffer (RCLB: 10mM Tris, pH7.6; 10mM NaCl; 5mM MgCl<sub>2</sub>) was added to a total volume of 50mL. This mix was centrifuged at 2000 rpm for 7 minutes. The supernatant was recovered either by using a pipette or pouring and leaving the pellet, consisting mainly of white blood cells. The pellet was resuspended and the procedure repeated a few times to remove the red blood cells. The cell suspension was incubated overnight at 37°C with 30 µl of proteinase K (20mg/ml of dH<sub>2</sub>O), 2ml RCLB and 13ml white cell lysis buffer (WCLB: 10mM Tris, pH7.6; 10mM EDTA, di-Sodium Salt; 50mM NaCl; 0.2% SDS; at 25°C). Proteinase K, a proteolytic enzyme, was added to breakdown the proteins by digestion before extracting the DNA with other solvents.

On the following day, 7ml of 6M NaCl was added to the cell suspension and shaken vigorously. This was followed by centrifugation at 5000 rpm for 45 minutes. The addition of 6M NaCl caused the protein and debris to pellet to the bottom of the tube. The supernatant was transferred to a 50ml Falcon Corning tube and ice-cold isopropanol (filled to the top of the 50ml corning tube) was added to precipitate the DNA out of solution. DNA was carefully fished out using a Gilson pipette and transferred into a labelled Nunc tube. The DNA was subsequently washed twice with 1ml of ice-cold 70% ethanol, centrifuged at 13 000 rpm for 60 seconds and air dried for a couple of hours. The DNA was suspended in 1ml of dH<sub>2</sub>O and incubated at 37°C overnight to dissolve.

#### **2.5.1.2 Phenol-chloroform extraction of genomic DNA**

The phenol/chloroform method of DNA extraction was used on all dried blood spots. This method is appropriate for the purification of DNA from small volumes (0.4ml) at concentrations  $\leq 1$  mg/ml, and takes advantage of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Phenol, the most hazardous chemical used in DNA extraction, efficiently denatures and dissolves proteins (Ausubel *et al.*, 1992), while chloroform stabilises the unstable boundary between an aqueous phase and a pure phenol layer. Hence, the phenol/chloroform mixture reduces the amount of aqueous solution retained in the organic phase, thus maximising the DNA yield (Ausubel *et al.*, 1992). This method involved several steps to isolate DNA. The dried blood spot was cut into quarters and placed in a 1.5ml microtube. 250µl of 0.1% Triton X-100, and 15µl 100mg/ml Proteinase K were added to the microtube and vortexed for 1 minute. The microtube was then incubated for 30

minutes at 50°C, followed by vortexing for 1 minute and incubation for an additional 30 minutes at 50°C. 25 µl of 10X SET Buffer were added to the microtube and mixed. 500µl 1:1 phenol/chloroform were added and mixed by inversion for one minute, followed by centrifugation at 13 000 rpm for 10 minutes. The supernatant was removed and transferred to a fresh tube. This was followed by the addition of 25µl 3M Na acetate and 250µl isopropyl alcohol. The solution was left at -20°C overnight and centrifuged at room temperature for 15 minutes on the following day. The pellet was washed with 500µl 70% ethanol, inverted twice and centrifuged at 13 000 rpm for 10 minutes. The tube was then inverted on tissue and the pellet allowed to air dry. The pellet was resuspended in 50µl dH<sub>2</sub>O and incubated at 37°C overnight to allow the DNA to dissolve.

#### **2.5.1.3 DNA spectrophotometry**

Quantification of DNA was performed on all DNA samples using a DU<sup>R</sup> 640 Spectrophotometer. Nucleic acids absorb UV light predominantly at 260nm, where as proteins absorb UV light predominantly at 280nm. By measuring a sample's 260nm and 280nm absorption, the purity of a DNA sample can be assessed. A pure solution of double-stranded DNA at 50µg/ml has an optical density (OD) of 1.0 at 260nm and an OD<sub>260</sub>/OD<sub>280</sub> ratio of 1.8. If there is contamination with protein or phenol the OD<sub>260</sub>/OD<sub>280</sub> ratio will be significantly less than the values given above (Ausubel *et al.*, 1992). Using these calculations, DNA was diluted to 10ng/µl with dH<sub>2</sub>O and the working solutions stored at 4°C.

Agarose gel electrophoresis was used to evaluate the quality and purity of the DNA samples. Visualisation was accomplished by the addition of ethidium bromide, a fluorescent dye, which intercalates between stacked base pairs and binds to DNA to display an increased fluorescent yield. The gel was run for 30 minutes at 92 Volts (constant current), then viewed under UV light on a Hoefer Mighty Bright Ultraviolet Transilluminator. The gel was photographed with a Polaroid camera.

## **2.5.2 Genetic Linkage Analysis**

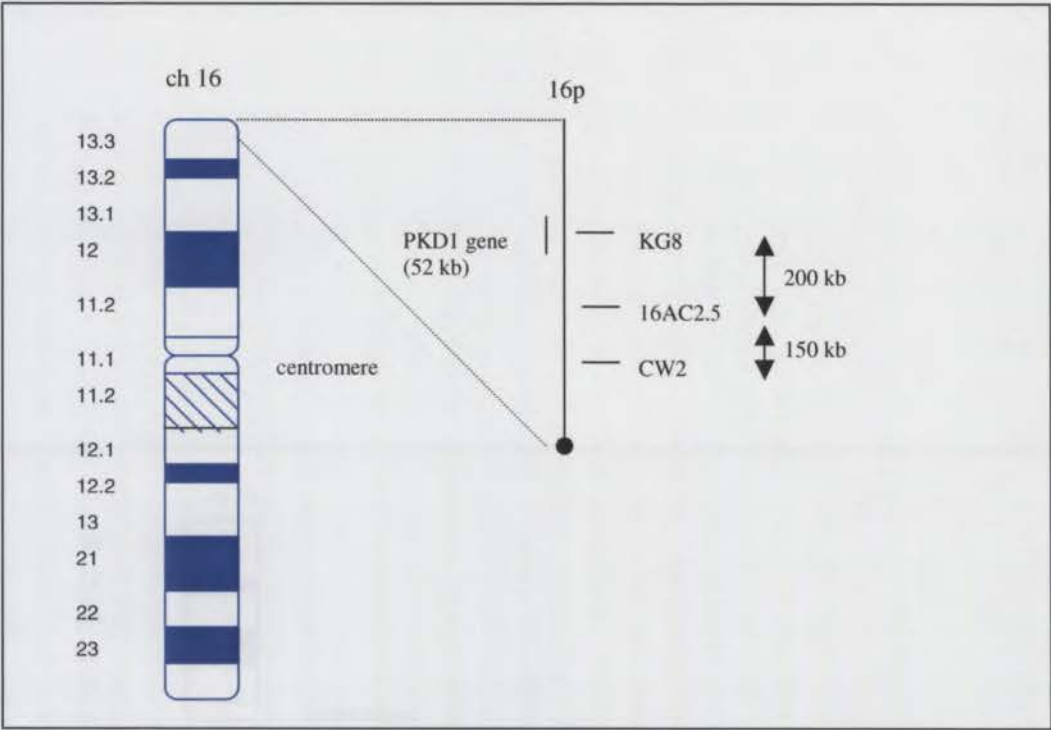
### **2.5.2.1 Selection of polymorphic markers**

Markers closely linked to the *PKD1* and *PKD2* loci were used to establish linkage. The polymorphic markers used for analysis of linkage to *PKD1* were D16S291 (16AC2.5) (Thompson *et al.*, 1992), KG8-*PKD1* (Consortium 1994), and D16S663 (CW2) (Somlo *et al.*, 1992; Torra *et al.*, 1996). KG8 is an intragenic marker located at the 3' end of the *PKD1* gene, whereas the other two microsatellites are centromeric to *PKD1* (Figure 2.6). Microsatellite markers used to show linkage to chromosome 4 were JSTG3, JSTG4, JV106, JV108 (Veldhuisen *et al.*, 1996 and personal communication with Dr Nadja Bogdanova) (Figure 2.7). Marker JV108 is telomeric to *PKD2* and markers JSTG3, JSTG4 and JV106 are centromeric to *PKD2*, at a symbolic distance of 0.005 cM (personal communication with Dr Nadja Bogdanova). Details of both sets of microsatellite markers are outlined in Table 2.11.

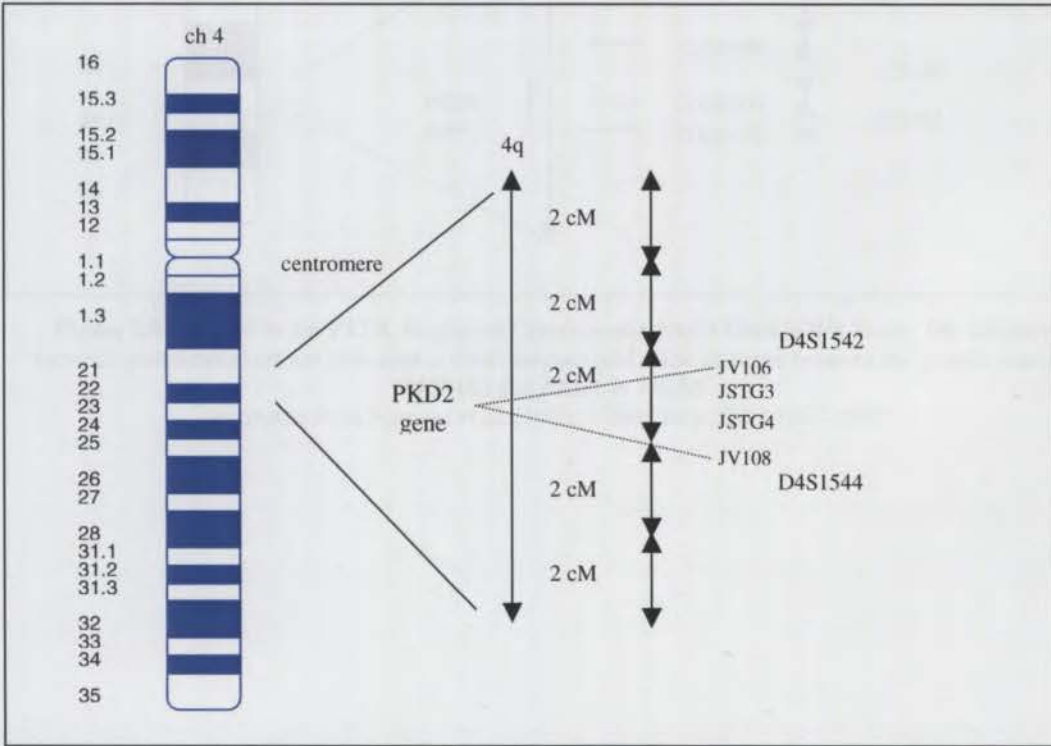
WA family 51 (A51) which did not reveal linkage to either *PKD1* or *PKD2*, was investigated in collaboration with Prof. Jing Zhou (Brigham and Women's Hospital and Harvard Medical School, Boston) to determine linkage to the novel Polycystic Kidney Disease 2-Like (*PKDL*) gene assigned to chromosome 10q24. Microsatellite markers used to study linkage to chromosome 10q24 included D10S603 which maps to the same interval as *PKDL*, and three adjacent markers D10S192, D10S185, D10S198 (Genethon markers) (Figure 2.8). Details of all microsatellite markers used in the present study are outlined in Table 2.10.

### **2.5.2.2 PCR reactions for genetic linkage analysis**

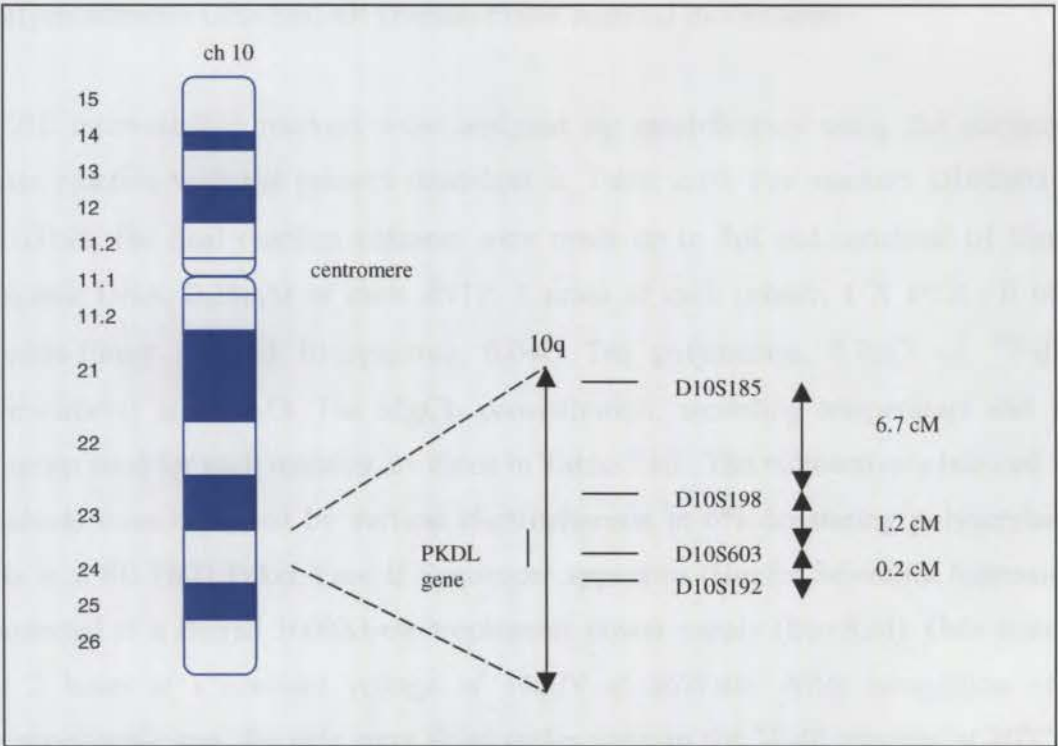
Microsatellite markers were analysed by amplification using the polymerase chain reaction with the primers described in Table 2.10. For *PKD1* linked markers, the final reaction volumes were made up to 10µl and consisted of 30ng of genomic DNA, 0.2mM of each dNTP, 10pmol of each primer (the forward primer had been fluorescently labelled using either FAM, HEX or TET dyes), 1 X PCR PE buffer (Perkin-Elmer Applied Biosystems), 0.5U of Taq polymerase, and dH<sub>2</sub>O. For *PKD2*, the final reaction volumes were made up to 15µl and consisted of 50ng of genomic DNA, 0.2mM of each dNTP, 15pmol of each primer (the forward primer had been fluorescently labelled using either FAM, HEX or TET dyes), 1 X PCR PE buffer



**Figure 2.6:** Markers in the PKD1 Region on Chromosome 16p13.3 Used in this Study. The distance between the markers are indicated in megabases (Mb).  
 Extracted from Torra *et al.* (1996) J Am Soc Nephrol 7:2142-2151



**Figure 2.7:** Markers in the PKD2 Region on Chromosome 4q21-22 Used in this Study. The distance between the markers JV106, JSTG3, JSTG4 and JV108 is 0.005cM (personal communication with Dr Nadja Bogdanova).



**Figure 2.8:** Markers in the PKDL Region on Chromosome 10q24 Used in this Study. The distance between genetic markers are indicated in centimorgans (cM). The distance between the genetic marker D10S185 and PKDL is 7.9cM.

Extracted from Nomura *et al.* (1998) J Bio Chem 273:25967-25973



(Perkin-Elmer Applied Biosystems), 0.75U of Taq polymerase, 10% glycerol and dH<sub>2</sub>O. The PCR products were separated by vertical electrophoresis in 6% polyacrylamide gels (6M Urea; 19:1 acryl:bisacrylamide), using the ABI 373 Genetic Analyzer (Perkin-Elmer Applied Biosystems). The run parameters were set at Filter set B, field 2500 volts, power at 30 watts, a current of 40 mAmps, and a laser setting of 40 mWatts. Samples were prepared by adding 2.5µl deionised formamide, 0.5µl Blue dextran EDTA (1mg Blue dextran per 1ml EDTA [20-50mM, pH 8.0]), 0.5µl of the internal size standard labelled with a uniquely coloured fluorescent dye (TAMRA GS500) and between 0.5µl and 1.0µl of PCR product. The standard creates a size calibration curve for each lane, allowing a calculation of the size of each PCR product by the automatic analysis software GENESCAN (Perkin-Elmer Applied Biosystems).

*PKDL* microsatellite markers were analysed by amplification using the polymerase chain reaction with the primers described in Table 2.10. For markers D10S603 and D10S198, the final reaction volumes were made up to 5µl and consisted of 30ng of genomic DNA, 0.25mM of each dNTP, 3 pmol of each primer, 1 X PCR PE buffer (Perkin-Elmer Applied Biosystems), 0.04U Taq polymerase, 0.1µCi of <sup>32</sup>P-dCTP (Geneworks) and dH<sub>2</sub>O. The MgCl<sub>2</sub> concentration, annealing temperature and PCR program used for each reaction are listed in Table 2.10. The radioactively labelled PCR products were separated by vertical electrophoresis in 6% denaturing polyacrylamide gels in a SE 1600 Poker Face II Sequencer apparatus (Hoefer Scientific Instruments) connected to a Biorad 3000Xi-electrophoresis power supply (Bio-Rad). Gels were run for 2 hours at a constant voltage of 1400V at 46Watts. After completion of the electrophoretic run, the gels were dried under vacuum for 30-40 minutes at 80°C and exposed overnight to Agfa X-ray film for autoradiography. Allele assignment was done manually.

For markers D10S192 and D10S185, the final reaction volumes were made up to 10µl and consisted of 20ng of genomic DNA, 0.25mM of each dNTP, 3 pmol of each primer (the forward primer had been fluorescently labelled using either FAM, HEX or TET dyes), 1 X PCR PE buffer (Perkin-Elmer Applied Biosystems), 0.04U Taq polymerase and dH<sub>2</sub>O. The MgCl<sub>2</sub> concentration, annealing temperature and PCR program used for each reaction are listed in Table 2.10. The PCR products were separated by vertical

electrophoresis in 6% polyacrylamide gels (6M Urea; 19:1 acryl:bisacrylamide), using the ABI 373 Genetic Analyzer (Perkin-Elmer Applied Biosystems) as described in the above paragraph.

Four different PCR amplification protocols were used for the above reactions.

**Protocol A:** initial denaturation at 94°C for 5 minutes, 15 cycles of 94°C for 20 seconds, 63-55°C for 60 seconds (0.5°C increment per cycle), followed by 18 cycles consisting of 94°C for 20 seconds, 55°C for 15 seconds and 72°C for 30 seconds. Final extension was at 72°C for 5 minutes.

**Protocol B:** initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 56°C for 1 minute and 72°C for 1 minute. Final extension was at 72°C for 10 minutes.

**Protocol C:** initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 20 seconds, 56°C for 15 seconds, 72°C for 30 seconds and a final extension at 72°C for 10 minutes.

**Protocol D:** initial denaturation at 95°C for 5 minutes, 10 cycles of 94°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds and 20 cycles of 89°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds. Final extension was at 72°C for 10 minutes.

The results of genetic linkage analysis are discussed in Chapter Four.

**Table 2.10: Details of the *PKD1* and *PKD2* Microsatellite Markers and Assay Conditions Used in the Present Study**

Gene	Accession Number	Sequence	MgCl <sub>2</sub>	Anneal °C	PCR Protocol	# Alleles	Size range
<b>1</b>							
16AC2.5	GDB # 180884	5' -GCAGCCTCAGTTGTGTTTCCTAATC-3' 5' -AGTGCTGGGATTACAGGCATGAACC-3'	1.5 mM	63-55°C	A	9	150-166
KG8	GDB # 377657	5' -CTCCCAGGGTGGAGGAAGGTG-3' 5' -GCAGGCACAGCCAGCTCCGAG-3'	1.5 mM	63-55°C	A	8	98-112
CW2	GDB # 269989	5' -GTCTTTCTAGGAATGAAATCAT-3' 5' -ATTGCAGCAAGACTCCATCT-3'	1.5 mM	63-55°C	A	4	117-123
<b>2</b>							
JV106	Pers. comm. with Dr Nadja Bogdanova	5' -CCGGGGTGACAGAGTAAGATT-3' 5' -GAAGGATAAAGGGCAAAATAAAAG-3'	2.0 mM	63-55°C	A	6	184-194
JSTG3	Pers. comm. with Dr Nadja Bogdanova	5' -CCTTTCCCATCCTTCACATTTC-3' 5' -GTAAAATTTCAAGGGGAAGCATT-3'	2.5 mM	63-55°C	A	9	141-157
JSTG4	Pers. comm. with Dr Nadja Bogdanova	5' -CCTCTGAACATCTTGGAATAATTGG-3' 5' -ATTCATATATTGATGGGATCCTCTCT-3'	1.5 mM	56°C	B	8	128-142
JV108	Pers. comm. with Dr Nadja Bogdanova	5' -AGCTTAGGCAACATAGAGA-3' 5' -AGGTAAAGATGTCAGTAATGTA-3'	1.5mM	63-55°C	A	5	105-113
<b>L</b>							
D10S603	GDB# 200082	5' -GCTGGATTATCCTCGGTAAC-3' 5' -GTTTCATCTCCCAAGGCAA-3'	1.5 mM	56°C	C	11	232-256

D10S192	GDB # 371860	5'-GGCTTACCTAGACCTTCATA-3' 5'-GCCTGAGACAATTCTTCTTC-3'	1.5 mM	55 <sup>0</sup> C	D	10	180-198
D10S198	GDB # 188036	5'-TGAGGGACTCATCTTCTGTT-3' 5'-GTCTGTGATCCCCATGTTAG-3'	1.5 mM	56 <sup>0</sup> C	C	8	185-203
D10S185	GDB # 187826	5'-TCCTATGCTTTCATTGCCA-3' 5'-CAAGACACACGATGTGCCAG-3'	1.5 mM	55 <sup>0</sup> C	D	8	143-159

*Pers. Comm. – personal communication*

### 2.5.3 *Analysis of Genetic Variants within the Renin-Angiotensin System (RAS)*

#### 2.5.3.1 The Alu I/D polymorphism in the ACE gene

The ACE I/D polymorphism (Rigat *et al.*, 1992) was analysed in 307 ADPKD patients (9 patients were excluded because amplification of the ACE I/D polymorphism was unsuccessful). A 190 bp fragment for the deletion (designated D) and a 490 bp fragment in the presence of the insertion (designated I) in the dipeptidyl carboxypeptidase 1 (DCP1) gene (Genbank accession # X62855) was amplified from genomic DNA with the following primers: 5'-CTGGAGACCACTCCCATCCTTTCT-3' and 5'-GATGTGGCCATCA-CATTTCG TC AGAT-3'. The final reaction volume was made up to 10µl and consisted of 30ng of genomic DNA, 2mM of MgCl<sub>2</sub>, 0.2mM of each dNTP, 4 pmol of each primer, 1 X PCR PE buffer (Applied Biosystems), 0.25U of Taq polymerase, 5% DMSO and dH<sub>2</sub>O. PCR conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 15 seconds, 57°C for 30 seconds and 72°C for 30 seconds. Final extension was at 72°C for 5 minutes (Rigat *et al.*, 1992).

To eliminate the possibility of mistyping ID heterozygotes as DD homozygotes, all samples found to be DD homozygotes were re-amplified with an insertion-specific primer (Shanmugam *et al.*, 1993). This reaction was again carried out in a final reaction volume of 10µl with 30ng of template DNA and the following primers; 5'-CTGGAGACCACTCCCATCCTTTCT-3' and the insertion specific primer 5'-TTTGAGACGGAGTCTCGCTC-3' (Rigat *et al.*, 1992). The annealing temperature was 61°C.

The PCR products were separated by horizontal electrophoresis on a 3% agarose gel [(4g of agarose powder and 2g Metaphor Agarose in 200ml of 1 X TAE Buffer (0.04M Tris-acetate; 0.001M EDTA)], stained with ethidium bromide (0.05µl/ml; Sigma) and visualised under ultraviolet light on a Hoefer Mighty Bright Ultraviolet Transilluminator. The gel was photographed with a Polaroid camera.

### 2.5.3.2 The Dinucleotide Repeat at the AGT Locus

The angiotensinogen 3' dinucleotide repeat (Kotelevtsev *et al.*, 1991) was analysed in a total of 316 ADPKD patients. A 167 bp DNA fragment spanning the region between nucleotides 69 and 236 in the angiotensinogen gene (Genbank Accession # X62854) was amplified from genomic DNA using the following primers: 5'-GGTCAGGATAGATCTCAGCT-3' and 5'-CACTTGCAACTCCAGGAAGACT-3' (Jeunemaitre *et al.*, 1992). The forward primer had been fluorescently labelled using either HEX or FAM dyes. The final reaction volume was made up to 10µl and consisted of 40ng of genomic DNA, 2mM of MgCl<sub>2</sub>, 0.2mM of each dNTP, 10 pmol of each primer, 1 X PCR PE buffer (Applied Biosystems), 0.5U of Taq polymerase, 0.66ng of gelatin and dH<sub>2</sub>O. A touchdown PCR cycling program was used, which consisted of an initial denaturation at 94°C for 5 minutes, 15 cycles of 94°C for 20 seconds, 63-55°C for 60 seconds (0.5°C increment per cycle), followed by 18 cycles of 94°C for 20 seconds, 55°C for 15 seconds and 72°C for 30 seconds. Final extension was at 72°C for 5 minutes.

The PCR products were separated by vertical electrophoresis in 6% polyacrylamide gels (6M Urea; 19:1 acryl:bisacrylamide), using the ABI 373 Genetic Analyzer (Perkin-Elmer Applied Biosystems). The run parameters were set at Filter set B, field 2500 volts, power at 30 watts, a current of 40 mAmps, and a laser setting of 40 mWatts. Samples were prepared by adding 2.5µl deionised formamide, 0.5µl Blue dextran EDTA (1mg Blue dextran per 1ml EDTA [20-50mM, pH 8.0]), 0.5µl of the internal size standard labelled with a uniquely coloured fluorescent dye (TAMRA GS500) and between 0.5µl and 1.0µl of PCR product.

The length of the PCR product ranged between 163 – 183 bp. The correspondence between the eleven alleles observed in this study and those described by Kotelevtsev *et al.* (1991) and Brand *et al.* (1998) are shown in Table 2.11.

**Table 2.11 Designation of the AGT Dinucleotide Repeat Alleles**

This study		Kotelevtsev <i>et al.</i> (1991) and Brand <i>et al.</i> (1998)	
Allele	Length (bp)	Allele	Length (bp)
1	183	1	133
2	181	2	131
3	179	3	129
4	177	4	127
5	175	5	125
6	173	6	123
7	171	7	121
8	169	8	119
9	167	9	117
10	165	10	115
11	163	11	113

### 2.5.3.3 The Tetranucleotide Repeat in the Renin Gene

The renin tetranucleotide repeat (Edwards *et al.*, 1992) was analysed in 316 ADPKD subjects. A DNA fragment spanning the region between nucleotides 1898 and 2261 in the human renin gene on chromosome 1q32 (Genbank Accession # M10152) was amplified from genomic DNA using the following primers: 5'-AGAGTACCTTCCCTCCTCTACTCA-3' and 5'-CTCTATGGAGCTGGTAGAACCTGA-3' (Edwards *et al.*, 1991). The final reaction volume was made up to 6µl and consisted of 40ng of genomic DNA, 1.5mM of MgCl<sub>2</sub>, 0.2mM of each dNTP, 10 pmol of each primer, 1 X PCR PE buffer (Applied Biosystems), 0.05U of Taq polymerase, 0.1µCi of <sup>32</sup>P-dCTP (Geneworks), 0.66ng of gelatin and dH<sub>2</sub>O. PCR cycling conditions consisted of an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 95°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute and a final extension at 72°C for 10 minutes. The radioactively labelled PCR products were separated by vertical electrophoresis in 6% denaturing polyacrylamide gels in an SE 1600 Poker Face II Sequencer apparatus (Hoefer Scientific Instruments) connected to a Biorad 3000Xi-electrophoresis power supply (Bio-Rad). Gels were run for 2.5 hours at a constant voltage of 1400 V. After completion of the electrophoretic run, the gels were dried under vacuum for 30-40 minutes at 80°C and exposed overnight to Agfa X-ray film for autoradiography. Allele assignment was done manually. The length of the PCR product ranged between 251-271 bp. The four alleles observed in this study (alleles 4, 3, 2 and 1) correspond to alleles 8, 10, 11 and 12 of the Edwards *et al.* (1992) nomenclature (Table 2.12).

**Table 2.12 Number and Length of the Renin Tetranucleotide Repeat**

Number, length and frequencies of the renin tetranucleotide repeats			
This study		Edwards <i>et al.</i> (1992)	
Allele	Length	Allele	Length
1	271	12	271
2	267	11	267
3	263	10	263
4	255	8	255

**2.6 GENERAL DATABASE DESIGN AND MANAGEMENT**

All data entry, maintenance and management relating to this study was performed by the author.

**2.6.1 Database Design**

The database for the overall study was constructed using the software packages Microsoft Excel™ v5.0 and Access™ v2.0. Family trees and genetic data were drawn and stored using the Cyrillic™ package. Genotype data from ABI 373A automated sequencers were downloaded and exported in Microsoft Excel v5.0 format.

**2.6.2 Data Entry and Checking**

All data were entered in the database by the author. Every possible effort was made to ensure that the data entered were correct. This was achieved by randomly selecting files and cross checking data from the patient file and the database.

**2.7 STATISTICAL ANALYSIS**

Statistical analyses were principally carried out using SPSS version 10.0, but Microsoft Excel (Microsoft Corporation) was also used. Various statistical procedures were employed to address the multiple aims of this thesis and were performed by Dr Valerie Burke (University Department of Medicine, UWA), Prof. Paul Burton and Prof. Nick De Klerk (TVW Institute for Child Health Research, UWA) and by the author.

In all analyses both index cases and relatives were included to increase the sample size. It must also be noted that throughout this thesis there are differences in the number of individuals that are included in the statistical analyses [Tables titled ‘Number of Individuals Included in the .... Analyses....’] versus the number of individuals



presented in the general clinical characteristics [Tables titled 'Characteristics of ... ADPKD Patients Included in ... Analyses']. Examples of these differences are shown in Chapter Three [Tables 3.7 and 3.8], Chapter Four [Tables 4.4 and 4.9], Chapter Five [Tables 5.7 and 5.8], Chapter Six [Tables 6.2 and 6.3] and Chapter Seven [Tables 7.2 and 7.3]. While one of these Tables presents the general demographic and clinical characteristics of the patients, including the number of individuals who have reached CRF and ESRF, the other Table displays the number of individuals who are included in the statistical analyses based on the availability of data and type of statistical analyses.

### **2.7.1      *Frequency Data Analysis***

The chi-square test is the most commonly used method for comparing frequencies or proportions, as it can be used with two or more groups. The test is used to test the null hypothesis that proportions are equal or, equivalently, that factors or characteristics are independent or not associated (Dawson-Saunders and Trapp, 1994).

Chi-square analysis and cross-tabulation were used to examine and compare the frequency of clinical manifestations (hypertension, urinary tract infection, renal calculi, haematuria, dysuria, nocturia, herniae and extrarenal cysts) between countries and between PKD1 and PKD2 individuals (Chapters Three and Four). The analysis was also used to compare the frequency of hypertension, CRF and ESRF between patients classified on the basis of ACE genotypes, AGT and renin alleles, as well as between groups based on smoking history (duration and number of cigarettes per day), alcohol consumption, current physical activity and serum and dietary lipids.

Chi-square analysis and cross-tabulation were also used to examine and compare the age at development of hypertension between countries and between PKD1 and PKD2 individuals. Patients were divided into two groups based on age at development of hypertension, namely <40 years and >40 years. Because hypertension is known to develop as an early manifestation in ADPKD, while in the general population the risk of developing hypertension increases with age, an arbitrary cut-off at 40 years of age was used to ensure that a relationship between hypertension and renal impairment in this study was based on the ADPKD status of the individual and not advancing age.

Allele and genotype frequencies of genetic variants within the RAS were also analysed by Chi-square analysis. In addition, allele and genotype frequencies were also assessed by the Hardy-Weinberg Equilibrium (HWE) with the probability test in the overall sample and with Fisher's exact test within each of the three study populations. The estimates of allele frequencies and HWE included only one, randomly chosen individual per family.

HWE is the simple relationship between gene frequencies and genotype frequencies that is found in a population under certain conditions (*i.e.* when there are no disturbing forces such as selection, mutation or migration that would change gene frequencies over time and when there is random mating in very large populations).

### 2.7.2 *Linear Regression Analysis*

Decline in renal function was assessed using two approaches, namely age at onset of renal failure and cumulative renal survival. As a novel element of the study, we examined two important renal outcomes: chronic renal failure (CRF) and end-stage renal failure (ESRF), rather than ESRF alone as in most published studies. CRF was defined as a serum creatinine (SCr) value of 150  $\mu\text{mol/L}$  and ESRF was defined as the point at which a person required dialysis or a renal transplant. An arbitrary SCr value of 800  $\mu\text{mol/L}$  was chosen as a reliable indicator of ESRF if the above information was not provided.

Age at onset of CRF was examined in two ways: (1) by calculating mean age at CRF using observed values of SCr (*i.e.* analysis included only patients who had already reached CRF) and (2) by linear regression using the least squares method. The latter method involved fitting individual regression lines of time versus  $1/\text{Cr}$  values and extrapolating age at CRF. The least squares method is a way to determine the equation of a line that provides a "good fit" to the points. The equation for a straight line is:

$$Y = a + bX$$

$Y$  is the dependent variable,  $X$  is the predictor variable,  $a$  is called the intercept or constant and is the point on the vertical axis of a scatter plot where the line crosses the axis and  $b$  is the slope or coefficient. The slope of the line measures the amount of change in  $Y$  (dependent variable) for each unit change in  $X$  (independent variable or risk factor) (Dawson-Saunders and Trapp, 1994). The least squares method was employed to increase the power of the study by substantially increasing the size of the CRF group, since it could be used to predict age at CRF for cases with  $\geq 2$  available SCr measurements. No formal measure of consistency and accuracy of predicted values in individuals with the models used were applied. Empirically the results of the analyses obtained using observed versus extrapolated values appeared consistent although, in terms of accuracy, the use of regression models to obtain the extrapolated values resulted in a displacement to an earlier age.

Similarly, age at ESRF was examined in two ways: (1) by calculating mean age at ESRF using observed values of SCr (*i.e.* analysis included only patients who had already reached ESRF) and (2) by incorporating a dummy variable (*i.e.* 0 = no ESRF; 1 = with ESRF). However, given the multi-centre sources of the data and possible misclassification among those not identified as having ESRF, the two analyses were undertaken, again to provide confirmation. Extrapolation was performed by Prof. Paul Burton, (TVW Institute for Child Health Research, WA).

### 2.7.3 *General Linear Modelling*

General linear models (namely univariate methods) are statistical procedures used to estimate variance from data by calculating a statistic from the ratio of the two estimates. The first estimate is between-groups-variance, which is a measure of the effect of the independent variable combined with error variance. The second estimate is within-groups-variance, which is error variance by itself. The F ratio is the ratio of between-groups variance to within-groups variance. A significant F-value tells us that the population means are probably not all equal.

Univariate models, namely analysis of variance (ANOVA), were used to compare age at renal failure, kidney length, diameter of the largest cyst and number of cysts between countries (Chapter Three) and between PKD1 and PKD2 individuals (Chapter Four). To

compare ultrasound findings between groups, analysis of covariance (ANCOVA) was performed to statistically control for the influence of age on the dependent variable (*i.e.* length of the kidneys and diameter of the largest cyst). Adjustment for height and weight was not possible because data on these variables was not available in all individuals.

The relationship between ACE I/D genotypes and plasma ACE activity was assessed by univariate analysis. Correction for the use of ACE inhibitors was achieved by using a “dummy” variable, taking the value of 0 for lack of treatment and of 1 for treatment with ACE inhibitors. ANOVA was used to examine the relationship between: 1) polymorphic alleles observed in the three RAS genes (ACE, AGT and renin) and age at CRF and ESRF; 2) serum ACE activity and age at CRF; 3) ACE I/D polymorphism, and left ventricular mass and cystogenesis; 4) The relationship between environmental determinants (smoking, alcohol, physical activity and serum lipids) and age at development of renal failure (CRF and ESRF) and 5) the relationship between smoking, alcohol consumption and serum lipids, and cystogenesis).

The percentage contribution of the ACE I/D polymorphism to the variance of plasma ACE activity was also calculated from General Linear Models.

With the regard to the analyses examining the relationship between serum lipids and renal failure, no adjustments for hyperlipidaemia as increasing the risk of coronary was undertaken due to the small numbers. The possibility of adjusting for multiple variables would substantially reduce the number of degrees of freedom, which would consequently become a problem in interpreting the findings with such small samples.

ANOVA was used to compare observed and extrapolated age at CRF between sib, cousin and parent-offspring pairs adjusting for sex and presence of hypertension. Results are discussed in Chapter Seven. One-way ANOVA was also used to compare difference in age at development of hypertension (adjusting for age) between PKD1 and PKD2 individuals and between sib, cousin and parent-offspring pairs. Results are discussed in Chapter Four and Seven.

The analyses based on age at CRF and ESRF were initially performed adjusted for age and repeated without adjusting for age. The results did not change significantly. Subsequently, all analyses based on age at CRF and age at ESRF included in this thesis did not adjust for age. This issue was discussed with various statisticians at the Institute for Child Health Research and RPH, Perth, Western Australia and it was decided that the analyses should not be adjusted for age given that the analyses are based on age at renal failure, *i.e.* that variable has already been taken into account.

#### **2.7.4            *Kaplan-Meier Survival Analysis***

Analysis of survival was performed using the Kaplan-Meier time-to-event method (Kaplan and Meier, 1972). Survival analysis is a statistical method used routinely in medical research to determine whether a new medication, a new treatment or a new procedure will perform better than the one in current use. Long-term outcomes, including mortality and major morbidity are important, however their use may introduce major constraints if a subject has not reached the specific end-point during the follow-up period. Therefore, if a subject has not become a case by the end of his/her follow-up, we say that his/her survival time is censored. By censoring the data, two “dummy” variables are incorporated in the analysis. The “dummy” variables are used to define the status of the individual *i.e.* 1 = yes, reached end-point and 0 = no, not reached end-point. Survival analysis can be performed in two ways. The first includes only individuals who have reached the end-point (in our case, CRF and ESRF) and the second includes all individuals (censored data).

In this thesis, survival analysis was initially performed in patients who had reached the end-point (CRF and ESRF) and was subsequently repeated in individuals who had developed renal impairment and in those that had not (which allowed for censoring data). For CRF, the initial analysis included only patients who had reached CRF and where observed age at CRF was available. The subsequent analysis consisted of patients with observed and extrapolated CRF values. In the analysis, two “dummy” variables were introduced to indicate CRF status as 1 = yes, reached end-point and 0 = no, not reached end-point, allowing for censored individuals.

Similarly, survival analysis to ESRF was initially performed in patients who had reached ESRF (*i.e.* cases) only. It was subsequently repeated to include all patients with information on age at last examination (or age at last SCr measurement) or age at ESRF. Additionally this analysis also incorporated two “dummy” variables indicating ESRF status as 1 = yes and 0 = no. The repeated analysis also allowed for censoring.

Differences in the number of individuals included in the repeated analyses for CRF and ESRF is seen in this thesis. The repeated analysis for CRF included the subset of individuals where extrapolated age at CRF was estimated, whereas for ESRF, the analysis included the whole data set with information available for all patients with age at either last examination or last SCr measurement.

#### **2.7.5            *Cox Regression Analysis***

Cox regression analysis (Cox, 1972) was employed to determine the relative risk of developing renal failure (CRF; ESRF) related to smoking and alcohol consumption and adjusted for the effect of co-variables including country, hypertension, sex, age, blood cholesterol and triglycerides, and PKD gene linkage. From the regression equation, the adjusted relative risk for each of these variables was determined. For example, the relative risk for developing ESRF in ADPKD subjects smoking >20 cigarettes per day was 3.6, indicating that smoking has a detrimental effect in developing ESRF, independent of the patient’s sex, blood pressure, cholesterol, etc. This method was the most appropriate method as it accounted for time-dependent censored observations.

Cox regression analysis was used to calculate the risk of developing renal failure (CRF; ESRF) related to smoking (smoking status; smoking duration; and number of cigarettes per day) and alcohol consumption. The initial analysis calculated the crude relative risk for each parameter. The second analysis calculated the relative risk of each of the above parameters adjusted for each other and for additional covariates including sex, PKD gene, country and blood pressure.

#### **2.7.6            *Variance Component Modelling***

Multivariate component modelling was employed by Prof. Nick De Klerk (TVW Institute for Child Health Research, WA) to determine the extent to which genes and

environmental factors contribute to variation in the age at CRF and rate of deterioration of kidney function in ADPKD patients (see Chapter Seven). Information on the covariance among first degree (parent-offspring, siblings), second degree (uncle/aunt-nephew/niece) and third degree (first cousins) relatives was used to estimate the component of variance. The likelihood of the phenotype (age at CRF, slope of decline in renal function) of family members assumed all measurements for a family were multivariate normal, with means dependent on the sex of a family member, and a constant variance for all family members depending on the familial relationship (Knuiman *et al.*, 1996; Palmer *et al.*, 2001). The slope for each individual with >2 SCr values was calculated by fitting a linear regression through all the SCr points so that the slope was the regression coefficient from the equation.

ANOVA is the primary statistical tool for determining heritability, where the observed phenotypic variance is partitioned into components reflecting differences in unobserved genetic and environmental factors, both of which must be determined from covariances or correlations among relatives. This approach assumes an underlying linear model where the observed phenotype,  $Y$ , is a linear function of genetic and environmental factors. A simple equation to define this,

$$Y = F + \Sigma$$

where  $F$  is the fixed effects and  $\Sigma$  is the error variance. Phenotypic variance was partitioned into three components: 1) additive genetic effects ( $\sigma^2A$ ) (the additive effect of genes); 2) common family environment ( $\sigma^2C$ ) (environment shared by an entire family); 3) common sibling environment ( $\sigma^2CS$ ) (environmental exposures unique to siblings over and above the common family environment). Therefore the total phenotypic variance based on a conventional covariance structure was modified as:

$$(\sigma^2) = \sigma^2A + \sigma^2C_s + \sigma^2C$$

The conditional covariances within a family were specified as:

$$0.5\sigma^2A + \sigma^2C_s + \sigma^2C \text{ between two siblings;}$$

$$0.5\sigma^2A + \sigma^2C \text{ between a parent and a child}$$

Narrow sense heritability, which is the ratio of variance contributed by the additive effects of alleles at one or more loci to the total phenotypic variance was calculated and defined by the following formula.

$$h^2N_S = \sigma^2A/(\sigma^2A + \sigma^2C_S + \sigma^2C)$$

The fixed effects in our model were sex, country (incorporated to adjust for ascertainment bias), ACE I/D genotypes and smoking. In addition, to adjust further for ascertainment bias the proband was conditioned.

The software package FISHER ([www.biomath.medsch.ucla.edu/faculty/klange/software.html](http://www.biomath.medsch.ucla.edu/faculty/klange/software.html)) (Hopper and Mathews, 1994) was used to undertake multivariate modelling and to partition phenotypic variance (age at CRF) into genetic and non-genetic components by maximum likelihood methods (Knuiman *et al.*, 1996; Palmer *et al.*, 2001). Individuals included in the analysis were accepted as a family unit, therefore an individual was included in both sib and parent-offspring analyses and vice versa.

Statistical significance was defined at the nominal 5% level.

#### 2.7.7 *Multiple Comparisons*

Multiple comparisons were performed in this thesis primarily due to the small sample size and to ensure the robustness of the two models used to generate age at CRF. While it is preferred to adjust for multiple tests (for example the Bonferroni correction) to protect against Type I errors, the aims and goals of the study ultimately predict the methods used and the need to include an adjustment variable. It is suggested that if the study is based on a hypothesis testing model, then the need for a correction variable is crucial. Conversely, if the study is based on addressing questions, which will lead to a hypothesis then *post hoc* adjustments are not required.

This thesis aimed to address several questions (hypothesis generating), which may provide data leading to the formulation of a hypothesis/hypotheses. As a result, the need for using a correction variable such as the Bonferroni test was not required.



The issue of adjusting for multiple tests is a debatable area whereby many epidemiologists and statisticians argue to the extent to which a correction variable should be included. According to Savitz and Olshan (1995, 1998), they conclude that there is a danger of erroneous dismissal of meaningful results adjusting for such tests. Moreover, according to Rothman (1990) the need for not making adjustments for multiple comparisons is preferable as it will lead to fewer errors of interpretation when the data under evaluation are not random numbers but actual observations of nature. The issue of multiple comparisons is further discussed in Chapter Eight.

In this thesis the data was based on actual observations of the course of ADPKD and aimed at addressing several questions leading to the generation of a hypothesis. Nonetheless, the data enabled the detection of patterns on the natural progression of ADPKD.

#### 2.7.8 *Genetic linkage analysis*

To establish linkage to *PKD1*, *PKD2* or other, at least three affected members or two affected and two unaffected members of each family were included in the analysis.

Lod scores were calculated using GENEHUNTER version 1.1 (Kruglyak *et al.*, 1996) for multi-point analysis and MLINK version 5.10 (Ott 1974, 1991) for two-point analysis. Linkage analysis was performed by Associate Professor Joachim Hallmayer (Centre for Clinical Research in Neuropsychiatry, Graylands Hospital, WA).

Two-point linkage analysis involves calculating the likelihood (L) of observing a particular configuration of a disease phenotype and marker alleles in a family assuming no linkage and hence free recombination between the two loci (*i.e.*  $\theta$ , the recombination fraction, is 0.5). The likelihood is then compared with the likelihood of observing the same configuration of the two loci within the same family, assuming linkage over a selected range of recombination fractions (*i.e.*  $\theta$  ranges from 0.0 to 0.5).  $\log_{10}$  of the ratio of these likelihoods is then determined for each value of  $\theta$  within the range, and each of the resulting numbers is referred to as a lod score  $z(x)$ , where  $x$  represents a particular value of  $\theta$  within the selected range of recombination fractions (Ott 1974, 1991).

Multi-point analysis involves simultaneous analysis of linkage between a disease locus and several linked marker loci, resulting in a value, Z, referred to as a multilocus or multi-point lod score. This value quantitates statistical support for linkage of multiple loci to each other. Multi-point linkage analysis is an important step for maximising linkage information and/or localising the disease gene more precisely on an established map of markers. Furthermore, multi-point linkage analysis can increase the informativeness of the pedigree. An individual may not be informative (*i.e.* they may be homozygous) for a particular marker, but may well be informative (*i.e.* heterozygous) for the next closest marker on the genetic map. Hence, simultaneous use of several markers in a single linkage analysis can help maximise linkage information available in a pedigree (Ott 1974, 1991).

The gene frequencies for *PKD1* and *PKD2* were set at 0.001 and 0.0001 respectively (Torra *et al.*, 1996). A dominant model was employed, with liability classes (LC) based on ultrasound diagnosis. Liability classes were used to account for age-dependent penetrance, according to the cumulative frequency of age at onset curve obtained from published data (Parfrey *et al.*, 1990a; Bear *et al.*, 1992; Ravine *et al.*, 1992; Wright *et al.*, 1993; Peral *et al.*, 1993): LC1 = 0.64 (<20 years); LC2 = 0.92 (20-30 years); LC3 = 1 (>30 years). No difference in recombination fractions between sexes and the absence of genetic interference and spontaneous mutations were assumed in the calculations.

Family lod scores were included in a weighting formula used to estimate the likelihood of a family being linked to the *PKD1* locus (Hateboer *et al.*, 1999b). The probability of linkage to *PKD1* was taken as 0.86-1.00 (Peters and Sandkuijl, 1992).

$$P(PKD1) = \frac{(\alpha_{PKD1}) (10^{LodPKD1})}{[(\alpha_{PKD1}) (10^{LodPKD1}) + (\alpha_{PKD2}) + (1 - \alpha_{PKD1} - \alpha_{PKD2})]}$$

In families where linkage to *PKD1* and *PKD2* was inconclusive, the following formula was used to estimate the likelihood of a family being linked to the *PKD2* locus (Narod, 1991; Hateboer *et al.*, 1999b). The probability of linkage to *PKD2* was taken as 0.89-1.00 (Hateboer *et al.*, 1999b).

$$P(PKD2) = \frac{\alpha_2 10^{\text{LodPKD2}}}{\alpha_2 10^{\text{LodPKD2}} + \alpha_1 10^{\text{LodPKD1}} + (1 - \alpha_1 - \alpha_2)}$$

The prevalence estimates used for *PKD1* ( $\alpha_1$ ), *PKD2* ( $\alpha_2$ ) and  $1 - \alpha_1 - \alpha_2$  were 0.84, 0.15 and 0.01 respectively (Hateboer *et al.*, 1999b).

## 2.8 CHAPTER SUMMARY

This chapter has described the general epidemiological, molecular, database and statistical materials and methods used in this thesis. In order to address the multiple aims of this thesis three study populations were used: (1) Australian population (n=69 families, 126 subjects); (2) Bulgarian population (n=32 families, 128 subjects); and (3) Polish population (n=16 families, 70 subjects). Informed consent was obtained for all subjects, and all data collection activities were formally approved by an appropriate institutional ethics committee. The presence of ADPKD was defined by the presence of two cysts in each kidney however the number of cysts is age dependent. Information collected included ultrasound data, serial SCr measurements, serum total cholesterol and triglycerides, serum ACE activity measurements, serial blood pressure values, information on antihypertensive therapy, LVM measurements, medical history including age at onset of ADPKD, frequency and development of clinical manifestations, family history, information on diet and lifestyle and genetic information. Database management and statistical analysis was undertaken using a variety of software packages to address the multiple aims of this thesis.

**CHAPTER THREE: INTER-POPULATION  
DIFFERENCES**

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# CHAPTER THREE: INTER-POPULATION DIFFERENCES

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## 3.1 INTRODUCTION AND LITERATURE REVIEW

An important factor that may influence studies of ADPKD progression is inter-population differences. These can be classified as biological (for example, PKD locus and mutation heterogeneity, as well as differences in genetic background) and environmental variables (socioeconomic conditions, health care, diet and lifestyle). Some of these factors are significant in determining patterns of population health.

This thesis aims to determine whether inter-population differences influence the ADPKD phenotype by comparing clinical findings in patients from three populations: Australians, Bulgarians and Poles. The literature review discusses inter-population differences in regard to genetic and environmental factors, with emphasis on the three populations investigated in this thesis.

### 3.1.1 *Frequency and distribution of alleles*

There are widespread differences in the frequency and distribution of alleles between populations, which may be caused by population history, migrations, genetic drift and selection and by human activities (Cummings, 1994).

Social customs and rules are powerful forces that dictate and shape human mating patterns. Social pressure often limits mate selection to those with common bonds, such as language, religion, geography, and economic status. These social constraints prevent random mating and this behaviour can rapidly change genotype frequencies (Cummings, 1994).

In fact, differences in mutation allele frequencies for a variety of genetic diseases has been reported. Two examples of genetic diseases showing significant differences in allele frequencies between countries include phenylketonuria (Phenylalanine Hydroxylase database: [http://data.mch.mcgill.ca/cgi-bin/pahdb\\_new/mutstats.pre](http://data.mch.mcgill.ca/cgi-bin/pahdb_new/mutstats.pre)) and cystic fibrosis (CF) (Estivill *et al.*, 1997).

Over 1000 mutations and a large number of variants and polymorphisms have been identified in the CF transmembrane conductor regulator (CFTR) gene (The Cystic Fibrosis Genetic Analysis Consortium, CFGAC: <http://genet.sickkids.on.ca>). The  $\Delta F508$  mutation which accounts for the majority of mutant CFTR alleles (Kerem *et al.*, 1989) varies widely between populations, ranging from 30% in Ashkenazi Jews to 88% in Denmark (Cystic Fibrosis Genetic Analysis Consortium 1990). However, the frequency of other CFTR mutations also varies widely (Estivill *et al.*, 1997). Differences in the reported frequency between populations of the five most common CFTR mutations (*i.e.*  $\Delta F508$ , G542X, N1303K, G551D and W1282X) are shown in Table 3.1. In view of the inclusion in this thesis of individuals residing in Australia, Bulgaria and Poland, comparisons are based on these three samples. The Australian sample is a heterogenous group comprising patients of diverse ethnic backgrounds. The majority of individuals are however of British descent, therefore, comparisons in CF allele frequencies are based on the reported frequencies of these mutations in the United Kingdom. As shown in Table 3.1, inter-population differences exist in terms of mutation allele frequencies between the three countries. The  $\Delta F508$  mutation is more common in the United Kingdom, whereas the G542X and N1303K mutations are more common in Bulgaria. The G551D is absent in Bulgaria and is found to occur on 3 chromosomes in Poland. The frequency of this mutation is 3.1% in the United Kingdom. The W1282X mutation is found to occur on two chromosomes in Bulgaria, one chromosome in Poland and 17 in the United Kingdom (Estivill *et al.*, 1997).

**Table 3.1: Frequency of the Five Most Common Cystic Fibrosis Mutations**

CF Mutation	Mutation frequency (%) by country		
	Bulgaria	Poland	United Kingdom
$\Delta F508$	64.2	66.2	75.3
G542X	4.7	2.3	1.7
N1303K	5.1	2.0	45*
G551D	0	3*	3.1
W1282X	2*	1*	17*

\* indicates the number of chromosomes with the mutation only (Data extracted from Estivill *et al.*, 1997)

In ADPKD, genetic differences are known to exist between countries in terms of the PKD genes and allele frequencies in other genes of relevance to ADPKD, namely components within the renin-angiotensin system (RAS) and in terms of the prevalence of hypertension.

The proportion of families linked to PKD1 or PKD2 varies between countries. The most comprehensive data relating to locus heterogeneity in ADPKD come from Peters and Sandkuijl (1992) who report on a large set of 322 families from 10 European countries. The proportion of families linked to PKD1 range from 0.71 in Greece to 1.00 in regions in Italy, England and France. The results are shown in Table 3.2. In a smaller study, Bogdanova *et al.* (1995) reported a frequency of PKD1 around 67% in Bulgaria. A study by Ravine *et al.* (1992), which included families residing in Britain and Australia, reported a frequency of PKD1 between 85-90%. The majority of Australian families in their study were of British descent; therefore we would expect that approximately 85-90% of Australian families in this study be PKD1 linked. Our results from linkage analysis are discussed in Chapter Four.

**Table 3.2: The Proportion of European Families Linked to *PKD1***

Country	Centre	Proportion of <i>PKD1</i> linked families
Iceland	Reykjavik	0.72
Denmark	Copenhagen	0.60
The Netherlands	Leiden	0.83
Belgium	Brussels	0.74
Federal Republic of Germany	Bonn	1.00
Wales	Cardiff	1.00
UK	Manchester	1.00
	London	1.00
	Edinburgh	0.87
France	Paris	1.00
	Brest	1.00
Greece	Athens	0.71
Italy	Genova	0.94
	Genova	0.78
	Ferrara	1.00
	Torino	1.00
	Verona	1.00
	Roma	0.91
Spain	Madrid	0.85

*Extracted from Peters and Sandkuijl, 1992*

The RAS has been studied in ADPKD because of its importance in the control of blood pressure (Bell *et al.*, 1988; Chapman *et al.*, 1990; Chapman and Gabow, 1997), and the reported associations between genetic variants within the RAS and essential hypertension (Jeunemaitre *et al.*, 1992b; Caulfield *et al.*, 1994) and renal disease progression (Fogarty *et al.*, 1996; Baboolal *et al.*, 1997; Perez-Oller *et al.*, 1999). Inter-population differences in allele frequencies of polymorphisms, namely an insertion/deletion (I/D) polymorphism in the angiotensinogen converting enzyme (ACE)

gene and microsatellite polymorphisms in the angiotensinogen (AGT) and renin genes, within the RAS have been reported. In terms of the ACE insertion/deletion (I/D) polymorphism, variation in the I/D allele frequency exists between populations (Table 3.3). The frequency of the D allele ranges between 0.3 in Chinese and Pima Indians to 0.64 in American Blacks, and the I allele ranges between 0.36 in American Blacks and 0.7 in Chinese and Pima Indians.

**Table 3.3: Inter-population Differences in ACE I/D Allele Frequencies**

Population	I:D ratio	Reference
Chinese	0.7:0.3	Lee <i>et al.</i> 1994
Pima Indians	0.7:0.3	Foy <i>et al.</i> 1996
Japanese	0.57:0.43	Mizui <i>et al.</i> 1995
Caucasians	0.43:0.56	Raynolds <i>et al.</i> 1993; Lindpaintner <i>et al.</i> 1996; Baboolal <i>et al.</i> 1997; van Dijk <i>et al.</i> 1999
American Blacks	0.36:0.64	Bloem <i>et al.</i> 1996

Differences in allele frequencies of a microsatellite polymorphism within the AGT gene have been reported in European populations (Table 3.4). The two studies presented in Table 3.4 comprise individuals from European populations, showing slight variation in AGT allele frequencies. The allele frequencies in the studies by Kotelevtsev *et al.* (1991) and Brand *et al.* (1998) are based on 100 unrelated European Caucasians and 350 sibships selected from centres in France, Germany, the Netherlands and the United Kingdom respectively.



**Table 3.4: Inter-population Differences in AGT Dinucleotide Repeat Alleles**

AGT alleles	Kotelevtsev <i>et al.</i> 1991	Brand <i>et al.</i> 1998
1	0.01	0.00
2	0.02	0.05
3	0.03	0.03
4	0.03	0.04
5	0.09	0.10
6	0.17	0.12
7	0.40	0.37
8	0.07	0.06
9	0.13	0.16
10	0.04	0.01
11	0.03	0.03

Inter-population differences in allele frequencies of a microsatellite polymorphism in the renin gene are also found (Table 3.5). Allele 4 is the most common allele among the four populations however, a slightly lower frequency is found in Blacks. Differences in alleles 1 to 3 are also observed between different racial groups.

**Table 3.5: Inter-population Differences in Tetranucleotide Repeat Alleles**

Renin alleles	Whites	Blacks	Mex- American	Asian
1	0.09	0.08	0.03	0.04
2	0.13	0.15	0.15	0.05
3	0.09	0.08	0.05	0.15
4	0.78	0.73	0.77	0.77

*Extracted from Edwards et al., 1992*

Based on the reported differences in allele frequencies between populations, we would also expect differences in allele frequencies between the three populations included in this thesis.

### **3.1.2      *Hypertension***

Hypertension is a complex trait associated with cardiovascular morbidity and mortality (Ward, 1990 p. 81-100; Lifton, 1996). The reported association between hypertension and renal disease progression in ADPKD (Bell *et al.*, 1988; Gabow *et al.*, 1990, 1992b; Chapman and Gabow, 1997) makes it an important candidate in the study of modifying factors in ADPKD.

There are many risk factors and predictors of hypertension including family history and genetic factors, body weight, diet, alcohol consumption, physical inactivity, psychosocial and socio-economic factors. Subsequently differences in the prevalence of hypertension exist between countries.

Population studies have consistently revealed higher blood pressure levels in black communities than other ethnic groups (Cooper and Rotimi, 1994; Klag *et al.*, 1997). Black Americans of African origin have also been demonstrated to have higher blood pressure levels than black Africans, suggesting the environmental augmentation of an ethnic predisposition. In countries that are experiencing economic change, consistently higher levels of blood pressure and a higher prevalence of hypertension have been noted in lower socio-economic groups. This inverse relationship has been noted with levels of education, income and occupation. This most likely represents the initial stage of the epidemic of cardiovascular disease (World Health Organisation Expert Committee on Hypertension Control: <http://www.who.int/ncd/cvd/trs862.html#s324>).

Comparisons in the prevalence of hypertension and mortality due to cardiovascular disease between Australia, Bulgaria and Poland are presented in Table 3.6. The prevalence of hypertension in Poland and Bulgaria is significantly higher than in Australia. Mortality due to hypertensive diseases is also highest in Bulgarians, intermediate in Poles and lowest in Australians (Global Cardiovascular Infobase: Country Profile: <http://cvdinfobase.ic.gc.ca/>).

In ADPKD, differences in the frequency of hypertension have been reported (Milutinovic *et al.*, 1984; Delaney *et al.*, 1985; Parfrey *et al.*, 1990a; Choukroun *et al.*, 1995). This variation may be due to the combined effects of the factors involved in the development of hypertension, as well as differences in the definition of hypertension, stage of ADPKD progression and the sensitivity and reliability of diagnostic instruments. In this study, it is therefore expected that differences in the prevalence of hypertension will be observed between the three countries.

**Table 3.6: Mortality Data Attributed to Cardiovascular Disease and the Prevalence of Hypertension in Australia, Bulgaria and Poland**

<b>Mortality Data</b>	<b>Australia (%)</b>	<b>Bulgaria (%)</b>	<b>Poland (%)</b>
All CVD (1994-95)	43.3	62.2	50.4
<b>Percentage (%) of all CVD-related deaths</b>			
Ischaemic heart disease	55.5	33.0	20.0
Cerebrovascular disease	23.5	33.0	15.0
Hypertensive Disease*	2.1	4.5	3.8
Rheumatic heart disease	0.6	0.5	1.2
Other CVD	18.3	29.0	60.1
<b>Prevalence Data</b>			
Hypertension (1995-96)	16.0	40.0	44.5

*CVD – cardiovascular disease; Source: Global Cardiovascular Infobase: Country Profile: (<http://cvdinfobase.ic.gc.ca/>); \* hypertensive disease includes essential hypertension, hypertensive heart disease, hypertensive renal disease and hypertensive heart and renal disease (WHO International Classification of Diseases, p. 262-263, 1975)*

### **3.1.3 Health status**

The white population in Australia is one of the healthiest in the world. This is shown in the declining death rates, increasing life expectancy, a low rate of life-threatening diseases and for most people, ready access to health care when needed. However, the Aboriginal and Torres Strait Islander people continue to experience much poorer health than the general Australian population. As of 1994, 99% of the Australian population were served with safe water and as of 1990, 90% of the population had adequate sanitary facilities. Life expectancy at birth as of 1995-1997 for Australian males is 75.6 years and 81.3 for females (World Health Organisation Regional Office for the Western Pacific: <http://www.who.org.ph/chip/default.htm>).

By contrast, life expectancy for both Bulgarians and Poles is significantly lower than Australians. Life expectancy at birth as of 1997 is 68 years for males in Bulgaria and Poland and 75 and 77 years for females in Bulgaria and Poland respectively (Global Cardiovascular Infobase: <http://cvdinfobase.ic.gc.ca/>).

### **3.1.4            *Environmental factors***

Apart from biological variables, environmental factors are also expected to affect disease and the overall health of a population. The following sub-sections discuss some environmental factors, which have a significant impact on the overall health of a population. These include the economic situation of a population, health care systems and diet and lifestyle. Again, comparisons in these factors will be based on the following three countries: Australia, Bulgaria and Poland.

#### **3.1.4.1            The economic situation**

Australia is a high income capitalist country, with an economy based on agriculture and minerals. In 1991, the gross national product (GNP) per capita (US\$) was 17,120. By contrast, Poland and Bulgaria are lower middle earning countries, which have undergone transitional reforms in their economy, moving from a command to a market oriented economy, primarily based on the service sector, however agriculture and industry also remain important. In comparison to Australia, in 1991 the GNP per capita (US\$) was 1840 and 1790 for Bulgaria and Poland, reflecting the economic status of these countries (Global Cardiovascular Infobase Country Profile: <http://cvdinfobase.ic.gc.ca/>).

#### **3.1.4.2            Health care systems**

Health care systems have a vital and continuing responsibility to people throughout their lifespan. They are crucial to the healthy development of individuals, families and societies everywhere. In 1996-97, Australia spent US\$32.2 billion on health services. The amount spent was 8.4% of Australia's gross domestic product (GDP). Almost 69% of this amount was government expenditure, the remaining being sourced from the private sector (Australian Institute of Health and Welfare: <http://www.aihw.gov.au/>). Poland spent 4.6% of GDP on health in 1994 and Bulgaria 4.8%, which is well below the Central and Eastern European average of 5.9% and the Western European average of 7.8% in 1994.

During the communist period in Bulgaria, the health care system provided free and accessible health care to all Bulgarian individuals. A network of health services was established across the country and many communicable diseases were largely

controlled. However, the centrally controlled health system lacked the capacity to respond to worsening health indicators. With the change in government in 1989, many elements of this model of health care had become discredited in Bulgaria. The goals for the future reforms include a public/private mix of services to ensure quality of care, efficient self government, system sustainability, and promoting equity in the health and medical care system (WHO Regional Office for Europe: <http://www.euro.who.int/countryinformation>).

Prior to 1989, the health system in Poland was fully socialist, with all resources being governmental. The total population was entitled to comprehensive health services from regionalized networks of hospitals, polyclinics and primary health stations. After the termination of socialism the health care system has remained largely unchanged but with greater emphasis on primary health care. Primary health care is moving towards a family doctor model aimed at improving the training, skills and facilities of family physicians. This model is intended to improve the quality of care and to establish family doctors as gatekeepers to secondary and tertiary healthcare (WHO Regional Office for Europe: <http://www.euro.who.int/countryinformation>). Contracts with health insurance funds and patient capitation funding are also intended to expand the range of primary care services and to increase the autonomy and incomes of family doctors. However, private expenditure on health is a significant proportion of total expenditure on health and in particular in financing outpatient care. This has contributed to a widening gulf between free public care and services available to those who are able and willing to pay. The overall aim of health care system in Poland will be split between the Ministry of Finance and Insurance Funding. Although, it remains to be seen whether these changes can maintain universal access to a wide range of services while leading to a real improvement in the quality of health services, consumer choice and health gains.

#### 3.1.4.3 Lifestyle

Apart from genetics and the physical and social environments, behaviour also influences the health of the population. It is recognised that, at least in most western European countries, improvement in lifestyle has largely been confined to the more socially and economically privileged population groups, who are better placed to adopt health-promoting changes in behaviour. Lifestyle risk factors are generally inter-related. For

example individuals that smoke regularly generally drink more alcohol and exercise less. A decrease in these risk factors is associated with a decline in cardiovascular disease and cancer.

In Australia the overall prevalence of smoking has declined dramatically since the early 1980s. Data from the Australian Institute of Health and Welfare in 1995, reported that 27% of males and 20% of females were current smokers. The decline in tobacco consumption in Australia is a result of health education and the allocation of Commonwealth funds to develop and implement a Tobacco and Health Campaign, targeted at young people (Australian Institute of Health and Welfare: <http://www.aihw.gov.au>).

In Central and Eastern European countries the prevalence of smoking remains high. By mid 1990's, 41% of Bulgarian men and 17% of Bulgarian women smoked regularly. According to the Bulgarian Countrywide Integrated Non-communicable Disease Intervention (CINDI), smoking rates and smoking intensity were higher in 1996/97 than in 1986/87. In 1996/97, the proportion of regular smokers varied between 29% and 58% for men and between 17% and 25% for women in the 25-64 years age groups (Highlights on Bulgaria, 2001). Poles are also heavy smokers, however since 1992, the implementation of anti-smoking campaigns and the enforcement of health policies resulted in a shift from unhealthy to health lifestyle patterns. As a result, the prevalence of smoking among Poles after 1992 has declined. Smoking prevalence among adults in Poland has decreased from 46% in 1986 (60% Male; 34% Female) to 39% in 1993 (51% Male; 29% Female) (WHO Tobacco or Health Programme: <http://www.cdc.gov/tobacco/whofirst.htm>).

In 1995, over 6% of Australian males and 4% of females (aged >15 years) reported drinking alcohol at levels dangerous to their health (Australian Institute of Health and Welfare: <http://www.aihw.gov.au>). Similarly, 4.3% of the Bulgarian population aged >15 years and 5.2% of the population aged 15-64 years reported drinking alcohol at dangerous levels to their health. A similar proportion is also expected for other Central and Eastern European countries.

Physical inactivity is a risk factor for coronary heart disease. It is estimated that up to 50% of Australians are physically inactive (Australian Institute of Health and Welfare: <http://www.aihw.gov.au/>). According to 1996/97 estimates, between 8-15% of Bulgarian individuals performed physical activity and the proportion regularly undertaking sports varied between 4-8% (WHO Regional Office for Europe: <http://www.euro.who.int/Document/E73818.pdf>). Again, similar estimates can be expected for other Central and Eastern European countries.

#### **3.1.4.4 Diet**

Economical conditions remarkably influence the food supply and nutrition within countries. The most significant changes have occurred in transitional societies as a result of increasing globalisation. Most countries in Asia, Latin America, Northern Africa, the Middle East and areas of sub-Saharan Africa have adopted a higher fat Western diet. This is expected to have significant implications on the health of these countries (Popkin, 2001).

In contrast to the general health of Australians, the Western diet includes a large increase in the consumption of fat and added sugar in the diet. Of people aged 20-69 years, 47% of men and 39% of women had a blood cholesterol >5.5 mmol/L. Moreover, 18% of Australians were estimated as being obese (Australian Institute of Health and Welfare: <http://aihw.gov.au/>).

The economic conditions in Central and Eastern European countries have also influenced diet and nutritional patterns, especially in lower socio-economic groups. The diet of Bulgarians follows the pattern for southern Europe except that the availability of fruit and vegetables appears to be low. The consumption of animal fats is however thought to be increasing, while the consumption of food containing protective elements has decreased, especially during winter and spring. In 1996/97, the CINDI survey reported that the consumption of fish, fruit and vegetables was insufficient and wholemeal bread was poorly represented in the diet. In addition, the intake of salt has traditionally been high, which may contribute to the higher cerebrovascular mortality in Bulgaria (see Table 3.5). Obesity in Bulgaria varied between 12-24% in men and 14-

39% for women (WHO Regional Office for Europe: <http://www.euro.who.int/Document/E73818.pdf>).

Conversely, since the transition of Poland's economy from socialism to a market economy, subsidies for animal fats have been cut and taxes imposed. This has led to price increases resulting in a positive shift to healthy eating behaviour (Zatonski *et al.*, 1998), including a greater consumption of margarine and vegetable oils and fruits such as oranges, bananas, kiwis and grape fruits available all year round. As a result, these changes are expected to improve future health outcomes.

The literature review has provided substantial evidence of the differences between countries in terms of the biological and environmental variables. Therefore it is expected that these differences may modify disease progression in ADPKD.

### **3.1.5**      *Aim of the study*

To compare clinical findings (*i.e.* age at onset and rate of progression to renal failure, ultrasound findings and hypertension) between the patients grouped according to country of origin.

## **3.2**              **SUBJECTS AND METHODS**

### **3.2.1**      *Subjects*

The study included 322 patients: 124 from Australia, 128 from Bulgaria and 70 from Poland.

### **3.2.2**      *Statistical Analysis*

Statistical procedures are discussed in Chapter Two, section 2.7. The number of individuals included in the comparison between countries for age and cumulative renal survival is shown in Table 3.7.



**Table 3.7: Number of Individuals Included in the Comparisons between Countries for Age and Cumulative Renal Survival to CRF and ESRF**

Variable	Australian	Bulgarian	Polish	Number of Individuals
Age at/cumulative survival to:				
CRF (observed)	30	41	33	104
CRF (observed & extrapolated)	107	121	61	289
ESRF*	33	27	9	69
To ESRF (patients with & without ESRF)	124	128	70	322

*\* ESRF – patients who have reached renal replacement therapy or have had a renal transplant*

**3.3 RESULTS**

**3.3.1 General Characteristics of ADPKD Patients**

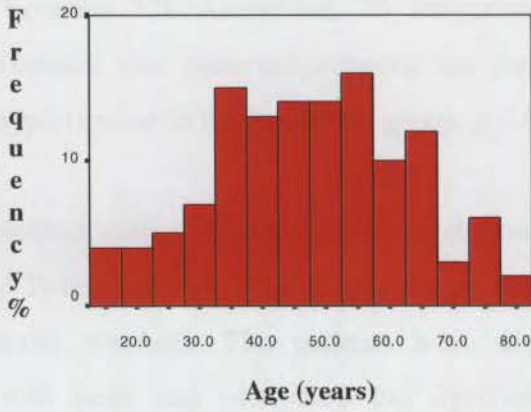
The general characteristics of the patients included in the comparison of phenotype severity between the three countries are provided in Table 3.8. The mean current age of the Australian sample was 47.4 (range: 14-81 years), Bulgarian 46.9 (range: 16-86 years) and Polish 40.9 years (range: 10-86 years)(see Figure 3.1).

**Table 3.8: Demographic and Clinical Characteristics of Patients Included in the Country Comparisons**

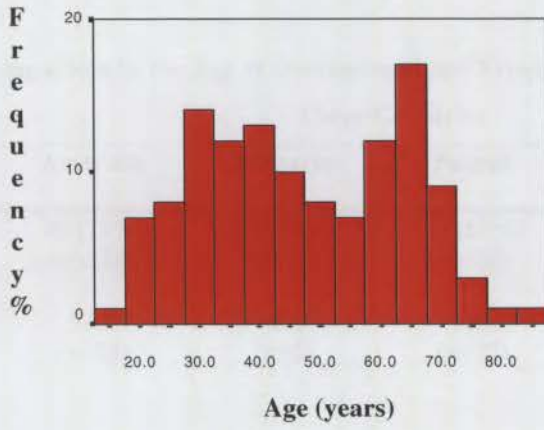
Variable	Country of residence			
	Australia	Bulgaria	Poland	Total
Number of families	69	32	16	116
Number of individuals	124	128	70	322
Mean current age (yr)	47.4±1.4	46.9±1.5	40.9±1.7	45.1±1.5
Number with CRF	47 (38%)	54 (42%)	32 (46%)	133 (41%)
Number with ESRF	33 (27%)	27 (21%)	9 (13%)	69 (21%)

*CRF - chronic renal failure; ESRF - end-stage renal failure*

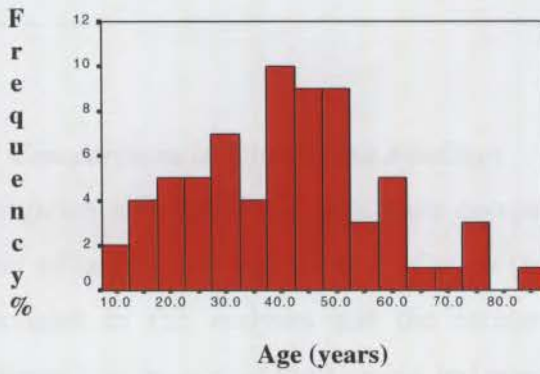
A



B



C



**Figure 3.1:** Age Distribution in the Three ADPKD Populations. A - Australian, B - Bulgarian and C - Polish populations

3.3.2            *Hypertension*

Hypertension was examined in two ways. The first analysis compared the frequency of hypertension between 118 Australian, 76 Bulgarian and 70 Polish individuals. A significant difference was observed between the three countries, revealing a higher frequency of hypertension in the Bulgarian group,  $p = 0.002$ .

The second method compared the age at development of hypertension between the Australian and Polish subjects. The Bulgarian group was excluded from this analysis because of small numbers. The patients were divided into two groups: age at development <40 years and >40 years. No significant difference in the number of patients who developed hypertension <40 or >40 years between Australia and Poland was found ( $p>0.05$ , Table 3.9)

**Table 3.9: Comparison in the Age at Development and Frequency of Hypertension between the Three Countries**

	Australia	Bulgaria	Poland	Total	<i>p</i> value
Presence of hypertension	86 (73%) (n=118)	67 (88%) (n=76)	43 (61%) (n=70)	196 (74%) (n=264)	<b>0.002</b>
Average age at development hypertension (yrs)	32.9 (n=58)	42.6 (n=5)	35.0 (n=27)	36.8 (n=90)	>0.05 (Aust vs Pol)
<40 years	44 (76%)	2 (40%)	19 (70%)	65 (72%)	>0.05 (Aust vs Pol)
>40 years	14 (24%)	3 (60%)	8 (30%)	25 (28%)	>0.05 (Aust vs Pol)

3.3.3            *Comparisons in Ultrasound Findings*

Australian, Bulgarian and Polish subjects were compared as regards to mean kidney length, diameter of largest cyst and number of cysts (adjusted for age). The number of individuals included in the analyses and the results are shown in Table 3.10. A significant difference in the size of the kidneys and number of cysts was found between countries,  $p<0.01$  (Table 3.10). Mean kidney length was greatest in the Polish group, and the mean number of cysts was highest in the Polish and Bulgarian samples. No significant difference in the diameter of the largest cysts was observed ( $p = 0.28$ ).

**Table 3.10: Comparison in Ultrasound Findings between the Three Countries**

Variable	Australia	Bulgaria	Poland	Total	p value
Mean kidney length (mm)	138.5±2.9 (n=73)	132.4±3.8 (n=43)	156.1±2.9 (n=69)	142.3±3.2 (n=185)	<b>&lt;0.01</b>
Mean diam of largest cyst (mm)	33.3± 1.9 (n=71)	27.9±3.1 (n=26)	33.5±1.9 (n=69)	31.6±2.3 (n=166)	0.28
Mean number of cysts	7.4±0.3 (n=69)	8.2±0.4 (n=43)	8.9±0.3 (n=69)	8.2±0.3 (n=181)	<b>0.01</b>

*Diam – diameter*

### 3.3.4 Renal Failure

The progression to renal failure was examined by comparing Australian, Bulgarian and Polish subjects as regards to mean age at renal failure (CRF or ESRF) and in terms of Kaplan-Meier cumulative survival curves. The analyses were repeated separately by sex.

#### 3.3.4.1 Age at Renal Failure

##### *Observed age at renal failure*

The initial analysis compared the mean age at CRF and ESRF between countries in subjects who had reached the respective stage of renal failure. The number of individuals and the results are shown in Table 3.11. No significant differences were found. The mean age at CRF was 46.7 years for Australia, 46.5 for Bulgaria and 43.8 for Poland,  $p = 0.53$ . The mean age at ESRF was 53.3 years for Australia, 56.5 for Bulgaria and 51.4 for Poland,  $p = 0.25$ .

**Table 3.11: Comparisons in Observed Age at CRF and ESRF between the Three Countries**

Age at renal failure	Australia	Bulgaria	Poland	Total	p value
CRF (observed age)	46.7±2.1 (n=30)	46.5±1.8 (n=41)	43.8±1.8 (n=33)	45.7±1.2 (n=104)	0.53
ESRF	53.3±1.7 (n=33)	56.5±1.7 (n=27)	51.4±3.2 (n=9)	53.7±1.3 (n=69)	0.25

*Extrapolated age at renal failure*

For patients where the observed age at CRF was unknown, this value was derived by extrapolation (as explained in Chapter Two, section 2.7). The data on all patients using either observed or extrapolated ages were re-analysed. The results are shown in Table 3.12. No significant differences were found for CRF ( $p = 0.36$ ).

**Table 3.12: Observed and Extrapolated Age at Renal Failure by Country**

Age at renal failure	Australia	Bulgaria	Poland	Total	<i>p</i> value
CRF (observed or extrapolated age)	41.4±1.4 (n=107)	39.4±1.3 (n=121)	38.4±1.7 (n=61)	39.7±0.8 (n=289)	0.36

**3.3.4.2 Cumulative Survival to Renal Failure**

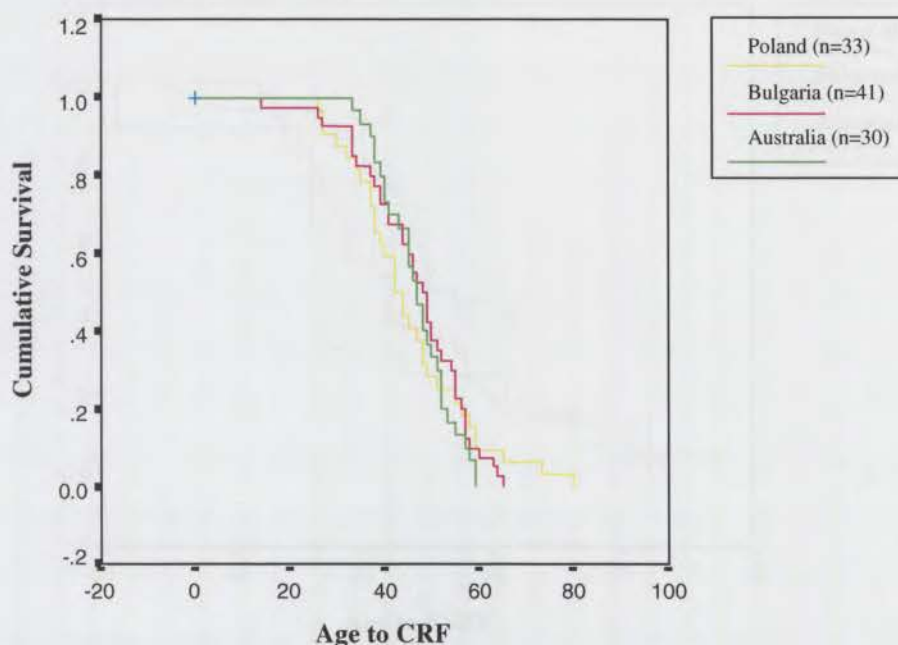
The second approach to comparing the deterioration of renal function between countries was based on Kaplan-Meier cumulative survival curves to CRF and ESRF. The results are shown in Figures 3.2-3.5.

No statistical difference in cumulative renal survival to CRF and ESRF was found between patients from the three countries (CRF:  $p = 0.82$ ,  $p = 0.81$ ; ESRF:  $p = 0.78$ ;  $p = 0.83$ ; Figure. 3.2-3.5).

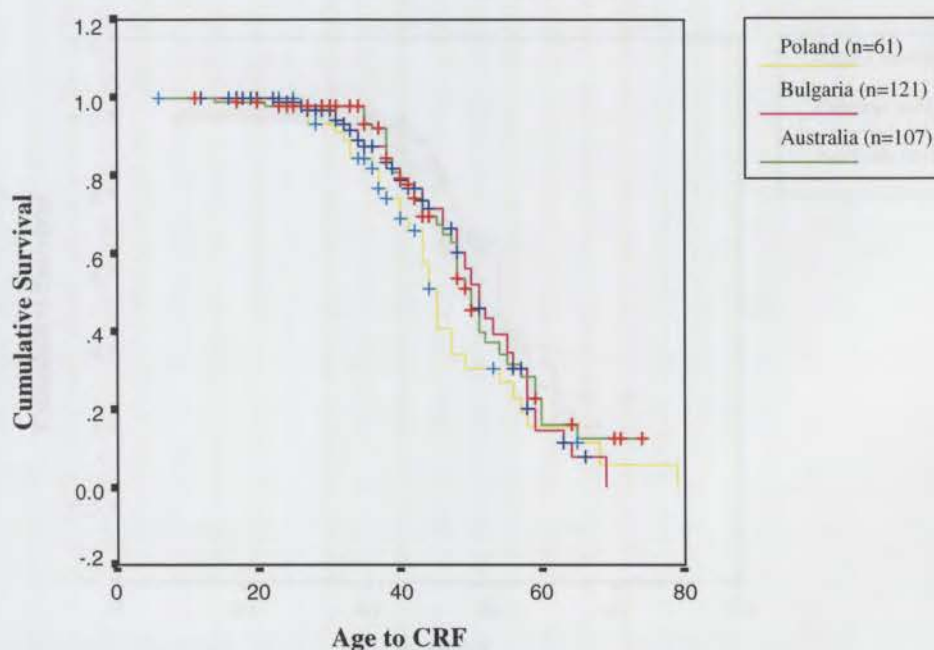
**3.4 DISCUSSION**

As the first component of this thesis, this part of the study aimed to examine whether inter-population differences affect renal disease progression in ADPKD. It is well recognised that populations differ in genetic composition (Rosser *et al.*, 2000) and in terms of diet and lifestyle and socioeconomic status, which are expected to impact on disease outcome and on the overall health of the population.

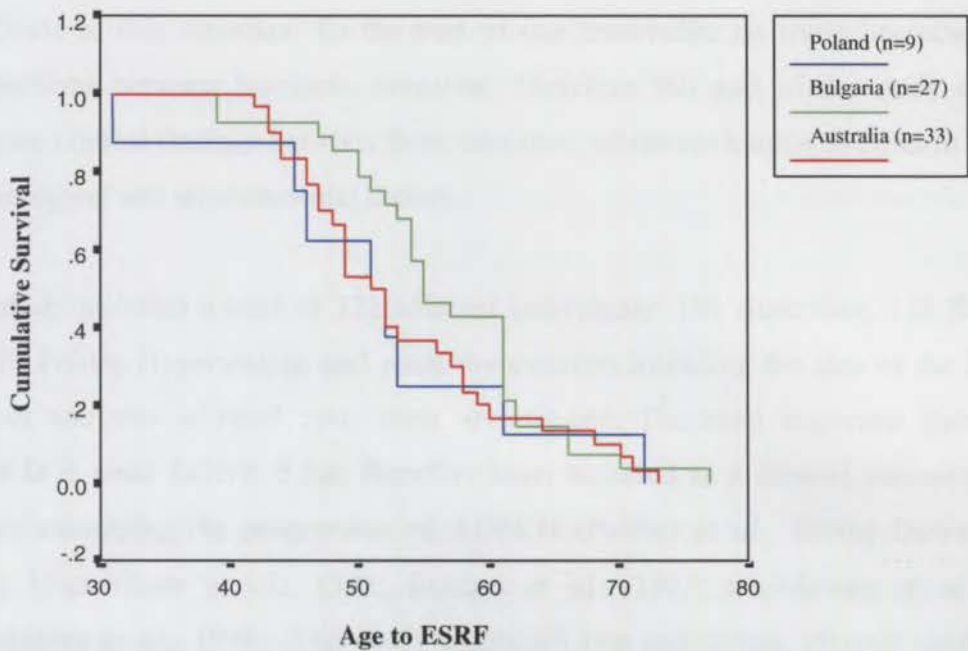
In ADPKD, several studies have identified factors including locus heterogeneity, hypertension, sex, germ line and somatic mutations (Ravine *et al.*, 1992; Gabow *et al.*, 1992b; Choukroun *et al.*, 1995; Peters and Breuning, 2001) as possible modifiers. Black race has also been suggested to be associated with a worse renal outcome in ADPKD in some studies (Yium *et al.*, 1994; Kimberling *et al.*, 1996; Heifner *et al.*, 1998) and not in others (Freedman *et al.*, 2000). However, the results of the studies investigating



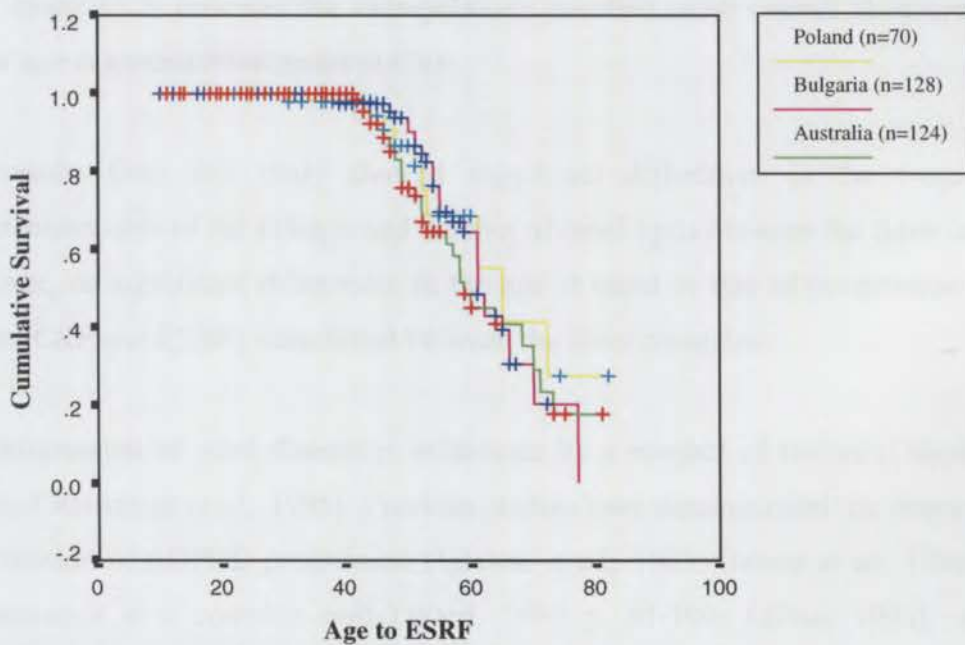
**Figure 3.2:** Survival Analysis to CRF (observed age) between the Three Countries. *No significant difference in renal survival to CRF (observed age) was found between the three countries ( $p=0.82$ )*



**Figure 3.3:** Censored Survival Analysis to CRF (observed and extrapolated age) between the Three Countries. *No significant difference in renal survival to CRF (observed and extrapolated age) was found between the three countries ( $p=0.81$ )*



**Figure 3.4:** Survival Analysis to ESRF in Patients with ESRF between the Three Countries. *No significant difference in renal survival to ESRF in patients with ESRF between the three countries ( $p=0.78$ )*



**Figure 3.5:** Censored Survival Analysis to ESRF between the Three Countries in all Patients. *No significant difference in renal survival to ESRF in all patients between the three countries ( $p=0.83$ )*

factors modifying ADPKD severity are inconclusive and apart from differences in study design, small numbers and ascertainment, inter-population differences may also contribute to this variation. To the best of our knowledge no study has made direct comparisons between European countries. Therefore this part of the study aimed to compare clinical findings between three countries, which are known to differ in terms of the biological and environmental factors.

The study included a total of 322 affected individuals: 124 Australian, 128 Bulgarian and 70 Polish. Hypertension and renal involvement including the size of the kidneys, number and size of renal cysts were investigated. The most important outcome of ADPKD is renal failure. It has therefore been included as a clinical parameter in all studies examining the progression of ADPKD (Parfrey *et al.*, 1990a; Davies *et al.*, 1991; Higashihara *et al.*, 1992; Roscoe *et al.*, 1993; Choukroun *et al.*, 1995; Higashihara *et al.*, 1998). This study examined two end points: chronic renal failure (CRF) and end-stage renal failure (ESRF), using two approaches, namely age at onset and cumulative renal survival. For CRF, the analyses were performed twice. The initial analysis included observed age, and the subsequent analysis included extrapolated age obtained from the individual curves of time versus  $1/\text{Cr}$  values. The findings were similar for the age at renal failure extrapolated from the individual regression lines of time versus  $1/\text{Cr}$ , however the extrapolations resulted in an overall shift towards an earlier age at around 39-40 years at CRF.

The results from this study showed significant differences in the frequency of hypertension, size of the kidneys and number of renal cysts between the three countries. However, no significant differences in the age at onset or rate of progression to renal failure (CRF and ESRF) were found between the three countries.

The progression of renal disease is influenced by a number of factors (Gabow *et al.*, 1992b; Choukroun *et al.*, 1995). Previous studies have demonstrated the importance of hypertension in ADPKD progression (Iglesias *et al.*, 1983; Gabow *et al.*, 1990; 1992). Hypertension is a complex trait (Ward, 1990 p. 81-100; Lifton, 1996), which is influenced by numerous factors including genes, sex, age, ethnicity and socioeconomic status (Whelton, 1994; Lifton, 1996). Subsequently, the prevalence of hypertension



varies widely between countries. Therefore it was not surprising that we observed differences in the frequency of hypertension between the three countries. The reported frequency in the Bulgarian sample was 88%, which may indeed reflect differences in the factors influencing blood pressure since the age and number of patients in this group is similar to the Australian sample, who reported a lower frequency of 73% (although the frequency of hypertension is still high). In Poland, the frequency of hypertension is relatively high (WHO Cardiovascular Infobase: <http://cvdinfobase.ic.gc.ca/>), however in this study, the Poles reported the lowest frequency of 61%. This however may reflect the younger age and hence lower number of Poles with ESRF.

Renal involvement *i.e.* the size of the kidneys and number and size of the cysts is also reported to be associated with the degree of renal impairment in ADPKD (King *et al.*, 2000). Previous studies have found a significant positive correlation between the rate of increase in renal cyst volume and the rate of decline in GFR in ADPKD (Gabow *et al.*, 1990; 1992b; King *et al.*, 2000). In this study the Poles showed significantly greater renal involvement in terms of larger kidneys and number of cysts. Kidney size was approximately 24 mm larger than the Bulgarians and 17 mm larger than the Australians. A significantly greater number of renal cysts were also found in the Bulgarians and Poles compared to the Australians, although no significant difference in the size of the largest cyst was found between the three countries. It is probable that the greater degree of renal involvement in the Poles may be in keeping with the two-hit model of cystogenesis proposed in ADPKD (Qian *et al.*, 1996; Brasier and Henske, 1997).

Based on the observed differences in the frequency of hypertension and ultrasound findings, we anticipated significant variation in the age at onset and rate of progression to renal failure between the three countries. Therefore, it was surprising that no significant difference in the age at onset and rate of progression of renal failure (CRF and ESRF) was found. However, these findings are in agreement with an earlier study that showed that racial variation does not affect renal disease progression (Freedman *et al.*, 2000). In their study, the age at first dialysis was compared between 57 black Americans and 180 white Caucasians. The mean age at dialysis was found to be similar between blacks and whites, regardless of family history (FH) (black FH positive, 63.8;

black FH negative, 66.3;  $p=0.66$ ; white FH positive, 60.8; white FH negative, 62.8;  $p=0.48$ ).

By contrast, previous reports have suggested that blacks with ADPKD enter ESRF at an earlier age than white counterparts (Yium *et al.*, 1994; Kimberling *et al.*, 1996; Heifner *et al.*, 1998). Yium *et al.* (1994) compared the age at ESRF between 23 black African American ESRF patients with ADPKD and the sickle-cell trait, and 49 white ESRF patients with ADPKD. Significantly earlier age at onset of ESRF was found in blacks (43.2 years) compared to whites (55.4 years). As a continuation of their study, Kimberling *et al.* (1996) described a black African American family with the sickle-cell trait and ADPKD who were found to enter ESRF 16 years (37.6 years) earlier than previously reported from their white male population (53.8 years). However, this family by chance may have inherited a mutation leading to a more severe clinical course of ADPKD. Moreover, the combined effects of the sickle-cell trait and ADPKD may have exacerbated renal disease progression. In sickle-cell trait, recurrent haematuria and medullary vascular changes may occur, which could also affect the course of ADPKD (De Jong and van Epps, 1985). Heifner *et al.* (1998) also compared renal survival in 64 African Americans and 611 Caucasians with ADPKD. Their study showed that median renal survival was 49 years in African Americans compared to 55 years in Caucasians, however, the results were non-significant. In addition, no information on whether the African Americans had the sickle-cell trait was indicated in their study. The findings from these studies are inconclusive because of the small number of blacks with ADPKD and because the majority of studies included blacks with the sickle-cell trait, which in itself may influence renal function over time regardless of the presence of ADPKD (Freedman *et al.*, 2000).

Based on these reports, further studies are required to determine whether inter-population differences are an important determinant of renal disease progression in ADPKD. In the case of our study, further follow-up is also necessary especially in larger samples since we did observe a higher frequency of the Polish individuals with renal failure and earlier age and rate of renal disease. This is particularly noticeable given that the mean age of the Polish patients is approximately 10 years younger than the Australian and Bulgarian groups. This empirical observation of a more severe

clinical course in this group would be consistent with the two-hit hypothesis. However, the size of the populations did not provide sufficient power to detect a significant between-group difference.

However, the effect of locus heterogeneity as one of the main determinants of phenotypic heterogeneity in ADPKD was not found in this part of the study. The observation that *PKD2* is a milder form of renal disease in ADPKD would have suggested that the Bulgarians would have reached renal failure later, since the reported prevalence of *PKD2* in Bulgaria is approximately 20-30% (Bogdanova *et al.*, 1995). Approximately 20% of the Bulgarian sample in this study is linked to the *PKD2* locus therefore any milder effects of *PKD2* in this group should have been expressed. However, Bogdanova *et al.* (1995) previously reported no significant difference in the clinical course, namely the frequency of hypertension and renal failure between *PKD1* and *PKD2* patients in their study. In fact, the *PKD2* families in this study belonged to the group of Bogdanova *et al.* (1995), hence these families may carry mutations which are specific to this group, leading to a more severe ADPKD phenotype. Moreover, the combination of genetic background and environmental factors may predispose these individuals to a more severe form of ADPKD.

Based on these findings we can only speculate that environmental, health care and other differences exert effects mostly in the preclinical stage, and that once a certain stage of kidney impairment is reached, progression is irreversible. However, this study does have a distinct selection bias towards patients and families with clinically manifest ADPKD. True differences and their full extent could be assessed in a population-based study of mutations in the PKD genes and prospective follow-up of the outcomes.

Based on the overall findings of this study, the lack of significant effect allowed us to combine the three groups as one and examine the influence of additional factors in this study.

# **CHAPTER FOUR: GENETIC LINKAGE ANALYSIS AND THE RELATIONSHIP BETWEEN PHENOTYPE SEVERITY AND LOCUS HETEROGENEITY**

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# CHAPTER FOUR: GENETIC LINKAGE ANALYSIS AND THE RELATIONSHIP BETWEEN PHENOTYPE SEVERITY AND LOCUS HETEROGENEITY

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## 4.1 INTRODUCTION AND LITERATURE REVIEW

Locus heterogeneity appears to account, at least in part, for the variable phenotype of ADPKD and the differences observed between affected families. At least three genes are believed to be involved in the development of the disease. In the majority of ADPKD families of European descent the gene responsible is *PKD1*, located on chromosome 16p13.3 (Reeders *et al.*, 1985). A second gene, *PKD2*, has been assigned to chromosome 4q21-22 (Kimberling *et al.*, 1993; Peters *et al.*, 1993). In a small number of families, linkage to either *PKD1* or *PKD2* has been excluded and the disease gene(s) remain to be localised (Bogdanova *et al.*, 1995; Daoust *et al.*, 1995; de Almeida *et al.*, 1995). More recently, two members of the *PKD2* gene family, namely *PKD2L* and *PKD2L2* have been identified and localised to chromosomes 10q24-25 and 5q31. These genes may represent candidates for the ADPKD genes in families unlinked to *PKD1* and *PKD2*. Variation in the frequency of the common genetic forms of ADPKD has been observed, with a higher frequency of *PKD2* in the Balkans (Bogdanova *et al.*, 1995), Iceland (Fossdal *et al.*, 1993), Spain (Coto *et al.*, 1995) and Cyprus (Constantinou-Deltas *et al.*, 1995).

The different genetic forms of ADPKD share the same main clinical features, however it has been suggested that mutations in *PKD1* confer a more severe clinical phenotype than *PKD2* in terms of age at onset, prevalence of complications and development of end-stage renal failure (ESRF) (Parfrey *et al.*, 1990a; Gabow *et al.*, 1992b; Ravine *et al.*, 1992; Roscoe *et al.*, 1993; Wright *et al.*, 1993; Constantinou-Deltas *et al.*, 1995; Coto *et al.*, 1995). Several studies have examined the relationship between phenotype and locus heterogeneity. The use of different clinical parameters as measures of disease severity has resulted in a wide, often overlapping, range of assessments. The variable results could also be due to differences in study design and sample size, as well as to

biological factors, such as the nature of mutations, genetic background, lifestyle and environment.

#### **4.1.1            *Locus Heterogeneity in ADPKD***

##### **4.1.1.1            PKD1**

The first gene associated with ADPKD, *PKD1*, was mapped to 16p near the  $\alpha$ -globin cluster by Reeders *et al.* (1985). Its location was later specified as 16p13.3 (Somlo *et al.*, 1992). Subsequent studies used recombination mapping and a new set of polymorphic markers that had been physically mapped to the ADPKD interval, to place the *PKD1* locus in an interval of approximately 500 kb at position 16p13.3 (Somlo *et al.*, 1992).

The *PKD1* gene was cloned in 1994 (The European Polycystic Kidney Disease Consortium, 1994). Its complete sequence and the predicted protein structure and function were published by the European Polycystic Kidney Disease Consortium, 1995; International Polycystic Kidney Disease Consortium, 1995 and the American Polycystic Kidney Disease Consortium, 1995. The *PKD1* protein, named Polycystin 1 (PC1) is predicted to be a large membrane-associated glycoprotein, containing 4302 amino acids, involved in cell-cell and cell-matrix differentiation (see Chapter One, section 1.5.2 for description of PC1).

##### **4.1.1.2            PKD2**

The existence of different genetic forms of ADPKD was suggested by studies of ADPKD families from Italy where two large kindreds failed to show linkage to chromosome 16 markers (Kimberling *et al.*, 1988; Romeo *et al.*, 1988). This was the first indication that ADPKD can be caused by mutations in a second gene.

The chromosomal location of *PKD2* was determined by Kimberling *et al.* (1993) who placed the gene on 4q, to an interval of approximately 9cM flanked by the markers D4S231 and D4S414 (Kimberling *et al.*, 1993). Refined genetic mapping of the second ADPKD locus was conducted in an international collaborative study (Peters *et al.*, 1993) including 2 families from Australia, 2 from the Netherlands, and 1 each from

Iceland, Denmark, Cyprus and Canada. No evidence of further locus heterogeneity was found in this study.

The *PKD2* gene was cloned by Mochizuki *et al.* (1996). Polycystin 2 (PC2) is predicted to be an ion channel protein (see Chapter One, section 1.5 for discussion on PC2).

#### **4.1.1.3 Possible additional loci**

Data on families where ADPKD is not linked to any of the known loci point to the existence of at least one additional gene responsible for the development of the disease. Evidence of such families has been reported from Bulgaria (Bogdanova *et al.*, 1995), Portugal (de Almeida *et al.*, 1995) and Quebec (Pei *et al.*, 1998). The genes involved remain to be identified.

The cloning of a third gene encoding a member of the polycystin superfamily, polycystin-L (*PKDL*) has been reported recently (Nomura *et al.*, 1998). *PKDL* has 50% amino acid sequence identity and 71% homology to polycystin-2 and bears striking sequence and structural resemblance to the pore-forming  $\alpha 1$  subunits of  $\text{Ca}^{2+}$  channels, suggesting that *PKDL* may function as a subunit of an ion channel. *PKDL* was assigned to 10q24 by fluorescence *in situ* hybridization and linked to marker D10S603 by radiation hybrid mapping (Nomura *et al.*, 1998).

#### **4.1.1.4 The polycystin complex**

The striking similarity in clinical presentation between the two most recognised forms of ADPKD suggests that PC1 and PC2 interact and may participate in a common pathway. Recent data do indeed suggest that PC1 and PC2 form a complex (discussed in Chapter One, section 1.5.3).

### **4.1.2 Ethnic Heterogeneity Revealed in Linkage Studies**

Differences in the frequency of the genetic forms of ADPKD have been demonstrated in European populations (Table 4.1). *PKD2* has been shown to account for about 10-15% of ADPKD in North-Western Europeans (Peters and Sandkuijl, 1992). However, in South-Eastern Europe (Bogdanova *et al.*, 1995; Constantinou-Deltas *et al.*, 1995) and

Iceland (Fosssdal *et al.*, 1993) the reported frequency of PKD2 is estimated to range between 30 and 40%.

There is limited information on the prevalence of ADPKD and the frequency of its different genetic forms in non-European populations. The reported frequency of PKD1 in Japan is estimated to be about 81% and 10% for PKD2 (Mizoguchi *et al.*, 2002). In a comparison in the phenotypes between PKD1- and PKD2- linked patients, Mizoguchi *et al.* (2002) reported no difference in clinical severity between the two groups. It is interesting to note that the study of ADPKD in Bulgaria included two Romani (Gypsy) families, both linked to PKD2 (Bogdanova *et al.*, 1995). A comparison in the clinical outcome between PKD1 and non-PKD1 linked patients in the Bulgarian study failed to reveal any significant differences in terms of age at onset of CRF and ESRF and in the prevalence of hypertension (Bogdanova *et al.*, 1995). Moreover, clustering of intracranial aneurysms (ICA) has been reported in one of the Gypsy families. A high incidence of ADPKD and ICA has been previously documented among Hungarian Gypsies who also present with a more severe phenotype (Tauszik *et al.*, 1989). The results of these studies suggest that the Bulgarian and Hungarian Romani patients may share the same founder mutation in PKD2, conferring a severe phenotype.



**Table 4.1: Estimates of the Frequency of the Different Genetic Forms in European Populations**

<b>Group/year</b>	<b>Number of Families</b>	<b>Geographic region or Ethnicity</b>	<b><i>PKD1</i></b>	<b><i>PKD2</i></b>	<b>non <i>PKD1</i></b>	<b>non <i>PKD1/2</i></b>	<b>non informative</b>
Parfrey <i>et al.</i> 1990a	17	Canadian	10 (60%)		2 (12%)		5 (28%)
Peters & Sandkuijl, 1992	328	Dutch, British, French, Greek, Italian, Spanish, Belgium, German, Danish, Icelandic	284 (86%)		44 (14%)		
Ravine <i>et al.</i> 1992	46	British	41 (89%)		5 (11%)		
Wright <i>et al.</i> 1993	15	Irish	11 (73%)		2 (13%)		2 (13%)
Fossdal <i>et al.</i> 1993	7	Icelandic	4 (57%)		3 (43%)		
Peral <i>et al.</i> 1993	31	Spanish	26 (84%)		3 (9.6%)		2 (6.4%)
Coto <i>et al.</i> 1995	17	Spanish	12 (70%)	5 (30%)			
Bogdanova <i>et al.</i> 1995	22	Bulgarian Gypsy	16 (80%)	3 (15%) 2 (100%)		1 (5%)	
Constantinou-Deltas <i>et al.</i> 1995	7	Cypriot	4 (57%)	3 (43%)			
Daoust <i>et al.</i> 1995	23	French-Canadian	7 (30%)			1 (5%)	15 (65%)
Torra <i>et al.</i> 1995	49	Spanish	44 (90%)	5 (10%)			

4.1.3                    *Phenotypic Differences Between PKD1 and PKD2 Patients*

One of the features of ADPKD is the considerable inter-familial variation of disease severity (Torra *et al.*, 1995, 1997). The existence of different genetic forms of ADPKD is one likely cause of this variation in phenotype. The differences in the predicted structure and functionally important domains of PC1 and PC2 suggest different physiological roles, which may contribute to the observed differences in disease severity between patients with germline mutations in one or the other gene.

Several studies have investigated the clinical characteristics of ADPKD in relation to locus heterogeneity. The results are summarised in Table 4.2 and presented in more detail in Table 4.3.

**Table 4.2: General Phenotypic Differences between PKD1 and PKD2**

Phenotypic features	PKD1	PKD2	Studies
ADPKD diagnosis	Earlier	Later	2, 3, 6, 7 (over page)
Prevalence of hypertension	Higher	Lower	2, 3, 5, 7 (over page)
Age at diagnosis of hypertension	Earlier	Later	5, 6, 7 (over page)
Renal cysts at diagnosis	More numerous	Fewer	2, 3, 5, 7 (over page)
Risk of progression to renal failure	Higher	Lower	1, 2, 4, 5, 6, 7 (over page)

The published data indicate that patients with mutations in *PKD1* are generally more severely affected compared to *PKD2*. However, the clinical parameters investigated and the reported outcomes vary substantially between studies. Note that all features other than the age of death or dialysis are frequently asymptomatic and hence are subject to ascertainment bias.

**Table 4.3: Studies that have Assessed Genotype/Phenotype Correlations in PKD1 and Non-PKD1 Families**

Group/ Year/ Number	Ethnic origin	Number of Subjects		Age at Diagnosis		Hypertension		Development of Renal Cysts		Extra-renal Cysts		Age at CRF		Age at ESRF		Median Age of Survival to ESRF	
				PKD1	PKD2	PKD1	PKD2	PKD1	PKD2	PKD1	PKD2	PKD1	PKD2	PKD1	PKD2	PKD1	PKD2
Parfrey <i>et al.</i> , (1990a) (1)	European-American	125	PKD1	earlier	later	24% <20yr	14% 20-39	46% <30yrs	11% <30yrs	not tested	not tested	40	>60	56.7	69.4	not tested	not tested
		27	non-PKD1			28% 20-39	yr								p<0.01		
						61% 40-59	25% 40-59										
						80% ≥60	67% ≥60										
Ravine <i>et al.</i> , (1992) (2)	English, Italian	285	PKD1	44.8	69.1	Odds ratio (0.52 non-PKD1 vs PKD1)		greater	lower	not tested	not tested	not tested	not tested	not tested	not tested	56.0	71.5
		49	non-PKD1											tested	tested		
Wright <i>et al.</i> , (1993) (3)	Northern Irish	49	PKD1	25	37 p<0.001	29%	12%	not tested	not tested	26%	58%	(29-53)	(58-67)	65%	25%	not tested	not tested
		17	non-PKD1											ESRF by 54 yrs	ESRF by 54 yrs		
Coto <i>et al.</i> , (1995) (4)	Spanish	77	PKD1	not tested	not tested	57%	8% <40yr;	81.%	17% <20yr	early	late	not tested	not tested	47.6	56.3	not tested	not tested
		36	PKD2			<40yrs;	33% >51yr	<20yr									
Bogdanova <i>et al.</i> , (1995) (5)	Bulgarian	95	PKD1	not tested	not tested	50.5%	31.5%	not tested	not tested	16%	5%	45.4	47.9	50.9	52.0	not tested	not tested
		57	PKD2				p>0.05						p>0.05		p>0.01		
Torra <i>et al.</i> , (1996) (6)	Spanish	146	PKD1	27.4	41.4	34.8 yrs	49.7 yrs	94% 30yrs;	67% <30yr;	similar &	similar &	not tested	not tested	53.4	72.7	52.0	71.0
		20	PKD2		p=0.0002		p=0.001	100%>30yr	100%	related to age	related to age				p<0.01		
Hateboer <i>et al.</i> , (1999b) (7)	Spanish, Welsh, Bulgarian, Dutch, English	333	PKD1	42.0	56.0	Odds ratio (0.25 non-PKD1 vs PKD1)		not tested	not tested	not tested	not tested	not tested	not tested	54.3	74.0	53.0	69.1
		291	PKD2														

#### 4.1.3.1 Age at onset of ADPKD

Age of onset of the disease has been documented in four studies, where age at diagnosis was chosen as the primary clinical indicator. The reported range was 25-45 years for PKD1 and 37-69 years for PKD2 (Ravine *et al.*, 1992; Wright *et al.*, 1993; Torra *et al.*, 1996; Hateboer *et al.*, 1999b). Although the data suggest an earlier onset in PKD1, there is a significant overlap in the reported ranges. Three studies have not recorded this parameter (Parfrey *et al.*, 1990a; Bogdanova *et al.*, 1995; Coto *et al.*, 1995).

#### 4.1.3.2 Cystogenesis

The age at which renal cysts develop was examined in four studies. In three of these studies, renal cysts were found in 46-94% of PKD1 subjects below 30 years of age (Parfrey *et al.*, 1990a; Coto *et al.*, 1995; Torra *et al.*, 1996), and in 100% above that age (Torra *et al.*, 1996), while in PKD2 the proportion ranged between 11% and 67% for subjects of the <30 age group (Parfrey *et al.*, 1990a; Coto *et al.*, 1995; Torra *et al.*, 1996). In three studies, the age at development of renal cysts was not investigated (Wright *et al.*, 1993; Bogdanova *et al.*, 1995; Hateboer *et al.*, 1999b).

The presence of extra-renal cysts was examined by four studies (Wright *et al.*, 1993; Bogdanova *et al.*, 1995; Coto *et al.*, 1995; Torra *et al.*, 1996) (Table 4.3). One study detected a higher prevalence of extra-renal cysts in PKD2 patients (Wright *et al.*, 1993) and two studies reported earlier development and higher prevalence of extra-renal cysts in PKD1 subjects (Coto *et al.*, 1995; Bogdanova *et al.*, 1995). Subsequently, another study showed that the prevalence of extra-renal cysts was similar in PKD1 and PKD2 patients and, in both groups, it was directly related to age (Torra *et al.*, 1996).

#### 4.1.3.3 Hypertension

Hypertension was assessed in seven studies and invariably found to be more common in PKD1 (Parfrey *et al.*, 1990a; Ravine *et al.*, 1992; Wright *et al.*, 1993; Coto *et al.*, 1995; Hateboer *et al.*, 1999b). Three studies specified age at development of hypertension, indicating that it occurred earlier in PKD1 (Parfrey *et al.*, 1990a; Bogdanova *et al.*, 1995; Torra *et al.*, 1996). Two reported the prevalence as a percentage of patients in specific age groups, also concluding on the earlier development of hypertension in PKD1 (Wright *et al.*, 1993; Coto *et al.*, 1995). In the remaining two studies, an odds

ratio was calculated, which varied between 0.25 and 0.52 for non-PKD1 versus PKD1 subjects (Ravine *et al.*, 1992; Hateboer *et al.*, 1999b).

#### 4.1.3.4 Decline in renal function

The rate of progression to kidney failure is clearly the most important clinical indicator of severity in ADPKD, which has been assessed in all studies.

The parameter reported by all studies was age at development of ESRF, which ranged between 47 and 57 years for PKD1 patients, and between 52 and 74 years for PKD2 (Parfrey *et al.*, 1990a; Ravine *et al.*, 1992; Wright *et al.*, 1993; Bogdanova *et al.*, 1995; Coto *et al.*, 1995; Torra *et al.*, 1996; Hateboer *et al.*, 1999b).

Very few studies have used age at chronic renal failure (CRF) as a measure of severity in comparing PKD1 and PKD2 subjects. Parfrey *et al.* (1990) and Bogdanova *et al.* (1995) observed that the mean age at CRF for PKD1 subjects was between 40 and 45 years. However, the average age at CRF for PKD2 patients in Bulgaria was reported as 48 years (Bogdanova *et al.*, 1995), whereas for Americans of European descent it was over 60 years (Parfrey *et al.*, 1990a). No significant difference in age at CRF between PKD1 and PKD2 subjects in Bulgaria was noted (Bogdanova *et al.*, 1995).

#### 4.1.3.5 Summary of clinical studies

Previous studies indicate that locus heterogeneity plays a role in phenotype heterogeneity. This effect has a number of important implications for diagnostic investigations and counselling in affected families, as well as for research. In the clinical setting, the more severe course of the disease in *PKD1*-linked families could mean that a better prognosis is suggested for non-*PKD1* families. However, the diagnosis in such families is rendered more difficult and postponed to a later age, due to the delayed development of symptoms. In terms of research, the effect of locus heterogeneity needs to be taken into account in any study aiming to examine factors modifying the ADPKD phenotype.

While the published studies provide cumulative evidence that PKD1 is generally more severe than non-PKD1, the number of studies where a comprehensive comparison has

been conducted is limited. These reports vary and overlap significantly in regard to the major compound measure of severity in ADPKD, namely progression to renal failure, precluding the direct use of the published information in subsequent research.

This makes it necessary to analyse linkage to the known PKD genes and conduct the comparisons within any specific study group, and incorporate the findings into further analyses performed on the same patient population. With this in mind, the aims of this part of my study were as follows:

- 1) Perform linkage analysis, assign families to *PKD1* or *PKD2*, and conduct further studies in families that fail to show linkage to the known loci;
- 2) Assess the relationship between locus heterogeneity and the ADPKD phenotype;
- 3) Incorporate this major variable into subsequent statistical analysis of other possible factors modifying disease severity;
- 4) Allow mutation detection to proceed in the most efficient way;
- 5) Make predictive testing based on linkage analysis possible.

## **4.2 SUBJECTS AND METHODS**

### **4.2.1 *Subjects for Genetic Linkage Analysis***

The study included 27 ADPKD families: 21 families from Australia and 6 Polish families. According to ethnic origin, the Australian families could be subdivided into 19 British, 1 Spanish and 1 Singaporean/Indian. The Australian families comprised 67 affected subjects, 17 unaffected relatives and 14 spouses, while the Polish families included 23 affected individuals, 7 unaffected relatives and 5 spouses. In addition, Dr Nadja Bogdanova (Institute for Human Genetics, Münster, Germany) conducted linkage analysis in 30 Bulgarian and a further 7 Polish families participating in the project, producing a total of 64 families.

### **4.2.2 *Subjects for Genotype/Phenotype Assessment***

Analysis of the clinical phenotype in relation to linkage status included 115 males and 107 females from the overall sample (Australia, Bulgaria and Poland). The age of these patients ranged between 9 to 85 years, with a mean age of 47.5 years. Based on the genetic linkage results, the patients could be subdivided into 184 (83%) *PKD1*-linked and 38 (17%) *PKD2*-linked (Table 4.9).

An additional 94 individuals, classified as 'other', could not be assigned to a specific linkage group, mainly due to small family size precluding linkage analysis. To increase the statistical power of further analyses, these individuals were tentatively assigned to the *PKD1* group, referred to as the "expanded" *PKD1* group. This assumption was based on the reported prior probability of linkage to *PKD1* in northern European populations of around 90% (Peters and Sandkuijl, 1992; Ravine *et al.*, 1992). The validity of this assignment was tested by conducting all analyses in duplicate, initially including the individuals where linkage data were available, and subsequently repeated on the expanded *PKD1* group. Details on the diagnostic and clinical assessment are provided in Chapter Two.

#### **4.2.3            *Genetic Linkage Analysis***

Laboratory and statistical methods for genetic linkage analysis are described in detail in Chapter Two, Section 2.5 and 2.7.

#### **4.2.4            *Statistical Analysis of Relationship between Linkage Status and Clinical Phenotype***

Statistical procedures are discussed in Chapter Two, section 2.7. Frequency data, ANOVA and survival analysis was performed by Dr Valerie Burke (University Dept. of Medicine, UWA) and by the author.

Decline in renal function was assessed using two approaches, namely age at onset of renal failure and cumulative renal survival. Age at CRF was examined in two ways: (1) observed values only and (2) observed and extrapolated values together (see Chapter Two, section 2.7). Kaplan-Meier survival curves (Kaplan and Meier, 1972) were used to calculate cumulative survival to CRF and ESRF and were compared using the log-rank test. The number of individuals included in the comparisons for age of onset and cumulative renal survival between PKD1 and PKD2 individuals is shown in Table 4.4.

**Table 4.4: The Number of PKD1 and PKD2 Individuals Included in the Analysis for Age at and Cumulative Survival to CRF and ESRF**

Variable	PKD1	PKD1 Expanded	PKD2
<b>Age at renal failure</b>			
CRF observed	61	92	11
CRF observed & extrapolated	174	240	33
ESRF	38	59	8
<b>Cumulative renal survival</b>			
To CRF observed & extrapolated	174	240	33
To ESRF	38	62	8

**4.3 RESULTS**

**4.3.1 Analysis of Linkage to PKD1 and PKD2**

A total of 27 families (21 WA and 6 Polish) were available for genetic linkage analysis. Twenty-three (85%) families showed evidence for linkage to *PKD1*. This included 18 (86%) WA and 5 (83%) Polish families. The multi-point linkage analysis results for the *PKD1* markers are shown in Table 4.5.

Four families (A24, A51, A71 and P118) showed negative lod scores for PKD1 and were subsequently run against the *PKD2* markers. Multipoint genetic linkage analysis was performed on these families and the probability of linkage to *PKD2* was calculated. The results are shown in Table 4.6. Of the WA families, one family (A24) was linked to *PKD2* (4.7%), one family (A51) failed to show linkage to either *PKD1* or *PKD2* (4.7%) and in one family (A71) the results were inconclusive (4.7%). Details on family A51 are discussed in section 4.5.2. One Polish family (P118) was not informative for the *PKD2* markers (17% of the 6 Polish families analysed in this study).



**Table 4.5: Multi-point Lod Scores for the *PKD1* Region Markers (16AC2.5-KG8-CW2)**

Family	Observed Maximum Lod score	Observed Minimum Lod score	Probability of linkage to <i>PKD1</i>	PKD group assignment
A3	0.7192	0.7170	0.96	<i>PKD1</i>
A4	1.1222	1.1208	0.99	<i>PKD1</i>
A5	0.3009	0.3009	0.91	<i>PKD1</i>
A6	1.0078	1.0078	0.98	<i>PKD1</i>
A7	0.2993	0.2984	0.91	<i>PKD1</i>
A9	0.3009	0.3009	0.91	<i>PKD1</i>
A10	0.2798	0.2790	0.92	<i>PKD1</i>
A12	0.3009	0.3005	0.91	<i>PKD1</i>
A13	0.2780	0.2779	0.91	<i>PKD1</i>
A15	0.7241	0.7237	0.97	<i>PKD1</i>
A22	0.2984	0.2983	0.97	<i>PKD1</i>
A24	-1.6668	-3.3889	<0.03	non- <i>PKD1</i>
A29	0.5568	0.5566	0.95	<i>PKD1</i>
A36	1.0242	0.9918	0.98	<i>PKD1</i>
A38	0.5796	0.5795	0.95	<i>PKD1</i>
A42	0.2991	0.2990	0.91	<i>PKD1</i>
A51	-2.3754	-3.6576	<0.01	non- <i>PKD1</i>
A52	0.2993	0.2993	0.93	<i>PKD1</i>
A55	0.5809	0.5809	0.95	<i>PKD1</i>
A61	0.5846	0.5844	0.95	<i>PKD1</i>
A71	0.4991	-0.9276	0.82	inconclusive
P110	0.2232	0.2176	0.90	<i>PKD1</i>
P111	0.2995	0.2989	0.91	<i>PKD1</i>
P112	0.5986	0.5978	0.96	<i>PKD1</i>
P113	0.5887	0.5875	0.95	<i>PKD1</i>
P117	1.7631	0.9222	0.99	<i>PKD1</i>
P118	-0.3413	-0.3519	0.70	not informative

**Table 4.6: Multi-point Lod Scores for the *PKD2* Region Markers (JV106-GSTG3-GSTG4-JV108)**

Family	Observed Maximum Lod score	Observed Minimum Lod score	Probability of linkage to <i>PKD2</i>	PKD group assignment
A24	0.2611	0.2605	0.99	<i>PKD2</i>
A51	-1.9825	-3.3567	0.13	non- <i>PKD2</i>
A71	-0.0544	-0.0581	0.09	inconclusive
P118	0.0000	0.0000	Not calculated	not informative

#### 4.3.2 Analysis of Linkage to *PKDL*

In family A51, linkage to either *PKD1* or *PKD2* was excluded with lod scores < -3. This family consisted of four affected individuals, namely the proband A51.1 (father) and three affected children (A51.3, A51.4 and A51.5). The proband (A51.1) had been diagnosed by ultrasonography at age 44 years when he presented with oedema and a

SCr of 128  $\mu\text{mol/L}$ . Hypertension was diagnosed at age 25 years. The proband's children were tested for the presence of cysts by abdominal ultrasonography at ages 16 (A51.3), 14 (A51.4) and 11 (A51.5) years respectively and found to have bilateral renal cysts. The two daughters underwent clinical examination at the time of ADPKD diagnosis and A51.4 was found to have borderline hypertension. The parents of the proband were tested by ultrasonography at ages 83 (father) and 85 (mother) years respectively and showed no evidence of polycystic kidneys, similarly the probands wife was also tested showing no evidence of cystic disease. The lack of renal cysts in the parents suggests that ADPKD in this family was the result of a new mutation in the proband.

Family A51 was analysed further for linkage to the *PKDL* gene on chromosome 10 (Table 4.7). The results were inconclusive: Markers D10S603 (*PKDL*) and D10S198 were not informative in this family. A negative lod score was observed for D10S185 and a positive lod score of 0.602 was obtained for D10S192.

Table 4.7: Chromosome 10 two-point Lod Scores for Family A51

Family	Theta	0	0.05	0.1	0.2	0.3
A51	D10S185	-3.380	-0.720	-0.440	-0.190	-0.08
	D10S198	0	0	0	0	0
	D10S603	0	0	0	0	0
	D10S192	0.602	0.535	0.465	0.318	0.170

4.3.3      *Summary of Linkage Analysis Results in the Entire Group of Families*

The overall number of families and affected individuals included in the linkage analysis is shown in Table 4.8. These individuals were used in the comparison of clinical outcome between PKD1 and PKD2 individuals (section 4.4).

**Table 4.8: Overall Number of Individuals Included in Genetic Linkage Analysis and the Classification of Individuals to the PKD Groups**

PKD linkage	Australia		Bulgaria		Poland		Total	
	Number families	Number affected subjects	Number families	Number affected subjects	Number families	Number affected subjects	Number Families	Number affected subjects
<i>PKD1</i>	18	58	23	81	11	45	52	184
<i>PKD2</i>	1	3	5	27	1	8	7	38
non-PKD1/2	1	4	2	8	0	0	3	11
non-informative	1	2	0	0	1	3	2	5
<b>Total</b>	<b>21</b>	<b>67</b>	<b>30</b>	<b>116</b>	<b>13</b>	<b>56</b>	<b>64</b>	<b>238</b>

## 4.4 COMPARISON OF CLINICAL PARAMETERS BETWEEN PKD1 AND PKD2

### 4.4.1 *General Characteristics of the Patients*

The general characteristics of the patients included in the comparison of phenotype severity, namely definite PKD1, PKD2 and other, are summarised in Table 4.9.

**Table 4.9: Characteristics of 316 ADPKD Patients included in the Comparisons between PKD1 and PKD2**

	PKD1	PKD2	Other (not analysed for linkage)	Overall
Number of Individuals	184	38	94	316
Mean current age (yr)	46±1.6	49±1.7	49±1.7	48±1.7
Number with CRF	75 (41%)	11 (29%)	43 (46%)	118
Number with ESRF	38 (21%)	8 (21%)	24 (26%)	70

### 4.4.2 *Onset and Diagnosis in PKD1 and PKD2 Individuals*

#### 4.4.2.1 *Reasons for ADPKD testing*

The reasons for ADPKD testing were compared between PKD1 and PKD2 patients. The number of individuals and results are shown in Table 4.10. As evident from the table,

the main reasons for ADPKD testing in *PKD1*-linked subjects included family history (32%) and the appearance of the first symptoms of the disease (35%). In comparison, only 12% of *PKD2* subjects were tested because of family history. Most individuals in this group underwent testing because of the presence of symptoms (38%) or as part of their participation in the present project (47%).

**Table 4.10: Reason for Diagnostic Testing in *PKD1*- and *PKD2*-linked Patients**

Reason for ADPKD testing	<i>PKD1</i> -linked (n=94)	<i>PKD2</i> -linked (n=32)
Family history	30 (32%)	4 (12%)
Symptoms	33 (35%)	12 (38%)
Participation in Project	13 (14%)	15 (47%)
Other	9 (10%)	1 (3%)
Family history and Symptoms	8 (8%)	0 (0%)
Screening and symptoms	1 (1%)	0 (0%)

*Values expressed as number and percentage (%)*

**4.4.2.2 Age at first symptom**

The age at first symptom(s) was compared between *PKD1* and *PKD2* patients. The number of individuals and results are shown in Table 4.11. The age at first symptoms was found to be significantly earlier in *PKD1*-linked individuals: 35.0 years for *PKD1* and 42.1 for *PKD2* ( $p = 0.03$ ).

**Table 4.11: Comparison in Age at First Symptom between *PKD1* and *PKD2* Linked Individuals**

	<i>PKD1</i> -linked (n=92)	<i>PKD2</i> -linked (n=36)	<i>p</i> value
Age at first symptom (yr)	34.9±1.7	42.1±2.8	0.03

The age at diagnosis was examined in the same group of 128 individuals, 92 *PKD1* and 36 *PKD2* (Table 4.12). The mean age at diagnosis was 33.7 years for *PKD1* and 38.4 for *PKD2* ( $p = 0.12$ ).

**Table 4.12: Comparison in Age at ADPKD Diagnosis between *PKD1* and *PKD2* Linked Individuals**

	<i>PKD1</i> linked (n=92)	<i>PKD2</i> linked (n=36)	<i>p</i> value
Age at diagnosis (yr)	33.7±1.9	38.4±2.6	0.12

**4.4.3      *Frequency of Clinical Manifestations in *PKD1* and *PKD2* Individuals***

The frequency of different symptoms and complaints was compared between PKD1 and PKD2 patients. The number of individuals and results are shown in Table 4.13. The frequency of back/flank pain was similar in the two groups ( $p>0.05$ ). Urinary tract infection, renal calculi and haematuria were recorded more frequently in PKD2 individuals but the difference was not statistically significant ( $p>0.05$ ). Dysuria and nocturia were more common in PKD1 patients ( $p<0.05$ ).

**Table 4.13: Frequency of ADPKD Clinical Manifestations in *PKD1* and *PKD2* Linked Individuals**

Clinical manifestation	<i>PKD1</i> linked (n=85)	<i>PKD2</i> linked (n=35)	<i>p</i> value
Back/flank pain	47 (55%)	20 (57%)	>0.05
Urinary tract infection (UTI)	37 (44%)	18 (51%)	>0.05
Renal calculi	17 (20%)	11 (31%)	>0.05
Haematuria	29 (34%)	16 (46%)	>0.05
Dysuria	33 (39%)	4 (11%)	<0.05
Nocturia	20 (24%)	4 (11%)	<0.05

Hypertension (defined in Chapter Two, section 2.3.4) was examined in two ways (Table 4.14). The first analysis compared the prevalence of hypertension between PKD1 and PKD2 subjects. The number of individuals and results are shown in Table 4.14. No difference was observed, with 75% of PKD1 and 72% of PKD2 patients showing evidence of hypertension ( $p>0.05$ ).

The second approach compared the age at development of hypertension between PKD1 and PKD2 patients. The number of individuals and results are shown in Table 4.14. The patients were divided into two groups, depending on age at development of

hypertension, namely before and after age 40 years. No significant difference in the number of patients who developed hypertension <40 or >40 years was found between PKD1 and PKD2 ( $p>0.05$ ).

**Table 4.14: Comparison in the Age at Development and Frequency of Hypertension between *PKD1* and *PKD2* Linked Individuals**

	<i>PKD1</i> linked	<i>PKD2</i> linked	<i>p</i> value
Presence of hypertension	116 (75%) (n=154)	21 (72%) (n=29)	>0.05
Average age at hypertension (yr)	35±2.0 (n=50)	40.8±2.8 (n=10)	0.35
<40 years	38 (76%)	6 (60%)	
>40 years	12 (24%)	4 (40%)	>0.05

**4.4.4            *Rate of Deterioration of Renal Function in *PKD1* and *PKD2* Individuals***

The effect of *PKD* genotype on progression to renal failure was examined by comparing PKD1 versus PKD2 with regard to mean age at renal failure (CRF or ESRF) and in terms of Kaplan-Meier cumulative survival curves.

The initial analysis was conducted in definite *PKD1*- and *PKD2*-linked patients, and was analysed by comparing the observed, as well as the extrapolated age (based on the individual regression lines of time versus 1/Cr values).

**4.4.4.1            *Age at renal failure***

*Observed age at renal failure*

The observed mean age at renal failure was initially compared between definite *PKD1* and *PKD2* subjects. The number of individuals and results are shown in Table 4.15. The results failed to show a significant difference between the two genetic forms of ADPKD. The mean age at CRF was 44.7 years for PKD1 and 50.1 for PKD2 ( $p = 0.25$ ). The age at ESRF was 52.0 years for PKD1 and 59.0 for PKD2 ( $p = 0.21$ ).

**Table 4.15: Comparison in Observed Age at Renal Failure between Definite *PKD1* and *PKD2***

Linked Subjects			
Age at renal failure	<i>PKD1</i> -linked	<i>PKD2</i> -linked	<i>p</i> value
CRF (observed age)	44.7±2.1 (n=61)	50.1±3.3 (n=11)	0.25
ESRF	52.0±2.3 (n=38)	59.0±3.5 (n=8)	0.21

The same analysis was repeated on the expanded PKD1 group (definite plus probable PKD1). The number of individuals and results are shown in Table 4.16. In the expanded PKD1 group, the mean age at CRF was 47.3 years, and the age at ESRF was 53.9 years. These results were essentially identical to those in the definite PKD1 group and consequently failed to show a difference between PKD1 and PKD2 ( $p = 0.46$  for CRF and  $p = 0.20$  for ESRF).

**Table 4.16: Comparisons in Observed Age at Renal Failure between the Expanded *PKD1* Group and *PKD2* Subjects**

Age at renal failure	<i>PKD1</i> (definite + probable)	<i>PKD2</i>	<i>p</i> value
CRF (observed age)	47.3±1.8 (n=92)	50.1±3.4 (n=11)	0.46
ESRF	53.9±2.1 (n=59)	59.0±3.5 (n=8)	0.20

To account for the effects of sex, we have repeated these calculations and compared separately PKD1 versus PKD2 for male and female patients. The results are shown in Table 4.17. No significant difference for age at CRF was observed between female and male PKD1 and PKD2 patients in the definite ( $p = 0.51$ ) and expanded PKD1 group ( $p = 0.43$ ). For ESRF, no significant difference in age at ESRF was found between male and female PKD1 and PKD2 patients ( $p = 0.06$ ), however this was found to be significant when the expanded PKD1 group was included in the analysis ( $p = 0.03$ ). In this analysis, it was also noted that PKD2 females reach ESRF approximately 17 years later than PKD2 males but the sample size was small.

**Table 4.17: Observed Age at Renal Failure in *PKD1* and *PKD2* Linked Subjects divided by Sex**

	Males		Females		<i>p</i> value *
	Observed CRF	ESRF	Observed CRF	ESRF	
<i>PKD1</i> definite	42.6±2.1 (n=34)	54.6±2.0 (n=22)	47.4±2.3 (n=27)	55.5±2.3 (n=16)	CRF: 0.51 ESRF: 0.06
<i>PKD1</i> expanded	42.7±1.6 (n=48)	54.1±1.5 (n=34)	47.9±1.7 (n=44)	53.8±1.8 (n=25)	CRF: 0.43 <b>ESRF: 0.03</b>
<i>PKD2</i>	50.0±5.4 (n=5)	51.3±4.7 (n=4)	49.7±4.9 (n=6)	67.0±5.4 (n=4)	

\* *p* values refer to comparison between respective *PKD1* group and *PKD2*

#### *Extrapolated age at renal failure*

Differences in the rate of progression to renal failure were further examined by comparing age at development of CRF extrapolated from the individual regression lines of time versus 1/Cr. The initial analysis included 174 definite *PKD1*-linked and 33 *PKD2*-linked subjects. The age at CRF was 38.9 years for *PKD1* and 42.7 for *PKD2* ( $p = 0.18$ ). The analysis was repeated on the expanded *PKD1* group (definite plus probable) comprising 240 individuals, where the age at CRF was 39.6 years, again not significantly different from *PKD2* ( $p = 0.19$ ).

The analysis was subsequently repeated by sex of the patients. The results are shown in Table 4.18. No significant difference in extrapolated age at CRF was found between male and female definite *PKD1* and *PKD2* subjects ( $p = 0.43$ ) and between male and females from the expanded *PKD1* group versus *PKD2* ( $p = 0.54$ ).

**Table 4.18: Extrapolated Age at Renal Failure in *PKD1* and *PKD2* Linked Subjects Divided by Sex**

	Males	Females	<i>p</i> value*
	Extrapolated age at CRF	Extrapolated age at CRF	
<i>PKD1</i> definite	38.8±1.9 (n=88)	40.0±1.9 (n=86)	0.43
<i>PKD1</i> expanded	38.6±1.4 (n=124)	40.6±1.2 (n=116)	0.54
<i>PKD2</i>	37.8±1.4 (n=17)	43.2±1.4 (n=16)	

\* *p* values refer to comparison between respective *PKD1* group and *PKD2*

#### *Cumulative survival to renal failure*

The second approach to assessing the effect of PKD genotype on deterioration of renal function was based on a comparison of Kaplan-Meier cumulative survival curves. The initial analysis included definite *PKD1* and *PKD2* subjects. The number of individuals



and results are shown in figures 4.1 and 4.2. A marginal difference in cumulative survival was detected, with faster progression to CRF observed in the PKD1 group ( $p = 0.05$ ; Figure 4.1). No significant difference in cumulative survival to ESRF was found ( $p = 0.11$ ; Figure 4.2).

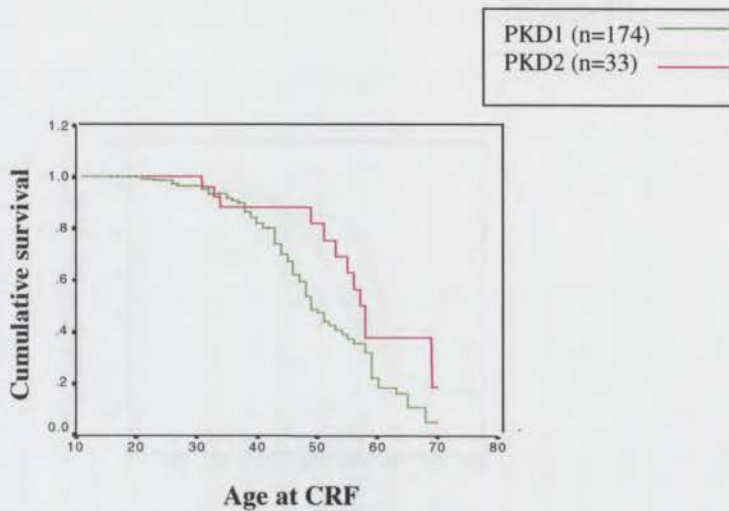
The analysis of Kaplan-Meier cumulative survival curves to CRF was repeated by comparing the expanded *PKD1* group and *PKD2* individuals. The results replicated those described in the previous paragraph, detecting a possibly faster rate of progression to CRF in PKD1 subjects ( $p = 0.05$ ; Figure 4.3), and no significant difference in rate of progression to ESRF ( $p = 0.08$ ; Figure 4.4).

#### 4.4.5 Cystogenesis in *PKD1* and *PKD2* Individuals

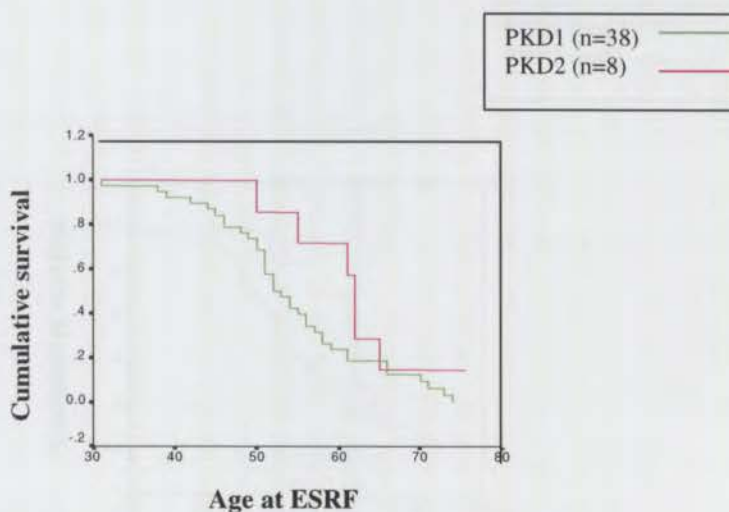
*PKD1*- and *PKD2*-linked subjects were compared as regards to mean kidney length, diameter of largest cyst and number of cysts (adjusted for age). The number of individuals and the results are shown in Table 4.19. The comparison of kidney length between PKD1 and PKD2 failed to reveal significant differences ( $p = 0.91$ ). No significant difference in the size of the largest cyst ( $p = 0.36$ ) and the mean number of kidney cysts ( $p = 0.37$ ) was found between *PKD1*-linked and *PKD2*-linked subjects. Similarly, no significant differences in the presence of extrarenal cysts were observed (Table 4.19).

**Table 4.19: Comparison in Ultrasound Findings between Definite *PKD1*- and *PKD2*- Linked Individuals**

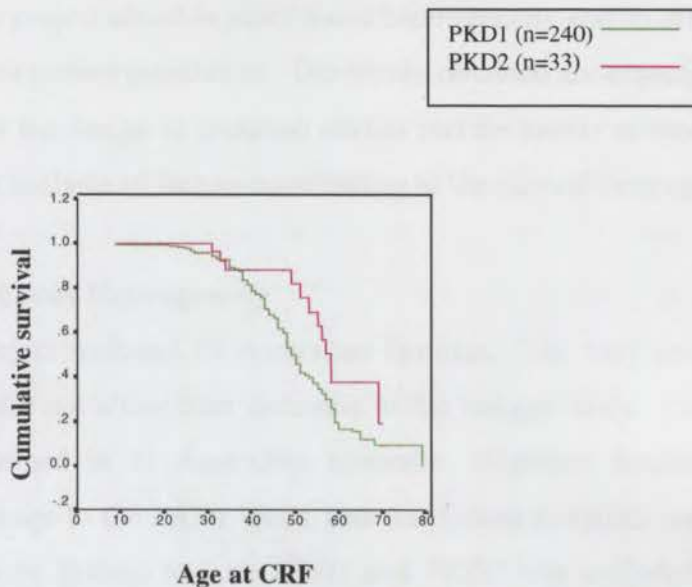
Variable	<i>PKD1</i> (definite) (N = 106)	<i>PKD2</i> (N = 36)	<i>p</i> value
Mean kidney length (mm)	139.9±3.8	140.8±6.4	0.91
Mean diam of lrgst cyst (mm)	25.2±1.7	28.5±3.0	0.36
Mean number cysts	7.5±0.3	8.0±0.6	0.37
Hepatic cysts	36 (34.0%)	9 (25.0%)	>0.05
Pancreatic cysts	5 (5.0%)	0	NS
Splenic cysts	1 (0.9%)	0	NS



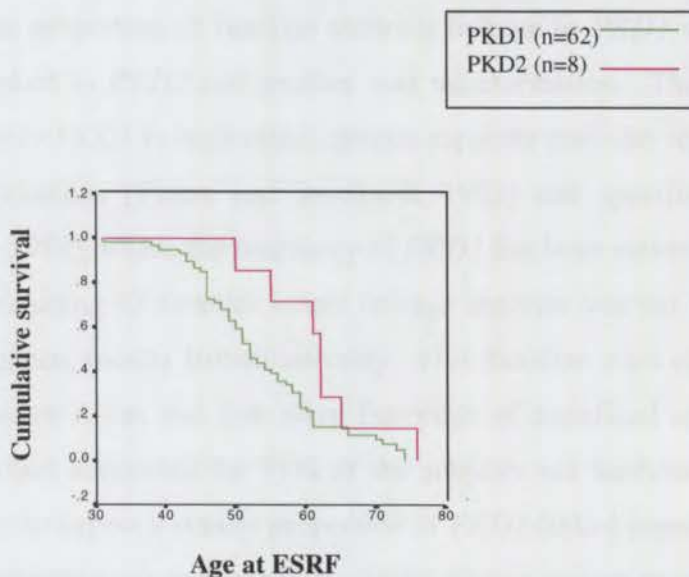
**Figure 4.1:** Survival Analysis to CRF in Definite PKD1 and PKD2 Subjects. A marginal difference in renal survival to CRF between definite PKD1 and PKD2 was found ( $p=0.05$ )



**Figure 4.2:** Survival Analysis to ESRF in Definite PKD1 and PKD2 Subjects. No significant difference in cumulative renal survival was found between PKD1 and PKD2 subjects ( $p = 0.11$ ).



**Figure 4.3:** Survival Analysis to CRF in PKD1 (definite plus probable) and PKD2 subjects. A marginal difference was found in renal survival to CRF between PKD1 (definite and probable) and PKD2 subjects ( $p=0.05$ )



**Figure 4.4:** Survival Analysis to ESRF in PKD1 (definite plus probable) and PKD2 Subjects. A marginal difference was found in renal survival to ESRF between PKD1 (definite and probable) and PKD2 subjects ( $p=0.08$ )

## 4.5 DISCUSSION

This part of the project aimed to study locus heterogeneity and its effect on the ADPKD phenotype in our patient population. The results obtained are expected to have practical implications for the design of mutation studies and for family counselling, as well as to assist in further analysis of factors contributing to the clinical heterogeneity of ADPKD.

### 4.5.1 *Locus Heterogeneity*

The overall project included 69 Australian families. The very small size of most of these families did not allow their inclusion in the linkage study. Linkage analysis was therefore performed in 21 Australian kindreds. Eighteen families (86%) provided evidence of linkage to the *PKD1* locus, one was linked to *PKD2*, one was inconclusive and in one family linkage to both *PKD1* and *PKD2* was excluded. This part of the study has allowed mutation analysis of the *PKD1* gene in Australian families to proceed successfully (see Appendix 9).

The overall project also included 13 Polish families with ADPKD, of which six were analysed in this study and the remainder at the Institute for Human Genetics in Münster, Germany. The proportion of families showing linkage to *PKD1* was 86%, whilst one family was linked to *PKD2* and another was uninformative. The high proportion of families linked to *PKD1* in both ethnic groups supports previous reports on North-West European populations (Peters and Sandkuijl, 1992) and specifically on the British (Ravine *et al.*, 1992), where the frequency of *PKD1* has been estimated at about 90%.

Among the remaining 48 families where linkage analysis was not feasible, 34 reported Northern European, mostly British ancestry. Five families were of Southern European descent, five were Asian and four were European of undefined origin. The Northern European families accounted for 71% of the subjects not analysed for linkage, which gives us reason to expect a similar proportion of *PKD1*-linked cases. We have therefore conducted most statistical analyses in duplicate, first including the patients with definite linkage data, and subsequently increasing the sample size by using an "expanded" *PKD1* group, to which the undefined subjects were added.

In one family (A51) linkage to either *PKD1* or *PKD2* was definitely excluded by the negative lod scores (below  $-3$  for both loci). The structure of this family is interesting,

as both parents of the proband appear unaffected on abdominal ultrasound examination. Further genetic examination eliminated the possibility of false paternity or adoption, suggesting that the disease is caused by a new mutation.

Further investigations of family A51 were conducted in collaboration with Prof. Jing Zhou (Brigham and Women's Hospital and Harvard Medical School, Boston, USA) and aimed to analyse linkage to the novel *PKDL2* gene assigned to chromosome 10q24. The study included a total of six non-*PKD1*/non-*PKD2* families. The results in A51 were inconclusive, with two uninformative markers (D10S198, D10S603), one giving a negative (D10S185) and one (D10S192) a positive lod score.

Given the small genetic distance of 0.2 cM between D10S192 and *PKDL*, linkage of our family to this locus remains a possibility. However, the cumulative lod scores for all six families excluded linkage to *PKDL*. The results of this study can be found in Appendix 9. Subsequently, the *PKDL* region has also been excluded in two Dutch, one Belgian and two Canadian families (Veldhuisen *et al.*, 1999). Nonetheless, given the remarkably high homology to Polycystin 2, *PKDL* remains a good candidate gene for future studies of families that are not linked to the two common loci.

The gene responsible for ADPKD in family A51 thus remains unidentified. This family adds to the list of other non-linked ADPKD pedigrees, identified in Bulgaria (Bogdanova *et al.*, 1995), Canada (Daoust *et al.*, 1995) and Portugal (de Almeida *et al.*, 1995), indicating that our knowledge of the complexity of the genetic basis of ADPKD is still incomplete.

#### **4.5.2      *Clinical Heterogeneity***

Germline mutations in the known PKD genes are considered to be the major causal factor in the development of the ADPKD phenotype. The differences in the structure and proposed functions of Polycystins 1 and 2 (discussed in Chapter One) suggest that mutations in one or the other gene could be expected to result in differences in phenotype severity. The effect of locus heterogeneity on the clinical manifestations of ADPKD has been addressed in a number of investigations (Parfrey *et al.*, 1990a; Ravine *et al.*, 1992; Gabow *et al.*, 1992b; Bear *et al.*, 1992; Bogdanova *et al.*, 1995; Coto *et al.*,

1995; Torra *et al.*, 1996; Hateboer *et al.*, 1999b). By far the largest set of data published so far come from a recent collaborative study comprising 333 *PKD1*-linked and 291 *PKD2*-linked patients, and 398 geographically matched controls (Hateboer *et al.*, 1999b). Most of the subjects included in the Hateboer study have been investigated previously by different groups (Ravine *et al.*, 1992; Bogdanova *et al.*, 1995; Coto *et al.*, 1995; San Millan *et al.*, 1995; Torra *et al.*, 1996).

The findings of the published studies point to a generally more severe clinical course of the disease in individuals carrying mutations in the *PKD1* gene. However, as discussed previously, the variation in the selected clinical parameters, and the wide, often overlapping, ranges of the values obtained, preclude the direct use of the published information in subsequent studies.

In this study, phenotype severity was examined using a comprehensive set of clinical indicators. The analyses first included individuals assigned to a specific linkage group and subsequently were repeated on an expanded PKD1 group, including the subjects with unknown linkage status. The findings will be discussed in the order of their presentation.

Most studies examine the age when the diagnosis of ADPKD was first established and report an earlier age in *PKD1*-linked subjects as compared to non-*PKD1* (Parfrey *et al.*, 1990a; Ravine *et al.*, 1992; Wright *et al.*, 1993; Torra *et al.*, 1996; Hateboer *et al.*, 1999b). However, the comparison of the reported mean ages shows a substantial overlap, with the mean age for PKD1 ranging from 25 to 45 years and that for PKD2 from 37 to 69. In this study, the mean age at diagnosis was 33.7 years for PKD1 and 38.4 for PKD2, falling within the above ranges but failing to show a significant difference between PKD1 and PKD2 ( $p = 0.12$ ).

However, the reasons for the first diagnostic examination can vary and may not always be related to the onset of clinical symptoms. This is indeed supported by our findings in the study of the reasons for the first examination, where nearly half of the PKD1 patients and only 12% of PKD2 individuals underwent testing because of family history. Similar proportions of PKD1 and PKD2 subjects were investigated because of

the presence of symptoms, and participation in this research project was the reason for examination in a large fraction of the PKD2 individuals. The results may suggest a higher level of concern about the disease in *PKD1*-linked families, which if true for other studies, may account for the very early age at diagnosis of PKD1.

This study also compared the mean age at development of first symptoms of the disease, which was 34.9 years for PKD1 and 42.1 years for PKD2 subjects ( $p = 0.03$ ). The findings confirm an earlier age at onset of the disease in *PKD1*-linked families. However, it should be noted that age at diagnosis is not a reliable clinical indicator of severity, since it may be influenced by many factors directly or indirectly related to the disease. Examples of some factors include differences in clinical practice between clinicians, as well as the psychological state of the individual.

No difference in the frequency of back/loin pain between PKD1 and PKD2 was observed in this study. The remaining symptoms, namely urinary tract infections, renal calculi and haematuria, appeared to be more common in the PKD2 group, however the results were not significant. Dysuria and nocturia were found to occur more frequently in PKD1 patients. Earlier reports have provided divergent results in respect to the frequency of urinary tract infection and renal calculi (Wright *et al.*, 1993; Torra *et al.*, 1996). Torra *et al.* (1996) failed to detect any differences, whereas Wright *et al.* (1993) found a higher prevalence of urinary tract infection and renal calculi in PKD2 subjects. The frequency of hypertension observed in our study deserves special attention. The presence and age at development of hypertension have been assessed by all studies of genotype/phenotype correlations in ADPKD (Table 4.3). Most studies have observed that hypertension is significantly more common, and develops earlier, in *PKD1*-linked families (Parfrey *et al.*, 1990a; Ravine *et al.*, 1992; Wright *et al.*, 1993; Coto *et al.*, 1995; Torra *et al.*, 1996; Hateboer *et al.*, 1999b). The large collaborative study of Hateboer *et al.* (1999b) reports that the risk of hypertension is 4 times higher in PKD1 than PKD2.

In this study, the frequency of hypertension was essentially identical in the 2 linkage groups. The proportion of hypertensive individuals in the PKD1 group was 75%, and in the PKD2 group it was 72%. The age at development tended to be earlier in the PKD1

group, although the differences were not statistically significant. This comparison is of limited value, since information was available for a small number of subjects and the analysis included only 60 patients (50 PKD1 and 10 PKD2). The proportion of individuals who had developed hypertension before age 40 years was 76% for PKD1 and 60% for PKD2. The only other study that has reported a lack of difference between PKD1 and PKD2 is that of Bogdanova *et al.* (1995), describing ADPKD in Bulgaria. In discussing our results, one has to take into account the fact that the individuals participating in the present project included those that were involved in the Bogdanova *et al.* (1995) study. Moreover, the Australian and Polish groups of patients have contributed one PKD2-linked family each, therefore the majority of PKD2 patients discussed here actually belong to the Bulgarian ADPKD families. Therefore the agreement between the conclusions is not surprising. One can speculate on the reasons for these findings. The rate of morbidity and mortality from cardiovascular disease in Eastern Europe is high and an upward trend has been recorded in recent years (<http://www.americanheart.org/statistics/biostats/bioin.htm>). It has been demonstrated that ADPKD has a more severe clinical course in families with a history of essential hypertension (Geberth *et al.*, 1995). The combination of background factors, such as high prevalence of essential hypertension and unfavourable life style, could contribute to the pattern observed in this ADPKD study.

In comparing ultrasound findings (adjusted for age) between PKD1 and PKD2, this study showed no difference in the mean length of the kidneys. The findings are similar to previous studies that also showed no difference in kidney length between PKD1 and PKD2 (Wright *et al.*, 1993; Torra *et al.*, 1996). In one study, the influence of age was noted (Torra *et al.*, 1996). No significant difference between PKD1 and PKD2 subjects in the development of extrarenal cysts was observed. The effect of genotype on extrarenal cystogenesis has been investigated previously, with controversial results. Torra *et al.* (1996) failed to detect any differences, whereas Wright *et al.* (1993) found a higher prevalence of hepatic cysts in PKD2 subjects and Bogdanova *et al.* (1995) found extrarenal (mainly hepatic) cysts to be markedly more common in PKD1.



#### 4.5.3 Renal Failure

Renal failure is the most important compound measure of severity of kidney disease, as it is the major cause of disability, poor quality of life and mortality, and requires expensive renal replacement therapies. It has therefore been included as a clinical parameter in all studies of genotype/phenotype correlations in ADPKD (Ravine *et al.*, 1992; Wright *et al.*, 1993; Bogdanova *et al.*, 1995; Coto *et al.*, 1995; Torra *et al.*, 1996; Hateboer *et al.*, 1999b).

To increase the power of the analyses in this, as well as in the other parts of the study, we assumed that most individuals where linkage analysis was impossible in fact belong to the PKD1 group. This increased the size of the PKD1 group from 61 definite (Table 4.15) to 92 definite plus probable PKD1 (Table 4.16) in the comparisons of observed age at renal failure, and from 174 to 240 individuals respectively in the comparisons of extrapolated age at CRF. This assumption was based on the North European descent of most of the individuals who could not be analysed for linkage, and on the reported prior probability of linkage to *PKD1* for these populations of around 90% (Peters and Sandkuijl, 1992; Ravine *et al.*, 1992). This assumption is supported by the comparison between the two PKD1 groups, definite versus expanded, as regards the age at renal failure. Similar values were obtained, with CRF reached at 44.7 years in the definite and 47.3 years in the expanded group, and ESRF at 52.0 years in the definite and 53.9 years in the expanded PKD1 group.

The findings were also similar for the age at renal failure extrapolated from the individual regression lines of time versus 1/Cr values, however it was noted that the extrapolations resulted in an overall shift towards an earlier age (for example, around 39-40 years extrapolated versus 45-47 years observed, in the case of CRF).

As indicated above, the observed average age at CRF in our definite (61 individuals) and expanded PKD1 group (92 individuals) was 46 years. This can be compared to only a limited number of previous studies, since CRF is a parameter, which has rarely been examined. Our findings coincide with those of Bogdanova *et al.* (1995), who observed an average age at CRF of 45.5 years, and are close to Parfrey *et al.* (1990a) and Wright *et al.* (1993), who observed an average age at CRF of 40 years.

Much more comprehensive comparisons to other studies are possible for ESRF, a universal indicator included in nearly all published investigations. Our group of PKD1 patients had reached ESRF at a mean age of 52.0 years (53.9 years for the expanded group). This falls within the narrow 50.9 to 56.7 years range provided by the studies of Parfrey *et al.* (1990a), Bogdanova *et al.*, (1995), and Torra *et al.* (1996) and coincides with the largest published study of 333 PKD1 patients (Hateboer *et al.*, 1999b), indicating a mean age at ESRF of 54.3 years. An earlier mean age of 47.6 years was reported in a group of 77 Spanish PKD1 patients (Coto *et al.*, 1995) and perhaps in the Irish study, where 65% of the 49 PKD1 individuals had reached ESRF by age 54 years and the mean age may be earlier (Wright *et al.*, 1993). These findings suggest that, in our group of patients, the course of the disease and its progression towards renal failure in PKD1 were comparable to those observed by most other investigators in a number of countries.

However, the comparisons for PKD2 lead to different conclusions. In our study, PKD2 patients reached CRF at a mean age of 50.1 years, and ESRF at a mean age of 59.0 years. This is in marked contrast to the 70 to 74 years range of the mean age at ESRF reported by Parfrey *et al.* (1990a), Torra *et al.* (1996) and Hateboer *et al.* (1999b) and to the Wright *et al.* (1993) finding that only 25% of PKD2 patients reach ESRF by age 54 years. Our observations are similar to those in the Spanish group of PKD2 patients (Coto *et al.*, 1995) who reached ESRF at 56.3 years, but where the age at ESRF was also earlier in the PKD1 group.

The present results also coincide with the findings of Bogdanova *et al.* (1995), which, as discussed earlier, is not surprising since our PKD2 group consists mostly of the Bulgarian patients originally investigated by Bogdanova *et al.* (1995). In the initial Bulgarian study, the mean age at ESRF in PKD2 was estimated at 52 years. The increase to 59 years was caused by exclusion of two Bulgarian PKD2 families in this study (clinical data were unavailable at the time of analysis). In one of the families, the clinical course of the disease was particularly severe, with chronic renal failure present in two affected individuals aged 18 and 34 respectively (Bogdanova *et al.*, 1995). Moreover, this study included an additional Bulgarian PKD2 family (B7808) that was

not included in the study of Bogdanova *et al.* (1995). In this family, the age at ESRF in three affected individuals was 50, 62 and 63 years respectively.

In discussing the results, one should take into account the small numbers: a total of eight PKD2 individuals in this study had actually reached ESRF. These individuals belong to a total of 5 families, therefore one cannot rule out the possibility that the severe clinical course in our PKD2 group (and primarily in the Bulgarian patients) is caused by the random occurrence of particularly severe *PKD2* mutations.

On the other hand, it remains possible that, in this population and particularly in the Gypsy families, the generally more benign course of PKD2 is modified by genetic background and environmental effects. This speculation can be taken further in a discussion of the clinical course of ADPKD in a Gypsy community in Hungary, where the disease has an incidence about 20-fold in excess of that reported globally (Forrai *et al.*, 1989) and a severe clinical course with early onset of renal failure and about 15% incidence of intracranial aneurysms. The geographic location of that "endemic" region, and the history of migrations of the affected Gypsy families suggest that they may share a common founder mutation with the Gypsy *PKD2* families from Bulgaria, namely R306X (Veldhuisen *et al.*, 1997). Neither the nature, nor the localisation of this mutation in the *PKD2* gene differ significantly from the majority of PKD2 mutations. If the hypothesis of a common founder mutation is confirmed, the R306X mutation cannot provide an adequate explanation for the severe phenotype observed in these patients and other factors, genetic or more likely environmental, have to be invoked.

Marginally significant difference was revealed by the analysis of Kaplan-Meier cumulative survival curves, which showed decreased survival of PKD1 subjects (definite as well as expanded PKD1 group) to CRF ( $p = 0.05$ ) but not ESRF ( $p = 0.11$  for definite and  $p = 0.08$  for expanded PKD1 group). With respect to CRF (Figures 4.1 and 4.3) there appears to be more rapid progression to CRF in PKD1 patients, which then slows in pace to match the rate of renal deterioration in PKD2 patients. The results for CRF, which are based on more data points in Figures 4.1 and 4.3, may have conferred the increased power necessary to detect a true difference between the two genotypes, in terms of the reduced chance of making a type II error (defined as less

chance of a false negative result). For ESRF (Figures 4.2 and 4.4), it is difficult to draw any conclusions, as the sample size was smaller. Whether, the observed differences between CRF and ESRF are the product of a biological phenomenon, or an artefact resulting from the small size of PKD2 group remains to be determined. The small sample size of the PKD2 group is an obvious limitation in such studies, which significantly reduces power and introduces the risk of type II errors.

Nonetheless, since the data obtained for our PKD1 group are in excellent agreement with those reported from other studies, including the largest international multicentre study of Hateboer *et al.* (1999b), the reason for the lack of differences between PKD1 and PKD2 should be sought in the more severe PKD2 phenotype discussed above.

In our study we also compared PKD1 and PKD2 by sex of the patients. No significant difference for age at CRF (observed and extrapolated) was found between male and female PKD1 and PKD2 patients. However, our findings for age at ESRF led to different conclusions. Similar to Hateboer *et al.* (1999b), our study showed significantly better renal survival in PKD2 females compared to PKD2 males and PKD1 male and females. The lack of difference to age at ESRF between PKD1 male and females is not surprising since other studies have also shown no sex effect (Bear *et al.*, 1992; Simon *et al.*, 1996; Hateboer *et al.*, 1999b). However, the reasons for better renal survival in female patients with PKD2 are not known and one can only speculate on these findings. Perhaps the slightly better renal survival in PKD2 patients may have allowed the protective effect of female sex to manifest. Testosterone is known to stimulate fluid secretion and solute transport in Madin-Darby canine kidney cells, which may have a similar effect on human kidney cells. Hence the deleterious effect of male sex may be more easily detected in the milder PKD2 group (Hateboer *et al.*, 1999b). Nonetheless, the small sample size make interpretation of these results difficult.

In conclusion, the data from this study suggest that the differences in clinical severity between the two common genetic forms of ADPKD, PKD1 and PKD2, may not be as clear-cut as previously reported. The prognosis in PKD2 families should therefore be individualised and discussed carefully, avoiding over-optimistic statements. Moreover, there appear to be differences between the clinical findings in PKD2 patients in

different countries that may be masked in large collaborative studies (Hateboer *et al.*, 1999b). Further studies are required to identify factors, which may be contributing to the observed differences between the clinical findings in PKD2, as they may lead to important conclusions about factors affecting disease severity and hence better management of the disease.

**CHAPTER FIVE: RELATIONSHIP BETWEEN THE  
RENIN-ANGIOTENSIN SYSTEM AND ADPKD  
SEVERITY**

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# CHAPTER FIVE: THE RENIN-ANGIOTENSIN SYSTEM (RAS)

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## 5.1 INTRODUCTION AND LITERATURE REVIEW

Interactions between mutations in different genes may play a role in phenotypic diversity. These types of genes are termed modifier genes and, according to Gruneberg (1963) (as cited by Romeo and McKusick, 1994), they are defined as “those capable of modifying the manifestation of a mutant gene without having an obvious effect on the normal condition”. Modifier genes are apparent in all genetically mixed populations and they are commonly referred to as the “genetic background” in which the mutant gene finds itself (Romeo and McKusick, 1994).

Genes encoding the components of the renin-angiotensin system (RAS) can be regarded as likely modifiers of the ADPKD phenotype for a number of reasons:

- 1) Early activation of the RAS has been demonstrated in both experimental and clinical studies of ADPKD (Chapman *et al.*, 1990);
- 2) Hypertension plays an important role in the pathogenesis of ADPKD, and the RAS is known to be involved in blood pressure regulation (Bell *et al.*, 1988);
- 3) Angiotensin II, the end-product of the RAS, has growth-promoting effects that could contribute to cystogenesis in ADPKD (Norman *et al.*, 1987).

A number of studies have examined the association between genetic variants in the RAS, and pathological conditions related to, and including, ADPKD. The findings have been divergent and inconclusive. In interpreting this impasse, one has to take into account the fact that such studies rely on the use of polymorphisms in the RAS genes. These polymorphisms include an Alu element insertion in the angiotensin converting enzyme (*ACE*) gene, a missense mutation in the angiotensinogen (*AGT*) gene and a polymorphic microsatellite in the renin gene. The functional significance of these genetic variants is far from clear and they should, at this stage, be regarded at best as markers in linkage disequilibrium with other functionally relevant variants within the RAS genes. Given the complexity of gene regulation and the pathogenesis of the

examined conditions, the search for association can easily be compromised by factors such as lack of linkage disequilibrium, inter-ethnic variation and, of course, sample size. One of the aims of this project was to investigate the association between the RAS and the ADPKD phenotype, in a study design that took into account some of the probable confounding variables and examined a large sample of patients originating from three different countries.

### **5.1.1      *The Renin-Angiotensin System (RAS)***

The RAS plays an important role in the control of blood pressure and salt and water homeostasis. Angiotensinogen is produced in the liver and is found in the  $\alpha$ -globulin fraction of plasma. It serves as the substrate of an enzymatic reaction, catalysed by renin. Renin, produced by the juxtaglomerular cells in the kidney, in response to decreased blood volume and sodium chloride delivery to the macula densa of the distal tubule, converts AGT to angiotensin I (Ang I). Ang I is then cleaved by the ACE to yield angiotensin II (Ang II) (see Figure 5.1). ACE is present both in the blood plasma and in tissues, including endothelial, epithelial and neuroepithelial cells. Ang II is a potent vasoconstrictor exerting three effects on the kidney: 1) increased vascular resistance; 2) via increased aldosterone release from the adrenal cortex and; 3) growth-potential in renal and cardiovascular systems (Norman *et al.*, 1987). Ang II exerts its physiological effects on target tissues by interacting with two pharmacologically distinct subtypes of cell surface receptors, namely types 1 and 2. In humans, the angiotensin type 1 (AT1) receptor is present predominantly in vascular smooth muscle cells and seems to mediate the major cardiovascular effects of Ang II.

#### **5.1.1.1      *The Renin-Angiotensin System and ADPKD***

Clinical studies have shown that hypertension plays a fundamental role in the progression of renal disease, as both a cause and effect of kidney impairment (Oppenheimer, 1934; Iglesias *et al.*, 1983; Brazy *et al.*, 1989; Gabow *et al.*, 1990, 1992b; Klag *et al.*, 1996). In addition to its systemic vasoconstrictor effect, Ang II action is more pronounced on the efferent arteriole (compared to the afferent arteriole), hence causing a rise in intraglomerular pressure. It also influences kidney microcirculation by inactivation of the breakdown of bradykinin, a vasodilator (Isselbacher *et al.*, 1994).

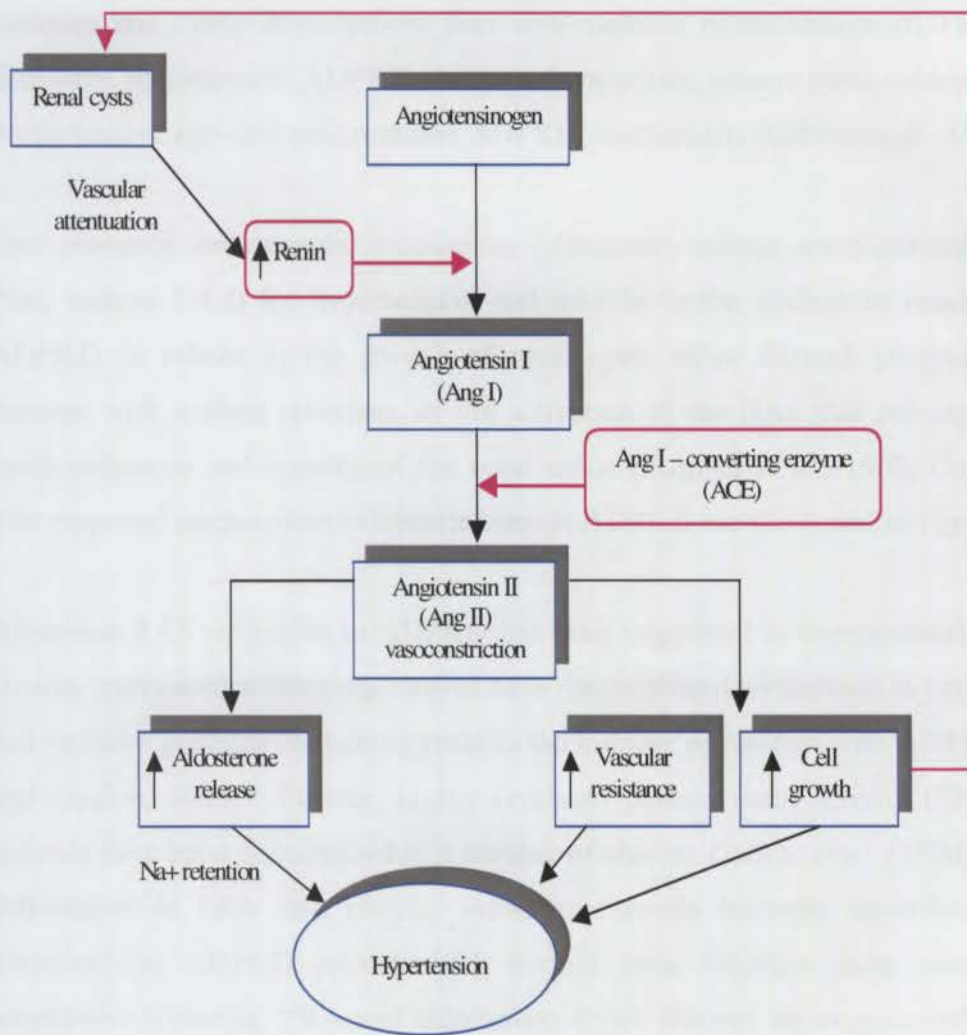


Hypertension occurs in 50-70% of ADPKD individuals prior to the onset of renal impairment (Hansson *et al.*, 1974; Gabow *et al.*, 1984, 1990; Milutinovic *et al.*, 1990; Chapman *et al.*, 1990; Gabow, 1993; Chapman and Gabow, 1997). ADPKD individuals with a concurrent family history of essential hypertension have been shown to present with a more severe clinical course of ADPKD (Geberth *et al.*, 1995b). Both male and female hypertensive ADPKD patients with normal renal function have greater renal volumes and cystic development than well-matched normotensive ADPKD subjects. Similarly, hypertensive ADPKD children demonstrate greater renal volumes than their normotensive age- and sex- matched ADPKD counterparts (Gabow *et al.*, 1990).

One proposed pathogenetic mechanism (alternative causes are discussed in Chapter One, section 1.4.2) for hypertension and its role in the decline of renal function in ADPKD, is related to the growth of renal cysts either through progressive tubular damage with sodium retention, or via activation of the RAS that subsequently cause renal ischaemia and scarring of the renal tissue (Ettinger *et al.*, 1969; Cornell, 1970). The proposed mechanisms of hypertension in ADPKD are illustrated in Figure 5.1.

Abnormal RAS activation in ADPKD has been suggested in experimental and clinical studies. Immunohistochemical studies have demonstrated an increase in juxtaglomerular and vascular granules containing renin in the kidneys of patients with ADPKD (Graham and Lindop, 1988). Further, higher levels of plasma renin activity (PRA) in these patients have been documented in a number of studies. Gabow *et al.* (1990) reported no differences in PRA and plasma aldosterone levels between normal subjects and normotensive ADPKD patients with normal renal function under baseline supine conditions. However, PRA and aldosterone levels showed an exaggerated response to upright posture in normotensive ADPKD patients as compared to normal subjects, possibly reflecting increased reactivity of the RAS even in the absence of hypertension in ADPKD patients (Chapman *et al.*, 1990). Elevated levels of PRA and aldosterone have been demonstrated in affected normotensive ADPKD family members as compared to normotensive unaffected members (Harrap *et al.*, 1991).

A higher renin concentration has been found in ADPKD kidneys, compared to normal kidneys and kidneys with renal artery stenosis. Renin messenger RNA is expressed in



**Figure 5.1:** The Renin-angiotensin System Pathway.

Modified from Bell *et al.* (1988) 34:683-690.

the tubulocystic epithelium of patients with ADPKD. These findings suggest that abnormal expression of the RAS system could enhance epithelial hyperplasia in growing renal cysts by a paracrine or autocrine mechanism (Torres *et al.*, 1992), which could be exerted through Ang II (Norman *et al.*, 1987). It may also stimulate the expression of other vasoactive factors and cytokines such as endothelin, transforming growth factor  $\beta_1$  (TGF- $\beta_1$ ), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF). These effects would lead to the increased production of extracellular matrix components and promote glomerular and interstitial infiltration by inflammatory cells (Egido, 1996). There is evidence that experimentally induced cystogenesis is enhanced by activation of the RAS, and is lessened by suppression of the RAS (Torres *et al.*, 1988). The effect of the RAS on cyst formation may also result from the altered sensitivity of tubular epithelial cells to Ang II (Wilson and Sherwood, 1991).

Some components of the RAS, namely the ACE and AGT, display heritable variation in their circulatory or cellular levels (Cambien *et al.*, 1988; Jeunemaitre *et al.*, 1992b; Costerousse *et al.*, 1993). This may provide the basis for a relationship between the constitutive over activity of the RAS and the development of vascular or renal damage in susceptible individual subjects.

#### 5.1.1.2 Angiotensinogen (AGT)

Angiotensinogen is the first component of the RAS and has been investigated as a candidate likely to be involved in the complex aetiology of essential hypertension. Two types of polymorphism in the *AGT* gene have been used in linkage and association studies. One is a simple dinucleotide (GT) repeat located in the 3'-flanking region of the *AGT* gene (Kotelevtsev *et al.*, 1991), which has been shown to display 11 alleles in Europeans and 13 alleles in Afro-Caribbeans (Caulfield *et al.*, 1995). This high degree of polymorphism makes the GT repeat a convenient intragenic marker for studies of the *AGT* gene.

The other type of variation in *AGT* involves two single nucleotide substitutions in the coding regions of *AGT* that result in missense mutations. These are 1) a methionine-threonine substitution at codon 174 (T174M), and 2) a threonine-methionine

substitution at codon 235 (M235T) (Jeunemaitre *et al.*, 1992b). M235T allele frequencies differ between racial groups, with the TT genotype being more common in Chinese than white Caucasians (Sanderson *et al.*, 1999). An association has been described between the M235T polymorphism and plasma AGT, with the highest levels found in subjects homozygous for the T allele, followed by MT heterozygotes and MM homozygotes (Jeunemaitre *et al.*, 1992b).

The evidence supporting the role of the *AGT* gene in essential hypertension has been controversial. A study of families with hypertension from France and USA (Jeunemaitre *et al.*, 1992b) has shown an excess of allele sharing in the microsatellite marker at the 3'-flanking region of the *AGT* gene in hypertensive sib pairs, and a significant difference in the distribution of T174M and M235T alleles between hypertensive subjects and unrelated controls. Caulfield *et al.* (1994) confirmed linkage of hypertension to the *AGT* locus in affected sib pairs, however they failed to replicate the positive association findings for the M235T polymorphism. More recently, Wang *et al.* (1999) also examined the role of *AGT* as a candidate gene for hypertension in Australian Anglo-Celtic Caucasians. However, both linkage and association findings excluded the *AGT* locus as a candidate for hypertension (Wang *et al.*, 1999). Further studies have produced conflicting results, with some supporting the association between M235T and essential hypertension (Hata *et al.*, 1994; Kamitani *et al.*, 1994; Iwai *et al.*, 1995; Nishiuma *et al.*, 1995), and others reporting a lack of association (Bennett *et al.*, 1993; Rotimi *et al.*, 1994; Brand *et al.*, 1998). A multi-centre European study, comprising 630 affected sib pairs, showed no evidence of linkage between the *AGT* locus and hypertension (Brand *et al.*, 1998).

Studies of the role of *AGT* in essential hypertension have also been conducted in animal models. The data obtained from linkage analysis in crosses between different strains of rats are also conflicting (Hubner *et al.*, 1994; Lodwick *et al.*, 1995).

The role of polymorphisms within the *AGT* gene in the deterioration of renal function has been studied in patients with IDDM and IgA nephropathy (Table 5.1). Two studies suggest an association (Fogarty *et al.*, 1996; Pei *et al.*, 1997), whereas three others report a lack of association (Hunley *et al.*, 1996; Tarnow *et al.*, 1996; Marre *et al.*,

1997). In view of the inconsistent results obtained in the studies of hypertension itself (paragraphs above), this controversy is not surprising.

To date, only three studies have examined the association between two polymorphisms, in *AGT* and in the angiotensin 1 receptor (AT1) gene, and ADPKD disease severity (Baboolal *et al.*, 1997; Saggar-Malik *et al.*, 2000; Konoshita *et al.*, 2001) (Table 5.6). The studies examined the effect of the M235T polymorphism in *AGT* and a nucleotide substitution of A with C at position 1166 in the AT1 gene on renal disease progression. The results from the three studies failed to show an association between these polymorphisms and renal disease in ADPKD subjects.

**Table 5.1 Studies that have Investigated the Relationship between the AGT M235T Polymorphism and Nephropathy in IDDM and IgA Nephropathy**

Condition	Group/Year	Ethnicity	Sample	Methodology and Laboratory analyses	Statistical Methodology	Results/Evidence for Association
Diabetes	Fogarty <i>et al.</i> (1996)	Northern Irish	* 95 IDDM with DN * 100 IDDM without DN * 80 blood donors	* Clinical data collected: SCr, UAE, HbA1c, BP, BP treatment, and DR * M235T polymorphism detected by PCR	* HWE and $\chi^2$ test to calculate M235T allele frequencies * T-test and ANOVA to compare between groups	* Association between M235T polymorphism & DN in IDDM patients
	Tarnow <i>et al.</i> (1996)	Danish	* 195 IDDM patients with DN * 185 IDDM patients without DN	* Clinical data collected: BP, BP treatment, DR, BMI, HbA1c, GFR * M235T polymorphism detected by PCR	* $\chi^2$ test to calculate M235T allele frequencies * T-test & ANOVA to compare between groups	* No association between M235T & T174M polymorphisms and DN in IDDM patients
	Marre <i>et al.</i> (1997)	French, Belgian	* 157 without IDDM DN * 104 with IDDM & MA * 126 with IDDM & proteinuria * 107 with IDDM & DN	* Clinical data collected: sex, age, ethnicity, weight, height, BP, onset of IDDM, current treatment, BP treatment, UAE, history of MI, cerebral stroke * M235T, ATIR, ACE I/D polymorphisms detected by PCR	* $\chi^2$ test to calculate allele frequencies * Parametric & non-parametric tests for categorical & continuous variables * LRA to calculate OR	* No association between M235T & DN in IDDM patients
IgA nephropathy	Hunley <i>et al.</i> (1996)	Caucasian	* 64 patients with IgA Nephropathy	* M235T & A1166C polymorphisms detected by PCR		* No association between the M235T & A1166C polymorphisms and nephropathy in IgA patients
	Pei <i>et al.</i> (1997)	Canadian	* 52 IgA patients with Ccr $\leq$ 3 ml/min/yr * 44 IgA patients with Ccr $\leq$ 6 ml/min/yr * 72 IgA patients with > 6 ml/min/yr * 100 healthy controls	* Clinical data collected: BP, Ccr, proteinuria, SCr, urine Cr, M235T & T174M polymorphisms detected by PCR	* HWE & $\chi^2$ test to calculate M235T allele frequencies * T-test & ANOVA to compare between groups * Ccr/time slope - LRA	* Association between polymorphisms in both AGT & ACE with IgA Nephropathy

IDDM – Insulin dependent diabetes mellitus; DN – diabetic nephropathy; SCr – serum creatinine; UAE – urinary albumin excretion; BP – blood pressure; DR – diabetic retinopathy; PCR – polymerase chain reaction; HWE – Hardy-Weinberg Equilibrium; ANOVA – analysis of variance; LRA – logistic regression analysis; OR – odds ratio; IgA nephropathy – Immunoglobulin A nephropathy

### 5.1.1.3 Renin

The role of the renin gene as a candidate likely to be involved in the development of essential hypertension has also been examined in association and linkage studies of experimental animals as well as humans. The polymorphisms used included restriction fragment length polymorphisms (RFLPs) and a polymorphic microsatellite repeat (ACAG)<sub>n</sub> in the human renin gene (chromosome 1q32), spanning the region between nucleotides 1898 and 2261 (Imai *et al.*, 1983; Shine *et al.*, 1984).

Differences in the number and frequency of alleles in the human renin polymorphic microsatellite exist between ethnic groups (Edwards *et al.*, 1992). Four alleles are found in Caucasians, Native Americans and Asians, while six alleles have been observed in Blacks (Brunner *et al.*, 1972; Kotchen *et al.*, 1982; Edwards *et al.*, 1992).

The role of the renin gene in blood pressure control has been investigated in animal models. A study using inbred salt-induced hypertension sensitive (S) and resistant (R) strains of rats displayed differences in the renin gene, namely a 1.2 kb insertion/deletion polymorphism in the first intron (Rapp *et al.*, 1989). This polymorphism was used to distinguish between the S and R allele, and to demonstrate that one dose of the S renin allele was associated with an increment in blood pressure of approximately 10 mmHg. Two doses of this allele increased blood pressure by approximately 20 mmHg (Rapp *et al.*, 1989). Similar results were obtained by Kurtz *et al.* (1990), who used RFLPs to analyze the association between renin and blood pressure in a cross between spontaneously hypertensive and normotensive rat strains (Kurtz *et al.*, 1990).

Despite the observations in animal models, several studies have failed to show a relationship between the renin gene and essential hypertension in humans. Soubrier *et al.* (1990) conducted an association study, with RFLP haplotypes in the renin gene compared in 120 normotensive and 102 hypertensive Caucasian subjects. This study found no association between renin and essential hypertension. Jeunemaitre *et al.* (1992a) found no evidence of linkage between renin and essential hypertension in a study of 98 affected sib pairs from 57 families with sustained hypertension.

There are only two published reports discussing polymorphic markers in the renin gene and their association with renal pathology (Angelico *et al.*, 1993; Deinum *et al.*, 1999). In the study by Deinum *et al.* (1999), four polymorphisms at the renin locus, consisting of two RFLPs (*TaqI* and *BglI*) and two microsatellites, the ([ACAG]<sub>n</sub> repeat and a CA-repeat located downstream of the renin locus) were studied in 199 patients with proteinuric IDDM and 192 normoalbuminuric IDDM controls. Diabetic nephropathy showed an association only with the *BglI* RFLP (Deinum *et al.*, 1999).

Evidence of increased renin activity found early in the clinical course of ADPKD (Chapman *et al.*, 1990; Gabow *et al.*, 1990; Harrap *et al.*, 1991; Torres *et al.*, 1992) suggests that genetic variation in the renin gene might play a role in modifying the ADPKD phenotype. This possibility has not been investigated in any study of either ADPKD patients or in individuals with other kidney disease leading to renal failure.

#### 5.1.1.4 The Angiotensin-Converting Enzyme (ACE)

Many studies have examined the role of ACE in pathological conditions, including ADPKD and other forms of renal disease. ACE catalyses the rate-limiting step of the renin-angiotensin pathway (Isselbacher *et al.*, 1994). The levels of plasma ACE activity remain relatively constant over time in a given individual (Tiret *et al.*, 1992), whereas wide (approximately 5-fold) variation is observed between individuals (Alhenc-Gelas *et al.*, 1991). Use of ACE inhibitors causes a rise in assayed plasma ACE levels, despite a fall in functional ACE capacity. Segregation and linkage analysis have provided evidence of strong genetic control over ACE levels, with a single major gene effect explaining a large part of the variability (Cambien *et al.*, 1988). An insertion (I) or deletion (D) of an Alu element in intron 16 of the ACE gene has been reported by Rigat *et al.* (1990). As with other polymorphisms, the allele frequencies of the Alu polymorphism in *ACE* show inter-population differences (Raynolds *et al.*, 1993; Lee, 1994; Mizuiri *et al.*, 1995; Bloem *et al.*, 1996; Foy *et al.*, 1996; Lindpaintner *et al.*, 1996; Baboolal *et al.*, 1997; van Dijk *et al.*, 1999). This is shown in Table 5.2.



**Table 5.2: Inter-population Differences in ACE Allele Frequencies**

Population	I:D ratio	Reference
Chinese	0.7:0.3	Lee <i>et al.</i> 1994
Pima Indians	0.7:0.3	Foy <i>et al.</i> 1996
Japanese	0.57:0.43	Mizuri <i>et al.</i> 1995
Caucasians	0.43:0.56	Raynolds <i>et al.</i> 1993; Lindpaintner <i>et al.</i> 1996; Baboolal <i>et al.</i> 1997; van Dijk <i>et al.</i> 1999
American Blacks	0.36:0.64	Bloem <i>et al.</i> 1996

A correlation between genotypes of the *ACE* Alu polymorphism and ACE activity levels has been found, with the highest levels of serum ACE observed in subjects homozygous for the D allele, followed by I/D heterozygotes and lowest levels found in I/I homozygotes (Rigat *et al.*, 1990). This association has subsequently been supported by a number of studies, conducted mostly in western European populations (Tiret *et al.*, 1992; Cousterousse *et al.*, 1993; van Dijk *et al.*, 1999), but has been disputed by others, such as Uemasu *et al.* (1997), who found no relationship between the I/D polymorphism and ACE levels in the blood serum. The studies that do report an association vary widely in terms of the estimates of the proportion of the variance in ACE activity explained by the Alu polymorphism in the *ACE* gene. These estimates range from 16% to 47% (Cambien *et al.*, 1988; 1994; Rigat *et al.*, 1990; Tiret *et al.*, 1992).

The Alu polymorphism appears to have little functional significance and has been shown in earlier studies to be a neutral polymorphism, in linkage disequilibrium with the *ACE* gene quantitative trait locus (QTL) that regulates ACE activity (Tiret *et al.*, 1992). Recent findings indicate that the QTL is likely to be located in the 3' region of the *ACE* gene (Zhu *et al.*, 2000). The latter study has identified a complexity of intragenic polymorphic haplotypes in the 3' region of *ACE*, with the I and D alleles of the Alu polymorphism occurring on diverse haplotype backgrounds, which had different and additive effects on ACE activity. The diversity of haplotypes and the observed inter-population differences (Zhu *et al.*, 2000) may account, at least in part, for the discrepant estimates of the proportion of the variance in ACE activity explained by the Alu I/D genotypes.

Regardless of this variation, the Alu polymorphism in ACE has been considered to be a useful polymorphic marker. It has been used in a number of studies examining the association between I/D genotypes and pathological conditions, such as left ventricular hypertrophy (Schunkert *et al.*, 1994), myocardial infarction (Nakai *et al.*, 1994), and stroke (Markus *et al.*, 1995). The effect of the ACE I/D genotype on renal pathology has been studied extensively in diabetic patients (IDDM as well as NIDDM), and in patients with IgA nephropathy. The findings of these studies are summarised in Tables 5.3, 5.4 and 5.5. Most studies compared the frequency of I/D genotypes between affected individuals with and without renal impairment, and matched controls, while some studies also included analysis of ACE activity (Schmidt *et al.*, 1995; Tarnow *et al.*, 1995; Freire *et al.*, 1998).

The findings in diabetic patients are inconsistent. Only three out of 7 studies of IDDM reported an association between I/D genotype and diabetic nephropathy, and the remaining 4 found no such association. In the case of NIDDM, a positive association between the presence of the D allele and nephropathy was detected in 5 of 7 studies (Fujisawa *et al.*, 1995; Mizuiri *et al.*, 1995; Dudley *et al.*, 1995; Doi *et al.*, 1996; Jeffers *et al.*, 1997). The I/D genotype was shown to have an effect on the severity of IgA nephropathy in 5 of 6 investigations, with the D allele associated with more rapid deterioration of renal function (Yoshida *et al.*, 1995; Gaciong *et al.*, 1995; Harden *et al.*, 1995; Hunley *et al.*, 1996; Pei *et al.*, 1997).

The Alu polymorphism in the ACE gene has also been examined in seven recent studies of ADPKD, with different clinical parameters used to measure ADPKD severity and search for an association with the I/D genotype. The findings are summarised in Table 5.6. The development of end-stage renal failure (ESRF) was the major parameter examined by Baboolal *et al.* (1997), Perez-Oller *et al.* (1999) and Konoshita *et al.* (2001). The three studies compared age at ESRF, as well as Kaplan-Meier survival curves in the different I/D genotype groups. More detailed comparisons, including a number of clinical parameters (kidney size, cysts in other organs, prevalence of UTI, cerebral aneurysms, and cardiac valve disease, etc.) were conducted in three studies (Uemasu *et al.*, 1997; van Dijk *et al.*, 1999, 2000). Saggar-Malik *et al.* (2000) investigated two end-points including chronic renal impairment (defined by a serum

creatinine >110  $\mu\text{mol/L}$ ) and ESRF. All studies included hypertension as a clinical parameter.

Four of these studies (Baboolal *et al.*, 1997; Perez-Oller *et al.*, 1999; Saggarr-Malik *et al.*, 2000; Konoshita *et al.*, 2001) examined only the differences in I/D genotype distribution between ADPKD subjects with different phenotype severity, whereas two (Uemasu *et al.*, 1997; van Dijk *et al.*, 1999) also included measurements of ACE activity. The recently published van Dijk *et al.* (2000) study which investigated I/D genotypes, was a follow-up of a previous study (van Dijk *et al.*, 1999), where ACE activity had been determined in the same patients.

Although the majority of studies concluded that there was no significant association between I/D genotype and renal failure in ADPKD, three (Baboolal *et al.*, 1997; Perez-Oller *et al.*, 1999; Konoshita *et al.*, 2001) suggested that the homozygous D/D genotype confers a greatly increased risk of early renal failure.

Baboolal *et al.* (1997) investigated 189 affected subjects from Australia and the UK, including 52 who had reached ESRF. Mean age at ESRF was estimated at 52 years for D/D homozygotes, 64 years for I/D heterozygotes and 59 for I/I homozygotes. The group with ESRF was divided further into three subgroups, namely those who had reached ESRF before age 40 years (11 individuals), between 41 and 49 years (21 individuals) and after 50 years (20 individuals). The comparison of the distribution of I/D genotypes between these three subgroups showed that D/D homozygotes predominated among the early ESRF group. Baboolal *et al.* (1997) estimated that this genotype resulted in a 17-fold increase in the relative risk of developing ESRF before age 40.

The second study suggesting an effect of the D allele on early kidney failure was that of Perez-Oller *et al.* (1999), which included 155 Spanish individuals with ADPKD. This study reported that renal survival was 48 years for D/D homozygotes, 53 for I/D heterozygotes and 51 years for I/I homozygotes. It also concluded that the D/D genotype was associated with early renal failure, which in this case was defined as ESRF developing before age 50 years. No estimate of relative risk was provided.

**Table 5.3 Studies that have Investigated the Relationship between the ACE I/D Polymorphism and IDDM**

Group/Year	Ethnicity	Sample	Methodology and Laboratory analyses	Statistical Methodology	Results/Evidence for Association
Doria <i>et al.</i> (1994)	White Americans	* 74 with DN * 77 normoalbuminuric controls	* Data collected: age at diagnosis & BP * ACE I/D by PCR	* ACE I/D frequency - $\chi^2$ * Test of association using Odds ratio	* Evidence that DNA sequence differences in ACE gene may contribute to susceptibility to DN in IDDM
Schmidt <i>et al.</i> (1995)	German & Polish	* 114 with DN * 134 normoalbuminuric controls	* ACE I/D by PCR * Serum ACE activity	* ACE I/D frequency - $\chi^2$	* Serum ACE correlated with I/D genotypes * No association between I/D polymorphism & DN in IDDM
Tarnow <i>et al.</i> (1995)	Danish	* 198 with DN * 190 normoalbuminuric controls	* ACE I/D by PCR * Plasma ACE activity	* ACE I/D frequency - $\chi^2$ * MLR	* No difference in ACE genotype frequency between patients with & without DN * Plasma ACE elevated in DN patients ( $p < 0.001$ )
Chowdhury <i>et al.</i> (1996)	British	* 166 (IDDM >15 yrs) * 341 newly diagnosed IDDM * 187 matched non-diabetic controls	* ACE I/D by PCR	* ACE I/D frequency - $\chi^2$	* No association between ACE I/D & IDDM
Marre <i>et al.</i> (1997)	Caucasian, Black and Asian	* 157 no DN * 104 MA * 126 proteinuria * 107 advanced DN	* Data collection: age, sex, ethnicity, IDDM – onset, treatment, dose, BP treatment, UAE, cardiovascular complications	* Continuous variables - $\chi^2$ , ANOVA * MLR to calculate OR	* Association between I/D & renal disease in inadequately controlled IDDM subjects
Barnas <i>et al.</i> (1997)	Austrian	* 63 with DN * 59 normoalbuminuric controls	* Data collection: age, sex, onset, prevalence of retinopathy, BP, SCr, Ccr, UAE, HbA <sub>1c</sub> , BP treatment * ACE I/D by PCR	* ACE I/D frequency - $\chi^2$ * t-test to compare data * LRA & MRA to calculate influence of prognostic factors on DN	* In patients with diabetes >20 years (n=90) * Association between I/D polymorphism & DN in IDDM
Beatriz <i>et al.</i> (1998)	Jewish	* 771 with DN * 89 normoalbuminuric controls	* ACE I/D by PCR * Ddel polymorphism by DGGE * Serum ACE activity	* ACE I/D frequency - $\chi^2$ * ANOVA	* Serum ACE correlated with I/D genotypes * The I/D polymorphism was not associated with DN in IDDM patients

DN – diabetic nephropathy; IDDM – insulin dependent diabetes mellitus; BP – blood pressure; PCR – polymerase chain reaction; UAE – urinary albumin excretion; SCr – serum creatinine; Ccr – creatinine clearance; MLR – multiple logistic regression; LRA – logistic regression analysis; ANOVA – analysis of variance; DGGE – ; HbA<sub>1c</sub> – hemoglobin A<sub>1c</sub>

**Table 5.4 Studies that have Investigated the Relationship between the ACE I/D Polymorphism and NIDDM**

Group / Year	Ethnicity	Sample	Methodology and Laboratory analyses	Statistical Methodology	Results/ Evidence for Association
Fujisawa <i>et al.</i> (1995)	Japanese	* 61 patients with MI * 136 patients without MI	* Data collection: BMI, HbA <sub>1c</sub> , total cholesterol	* Comparison between groups - students t-test	* D allele is an independent risk factor for MI in Japanese NIDDM patients (adjusted for age & smoking)
Mizuiru <i>et al.</i> (1995)	Japanese	* 80 with DN * 31 without DN * 76 healthy controls	* ACE I/D by PCR	* ACE I/D frequency and qualitative data - $\chi^2$	* DN patients showed excess of I/D genotype vs patients without DN (P<0.02) & less of the I/I genotype vs healthy controls (P<0.01) and patients without DN (P<0.01)
Dudley <i>et al.</i> (1995)		* 180 patients with MA * 2 matched controls without MA; 1 group matched for HBP and one was not.	* Data collection: 1) UAE, BMI, HbA <sub>1c</sub> , LDL, Triglyceride, HDL, BP, smoking, DR * ACE I/D by PCR	* Continuous variables - t-test; non-parametric data - rank test; categorical data - $\chi^2$ & McNemar's test	* D/D genotype associated with higher urinary albumin levels in NIDDM subjects but not DR
Doi <i>et al.</i> (1996)	Japanese	* 362 NIDDM patients * 105 healthy controls	* Data collection: 1) BMI, NIDDM duration, HbA <sub>1c</sub> , BP, DR, UAE	* ACE I/D frequency - $\chi^2$ * differences between groups - ANOVA	* Association between I/D polymorphism & DN but not DR
Nakajima <i>et al.</i> (1996)	Japanese	* 41 with normal albuminuria * 47 with MA * 54 with proteinuria	* Data collection: duration & NIDDM treatment, SCr, HbA <sub>1c</sub> , ACR * ACE I/D by PCR	* ACE I/D frequency - $\chi^2$	* No association between I/D polymorphism & albuminuria
Jeffers <i>et al.</i> (1997)	non-Hispanic	* 509 NIDDM patients	* Data collection: NIDDM duration, BP & treatment, UAE, CHD, BMI, cholesterol, HDL, Triglyceride, smoking, SCr, Uric acid	* I/D frequency & complications - $\chi^2$ * continuous & categorical variables- ANOVA & LRA	* D/D genotype associated with increased risk for DN but not CHD
Kimura <i>et al.</i> (1998)	Japanese	* 98 with DN * 110 without DN	* Data collection: diabetes duration, cholesterol, HBP, DR, MA, overt proteinuria, smoking * ACE I/D and PAI-1 polymorphisms by PCR	* ACE I/D effect on DN & macroangiopathy - LRA	* ACE D/D genotype & its interaction with PAI-1 are independent risk factors for macroangiopathy but not for DN in NIDDM subjects

MI – myocardial infarction; MA – microalbuminuria; NIDDM – non-insulin dependent diabetes mellitus; DN – diabetic nephropathy; UAE – urinary albumin excretion; BMI – body mass index; BP – blood pressure; DR – diabetic retinopathy; LDL – low density lipoprotein; HDL – high density lipoprotein, SCr – serum creatinine; CHD – coronary heart disease; ANOVA – analysis of variance; LRA – logistic regression analysis; PAI-1 – plasminogen activator inhibitor-1; HbA<sub>1c</sub> – haemoglobin A<sub>1c</sub>

**Table 5.5 Studies that have Investigated the Relationship between the ACE I/D Polymorphism and IgA Nephropathy**

Group	Ethnicity	Sample	Methodology and Laboratory analyses	Statistical Methodology	Results Evidence for Association
Harden <i>et al.</i> (1995)	White Caucasian	* 100 IgA nephropathy subjects * 100 normal subjects without renal disease	* Data collection: 1) BP, SCr, UPE * ACE I/D polymorphism by PCR	* Ccr/time slope - LRA	* ACE I/D polymorphism not linked with development of IgA nephropathy but is with earlier presentation and increased rate of progression in patients with the DD genotype
Yoshida <i>et al.</i> (1995)	Japanese	* 53 IgA nephropathy patients * 46 age-matched controls without proteinuria	* Data collection: UPE, BP, SCr, * ACE I/D polymorphism by PCR	* ACE I/D frequency - $\chi^2$ * Ccr/ time slope - LRA * effect of ACE I/D genotypes & ACE inhibitors - ANOVA	* D/D is a risk factor for progression of CRF in IgA nephropathy & predicts efficacy of ACE inhibition on proteinuria & on progressive deterioration of renal function
Zbigniew <i>et al.</i> (1995)	Japanese	* 241 renal allograft recipients * 109 healthy blood donors	* Primary renal diseases of recipients: glomerulonephritis (74.5%), reflux nephropathy (11.2%), PKD (8.2%) and others (6.1%) * ACE I/D detected by PCR		* Excess of D/D homozygotes in renal allograft recipients compared to healthy controls * ACE I/D polymorphism may be a risk factor for progression of renal disease
Fukushima <i>et al.</i> (1995)	Japanese	* 24 patients (Ccr > 80% of initial rate); 25 patients Ccr <50% of initial rate or with ESRF * 100 controls			* No association between ACE I/D polymorphism & IgA nephropathy
Hunley <i>et al.</i> (1996)	American Caucasians	* 64 IgA patients a) Non-progressors (SCr, <1.5 mg/dl) b) Progressors (SCr >4.5 mg/dl)	* Data collection: HBP, proteinuria, SCr * ACE I/D polymorphism detected by PCR	* interaction among clinical parameters - LRA * ACE I/D frequency - $\chi^2$	* ACE I/D polymorphism is associated with progressive deterioration in renal function in IgA nephropathy
Pei <i>et al.</i> (1997)	Caucasians	* 52 slow progressors (S) (SCr – 1.6mg/dl); 44 intermediate progressors (I) (SCr – 7.3 mg/dl; 72 fast progressors (F) (SCr – 8.8mg/dl) * 100 Controls	* Examined: 1) rate of deterioration of creatinine clearance and 2) maximal level of protein * Data collection: urinary Ccr, proteinuria, BP	* continuous variables - T-test or ANOVA; categorical variables - $\chi^2$ * Ccr/ time slope - LRA * genotype frequencies - HWE & $\chi^2$ * estimate risk - OR	* In multivariate analysis association between AGT M235T and ACE I/D polymorphisms were associated with disease progression

*IgA – immunoglobulin A; Ccr – creatinine clearance; ESRF – end-stage renal failure; SCr – serum creatinine; BP – blood pressure; UPE- urinary protein excretion; PCR – polymerase chain reaction; LRA – logistic regression analysis; ANOVA – analysis of variance; HWE – Hardy-Weinberg Equilibrium*

**Table 5.6: Studies that have Investigated the Relationship between the ACE I/D Polymorphism and ADPKD**

Study	Number of patients	Ethnicity	Measures of ADPKD severity	Characterisation of the ACE gene	Association between ACE and ADPKD	Problems of the study
Baboolal <i>et al.</i> (1997)	* 189 PKD1 patients (46 families)	British, Irish, Italian	* age at ESRF * cumulative renal survival to ESRF * hypertension	* ACE I/D genotypes	* Yes	* failed to demonstrate a dosage effect of the D allele * small numbers used to calculate relative risk to ESRF
Uemasu <i>et al.</i> (1997)	* 47 patients	Japanese	* I/Cr slope * Clinical manifestations renal size hypertension extrarenal cysts cardiac valvular disease	* ACE I/D genotype * Serum ACE activity	* No	* overall small sample * small number of DD genotypes, hence analysis performed between ID & II only  * linkage analysis not performed
Perez-Oller <i>et al.</i> (1999)	* 155 PKD1 patients (49 families)	Spanish	* cumulative renal survival to ESRF * hypertension	* ACE I/D genotype	* Yes	* lack of dosage effect of the D allele * no estimate of relative risk to ESRF
van Dijk <i>et al.</i> (1999)	* 73 untreated ADPKD patients	Dutch	* proteinuria and microalbuminuria * hypertension	* ACE I/D polymorphism * Serum ACE activity	* No	* neither renal survival nor age at ESRF examined * no estimate of relative risk to ESRF * only 2 SCr values taken in a 2 week interval
van Dijk <i>et al.</i> (2000)	* 49 ESRF patients (<40 yrs) * 21 PKD1 patients with ESRF (>60 yrs) * 59 unaffected controls	Dutch	* hypertension * renal function divided in 2 groups: 'fast' & 'slow' progressors	* ACE I/D polymorphism	* No	* small sample * neither renal survival nor age at ESRF examined * no estimate of relative risk to ESRF
Saggar-Malik <i>et al.</i> (2000)	* 176 patients	British	* SCr (<110 & >110 umol/L); hypertension and ESRF as outcome variables	* ACE I/D polymorphism	* No	* small sample of patients with ESRF, chronic renal impairment and with the DD genotype * linkage analysis not performed * renal survival not performed
Konoshita <i>et al.</i> (2001)	* 103 patients	Japanese	* age at ESRF * cumulative renal survival to ESRF	* ACE I/D polymorphism	* No	* combined ID & II groups, hence difficult to draw conclusion ( <i>i.e.</i> dosage effect of the DD allele) * small number of DD patients

*ESRF – end-stage renal failure; SCr – serum creatinine*

More recently a study conducted on 103 ADPKD Japanese patients also suggested that patients with the D/D genotype reach ESRF significantly earlier than I/D and I/I subjects. The study reported that the age at renal death and cumulative renal survival was 45 years for D/D homozygotes and about 55 years for I/D and I/I genotypes. However, this study only included a small number of D/D subjects ( $n = 10$ ) and both the I/D and I/I genotypes were combined in one group making it difficult to draw any firm conclusions on the effect of the D allele (Konoshita *et al.*, 2001).

The three studies failed to demonstrate a dosage effect of the D allele; paradoxically I/D heterozygotes in the studies of Baboolal *et al.* (1997) and Perez-Oller *et al.* (1999) had better renal survival than I/I homozygotes.

The remaining four studies published to date, namely Uemasu *et al.* (1997), van Dijk *et al.* (1999; 2000) and Saggar-Malik *et al.* (2000) included smaller numbers of ADPKD patients, *i.e.* 47 Japanese (Uemasu *et al.*, 1997), 73 Dutch (van Dijk *et al.*, 1999, 2000) and 176 British (Saggar-Malik *et al.*, 2000), however these studies used a wider range of clinical parameters to characterise the ADPKD phenotype. Both Uemasu *et al.* (1997) and van Dijk *et al.* (1999) included measurements of ACE activity levels. van Dijk *et al.* (1999) reported an increased rate of microalbuminuria among D/D homozygotes. As previously mentioned, Saggar-Malik *et al.* (2000) investigated the effect of the ACE genotype on renal failure by using two renal end-points. All four studies failed to replicate the positive findings discussed in the previous paragraphs and found no association between I/D genotype and rate of progression towards ESRF.

The results on studies investigating the relationship between the ACE I/D polymorphism and ADPKD have been controversial with some studies showing an association between the ACE I/D polymorphism and increased risk of developing renal failure (Baboolal *et al.*, 1997; Perez-Oller *et al.*, 1999), and others failing to find such an effect (Uemasu *et al.*, 1997; van Dijk *et al.*, 2000). The basic assumption of such studies is that the Alu genotypes are representative of the true biological variable, namely ACE activity. However, the estimates of the proportion of the variance in enzyme activity explained by the Alu polymorphism vary substantially (Cambien *et al.*, 1988; Rigat *et al.*, 1990; Tiret *et al.*, 1992; Cambien *et al.*, 1994) and individual and



population differences in the genetic control can be expected to have a confounding effect on studies where the I/D genotype is used as a substitute for ACE activity. Therefore, to address this problem this thesis includes 307 affected individuals (which is the largest sample to date) from three different populations and aims to investigate the relationship between ACE and ADPKD severity in terms of both plasma enzyme activity and I/D genotypes. The large sample size and the overall design of this study will provide substantial power to detect any association between the ACE I/D polymorphism and ADPKD.

#### **5.1.1.5 Genetic methodologies used to assess the association between the RAS and pathological conditions**

In an attempt to elucidate the role of RAS in the development of hypertension and renal disease, several studies have employed two genetic methods, namely linkage analysis and allelic association. In comparing the two procedures, allelic association is preferable in detecting a correlation in the population between a phenotype and a particular allele. This type of association is usually assessed as an allelic or genotypic frequency difference between cases and controls. An allele A at a gene of interest is said to be associated with the trait if it occurs at a significantly higher frequency among affected persons compared to unaffected controls. Moreover, allelic association is most meaningful when applied to functionally significant variations in genes that have a clear biological relation to the trait. An example of this is the apolipoprotein E (Apo-E) gene and late-onset Alzheimer's Disease (Corder *et al.*, 1994). Allelic association can also provide the statistical power needed to detect genes of small effect.

In contrast, linkage studies test whether a disease and an allele show correlated transmission within a pedigree. The disadvantage of traditional linkage studies is that they are only able to detect single genes or genes largely responsible for the trait (Lander and Schork, 1994).

The reported positive associations between the RAS polymorphisms and the various pathological conditions can arise for three reasons. Firstly, a positive association can occur if allele A is actually the cause of the disease. In this case, the same positive association would be expected to occur in all populations. Secondly, a positive

association can occur if allele A does not cause the trait but is in linkage disequilibrium with the actual cause, that is, A tends to occur on those chromosomes that also carry a trait-causing mutation. Lastly, positive associations can arise as an artefact of population admixture. In a mixed population, any trait present at a higher frequency in an ethnic group will show positive association with an allele that also happens to be more common in that group (Lander and Schork, 1994).

#### **5.1.1.6 Summary of the literature review**

The conflicting findings indicate that further examination is required to investigate the possible role of genetic components in the RAS in the variable nature of ADPKD. Since PKD gene type, allelic heterogeneity, sex and hypertension are likely to modify the phenotype in ADPKD, it is imperative that RAS association studies correct for these variables.

The aims of the study are:

- 1) To examine the effect of the ACE I/D polymorphism on ADPKD phenotype severity
  - a) on age and progression to renal failure (CRF and ESRF)
  - b) left ventricular mass
  - c) on renal cystogenesis
- 2) To examine the effect of the angiotensinogen gene on ADPKD phenotype severity
  - a) on age and progression to renal failure (CRF and ESRF)
- 3) To examine the effect of the renin gene on ADPKD phenotype severity
  - a) on age and progression to renal failure (CRF and ESRF)

## **5.2 SUBJECTS AND METHODS**

### **5.2.1 Subjects**

The study included 316 ADPKD patients, 159 males and 157 females, aged from 9 to 85 years (mean 45.2 years). The general characteristics of the patients are provided in Table 5.7. Details on the clinical assessment of the individuals are provided in Chapter Two. The linkage analysis methods and results are described in Chapter Four.

**Table 5.7: Characteristics of 316 ADPKD Patients Included in the RAS Analyses**

	Country of residence			
	Australia	Bulgaria	Poland	Overall
Total Number of Affected Individuals	116	133	67	316
Male/female	53:63	70:63	36:31	159:157
<b>Genetic linkage</b>				
<i>PKD1</i>	58 (50%)	81 (60%)	45 (65%)	184 (58%)
<i>PKD2</i>	3 (3%)	27 (20%)	8 (12%)	38 (12%)
Undefined	55 (47%)	25 (19%)	14 (21%)	94 (29%)
Mean current age	46.6 ( $\pm 1.4$ )	46.7 ( $\pm 1.5$ )	39.8 ( $\pm 2.0$ )	45.2 ( $\pm 0.9$ )
Hypertension	85 (73%)	70 (81%)	41 (61%)	197 (62%)
CRF	43 (37%)	51 (39 %)	24 (36%)	118 (37%)
ESRF	33 (28 %)	26 (38 %)	9 (13%)	68 (22 %)

*Values are expressed as means  $\pm$ SE and numbers (%)*

### 5.2.2 *Methods*

Plasma ACE activity and detection of the ACE I/D polymorphism and the angiotensinogen and renin microsatellites are described in detail in Chapter Two, sections 2.3.2 and 2.5.3.

#### 5.2.2.1 *Statistical Analysis*

Statistical procedures are discussed in Chapter Two, section 2.7. Frequency data, ANOVA and survival analyses were performed by Dr Valerie Burke (University Dept. of Medicine, UWA).

Decline in renal function was assessed using two approaches, namely age at onset and cumulative renal survival. The age at onset of CRF was examined in two ways: (1) observed values only and (2) observed or extrapolated values (see Chapter Two, section 2.7). Kaplan-Meier survival curves (Kaplan and Meier, 1972) were used to calculate cumulative survival to CRF and ESRF and were compared using the log-rank test. At the time of the study the number of individuals with available SCr data and included in the RAS analyses is shown in Table 5.8. As a result of follow-up data becoming

available at various times throughout the study period, the number of individuals included in each analysis differs according to the availability of SCr data.

Table 5.8: Number of individuals included in the analyses of the effect of RAS genes on age at onset and cumulative renal survival to CRF and ESRF

	Angiotensino gen	Renin	Angiotensin Converting Enzyme						
Variable	Overall	Overall	Overall	<i>PKD1</i> expanded	<i>PKD2</i>	<i>PKD1</i> expanded & by sex		<i>PKD2</i> & by sex	
						Male	Female	Male	Female
Age at renal failure									
CRF observed	-	-	117	-	-	-	-		
CRF observed & extrapolated	289	289	-	-	-	116	118		
ESRF	68	68	68	-	-	128	131		
Cumulative survival									
To CRF observed	-	-	-	-	-	-	-	-	-
To CRF observed & extrapolated	-	-	282	234	33	116	118	14	19
To ESRF	68	-	307	259	38	128	131	18	20

5.3

ANGIOTENSINOGEN CONVERTING ENZYME (ACE)

RESULTS

5.3.1

I/D Genotypes in the ACE Gene and Plasma ACE Activity

In the 307 individuals studied, the frequency of the insertion (I) allele was 0.45 and that of the deletion (D) allele was 0.55. A difference between the three populations was observed, with a lower frequency of the D allele in the Polish sample ( $p = 0.02$ ). The allele and genotype distribution is shown in Table 5.9. No deviation from Hardy-Weinberg equilibrium was seen in the overall sample, or in any individual population.

Table 5.9: Alu Polymorphism in the *ACE* Gene: Allele and Genotype Frequencies by Country

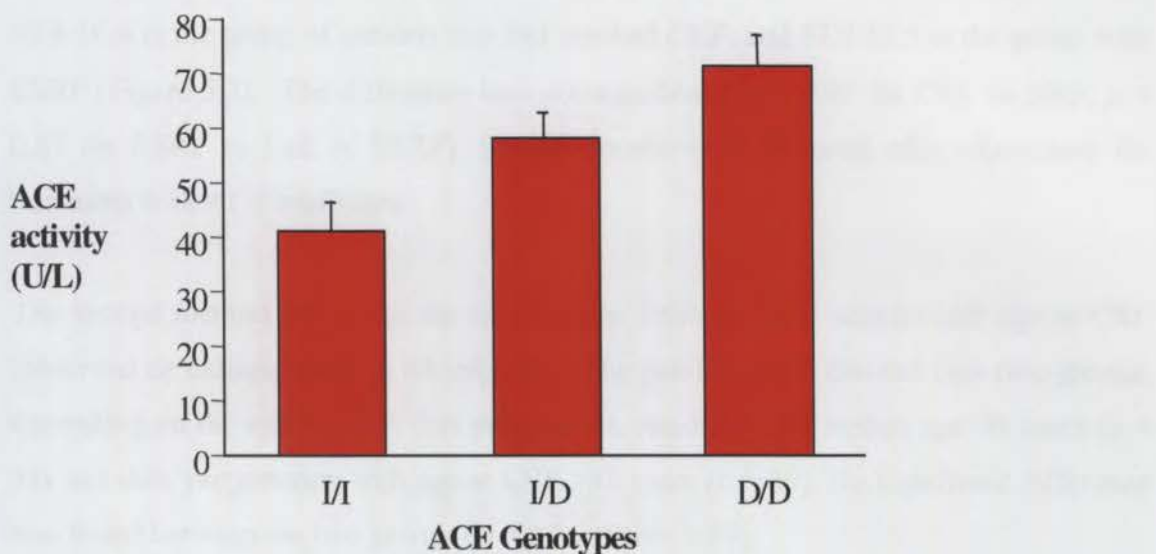
	Country			
	Australia	Bulgaria	Poland	Total
Allele Frequencies				
I	0.41	0.41	0.52	0.45
D	0.59	0.59	0.48*	0.55
Genotypes				
I/I	21 (18.1%)	13 (10.5%)	18 (26.9%)	52 (17.0%)
I/D	54 (46.6%)	75 (60.5%)	34 (50.7%)	163 (53.0%)
D/D	41 (35.3%)	36 (29.0%)	15 (22.4%)	92 (30.0%)
Total	116	124	67	307

\*The frequency of the D allele is significantly lower in the Polish group ( $p = 0.02$ )

The plasma ACE activity in all 92 Australian ADPKD patients fell within the reference interval (23-100 U/L). The measured enzyme activity correlated with the Alu genotype, with a mean value of 41.2 U/L observed in I/I homozygotes, 59.0 U/L in I/D heterozygotes, and 71.2 U/L in D/D homozygotes ( $p<0.006$ ; see also Figure 5.2). Similar results were obtained after adjustment for the use of ACE inhibitors in the control of hypertension: 43.5 U/L for II, 56.2 U/L for ID and 69.7 U/L for DD. The fraction of the variance of plasma ACE activity explained by the *ACE* I/D polymorphism was estimated at 14%.

### 1.3.3 Plasma ACE activity and Alu genotyping

The relationship between plasma ACE activity and the *ACE* genotype in 100 healthy subjects was studied in this study. The *ACE* genotype was studied in 100 subjects, and plasma ACE activity was measured in the same 100 subjects. The *ACE* genotype was studied in 100 subjects, and plasma ACE activity was measured in the same 100 subjects. The *ACE* genotype was studied in 100 subjects, and plasma ACE activity was measured in the same 100 subjects.



**Figure 5.2:** Correlation between Alu Genotypes and Plasma ACE Activity

### 5.3.2 *Plasma ACE Activity and Renal Function*

The relationship between plasma ACE activity and the development of renal failure was examined in two ways. The first analysis included the 92 Australian patients, grouped on the basis of kidney function into normal ( $n = 28$ ), CRF ( $n = 37$ ) and ESRF ( $n = 27$ ). Plasma ACE activity was compared between the three groups (Figure 5.3). The mean ACE activity levels were:  $61.7 \pm 3.8$  U/L in the group with normal renal function (NRF);  $59.8 \pm 6.4$  in the group of patients that had reached CRF; and  $57.9 \pm 5.5$  in the group with ESRF (Figure 5.3). The difference was not significant ( $p = 0.49$  for CRF vs NRF;  $p = 0.87$  for ESRF vs lack of ESRF). Similar results were obtained after adjustment for treatment with ACE inhibitors.

The second method examined the relationship between ACE activity and age at CRF (observed or extrapolated) in 84 subjects. The patients were divided into two groups, depending on the age at CRF: fast progressors, reaching CRF before age 40 years ( $n = 45$ ) and slow progressors, with age at CRF  $>40$  years ( $n = 39$ ). No significant difference was found between the two groups ( $p = 0.35$ ; Table 5.10).

**Table 5.10: Effect of Serum ACE Activity on Age at Chronic Renal Failure**

Age at CRF	Number	Mean sACE activity	<i>p</i> value
<40 years	45	$62.6 \pm 4.1$	0.35
>40 years	39	$56.3 \pm 5.5$	
Total	84	$59.7 \pm 3.4$	

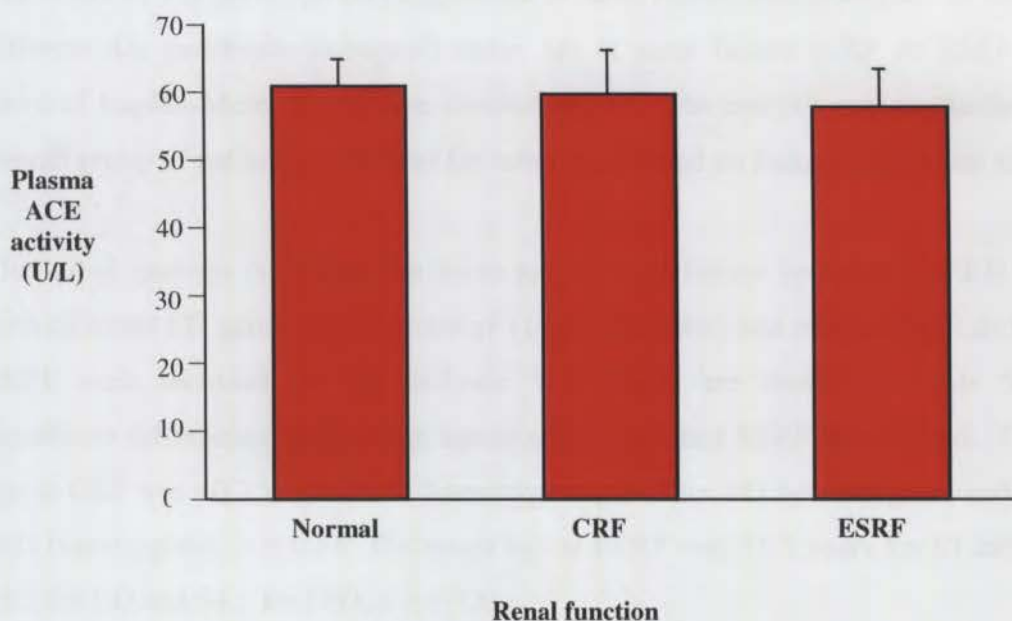
*Values are expressed as means  $\pm$  SE*

### 5.3.3 *I/D Genotypes in the ACE Gene and Renal Function*

#### 5.3.3.1 *I/D genotype and overall risk of renal failure*

The analysis included 307 ADPKD subjects, grouped according to renal function into normal ( $n = 190$ ), CRF ( $n = 117$ ) and ESRF ( $n = 68$ ). The distribution of I/D genotypes was compared between the three groups (Table 5.11). No significant differences were found:  $p = 0.58$  in the comparison of CRF versus normal renal function and  $p = 0.83$  for ESRF vs lack of ESRF.





**Figure 5.3:** Plasma ACE Activity in ADPKD Individuals with Normal Renal Function and Renal Impairment.

**Table 5.11: Comparison of *ACE* Genotypes between ADPKD Subjects with Normal Kidney Function and Renal Failure**

Renal Function	<i>ACE</i> Genotypes			Total
	I/I	I/D	D/D	
Normal renal function	31 (16.3%)	97 (51.2%)	62 (32.5%)	190
CRF	21 (18%)	66 (56%)	30 (26%)	117
ESRF	13 (19%)	35 (51.5%)	20(29.5%)	68

### 5.3.3.2 I/D genotype and rate of deterioration of renal function

The effect of I/D genotype on progression to renal failure was examined by comparing different *Alu* genotypes as regards mean age at renal failure (CRF or ESRF) and in terms of Kaplan-Meier cumulative survival curves. The analysis was conducted for the overall group of patients, as well as for subgroups based on linkage status and sex.

The initial analysis compared the mean age at renal failure between ADPKD subjects with different I/D genotypes. A total of 117 subjects who had reached CRF and 68 with ESRF were included in this analysis. The results are shown in Table 5.12. No significant differences in the mean age to either CRF and ESRF were found. The mean age at CRF was 40.7 years for I/I homozygotes, 39.7 for I/D heterozygotes and 39.1 for D/D homozygotes,  $p = 0.81$ . The mean age at ESRF was 51.5 years for I/I individuals, 55.5 for I/D and 54.5 for D/D,  $p = 0.42$ .

**Table 5.12: Comparison of Mean Age (in years) at Renal Failure between *ACE* Genotypes**

Age at Renal Failure	<i>ACE</i> Genotype			<i>p</i> value
	I/I (n=21)	I/D (n=66)	D/D (n=30)	
CRF (n=117)	40.7±1.9	39.7±1.1	39.1±1.5	0.81
	I/I (n=13)	I/D (n=35)	D/D (n=20)	
ESRF (n=68)	51.5±3.0	55.5±1.6	54.5±1.7	0.42

*Values are expressed as means ±SE*

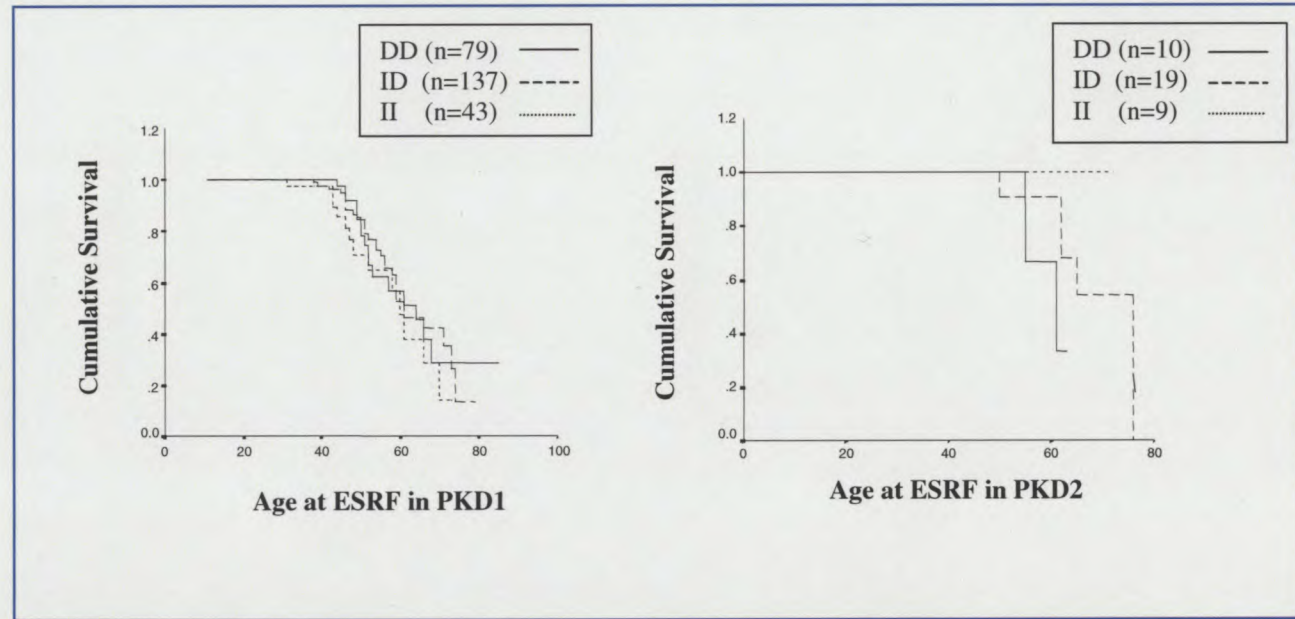
The second approach to assessing the effect of I/D *ACE* genotypes on deterioration of renal function was based on a comparison between the three genotypes using Kaplan-Meier cumulative survival curves. The number of individuals and results are shown in

Figure 5.4. No statistically significant differences were found in the overall patient population ( $p = 0.82$  for CRF and  $p = 0.75$  for ESRF).

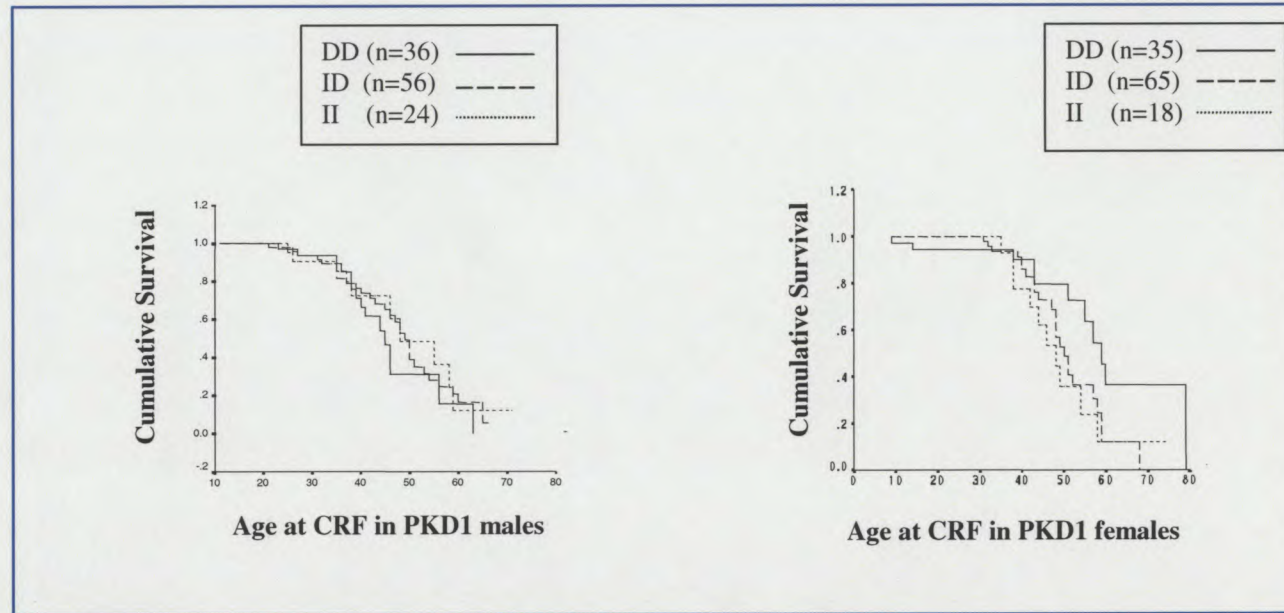
The same analysis was conducted separately for the PKD1 and PKD2 groups. In the PKD1 group, the analysis was initially performed on the definite PKD1 and subsequently repeated on the expanded PKD1 group. The number of individuals and the results of survival to CRF and ESRF are shown in Figures 5.5 and 5.6.

No significant effect of I/D genotype was detected on survival to CRF ( $p = 0.61$  for PKD1 and  $p = 0.53$  for PKD2) or to ESRF ( $p = 0.45$  for PKD1 and  $p = 0.16$  for PKD2). Similar results were obtained from the analysis of the definite and the expanded PKD1 groups.

The comparison between Kaplan-Meier cumulative survival curves in individuals with different I/D genotypes was also performed separately for male and female ADPKD subjects, linked to *PKD1* or *PKD2*. The number of individuals and the results are shown in Figures 5.7-5.10. No significant effect of I/D genotype was detected.

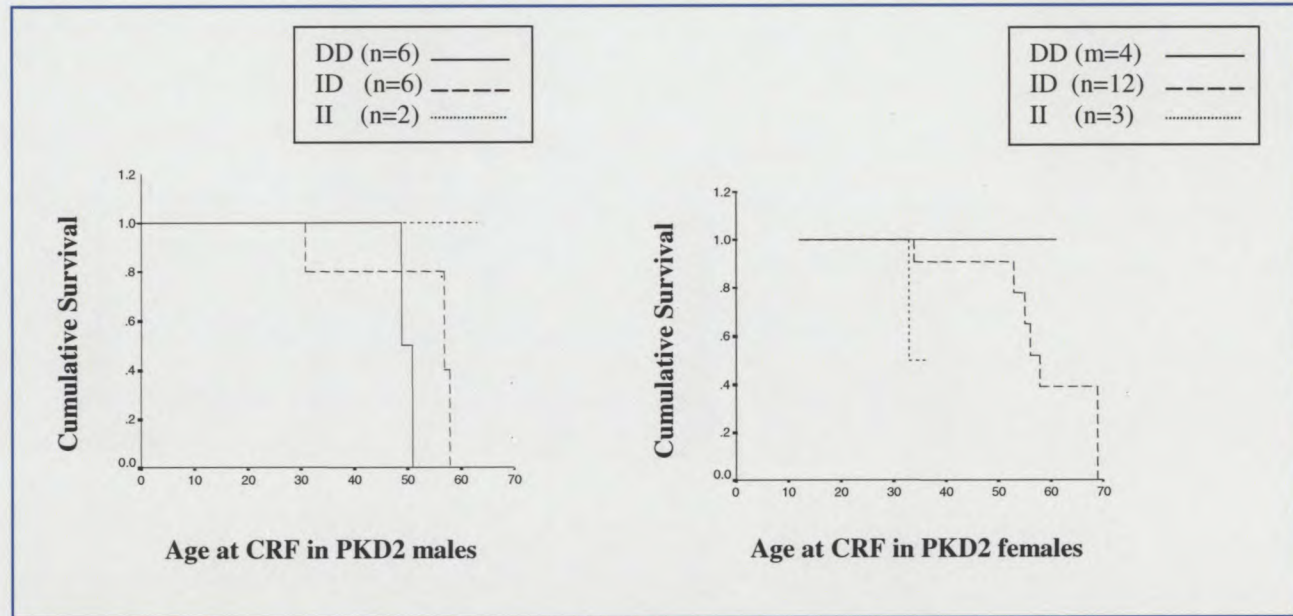


**Figure 5.6:** Analysis of the I/D Polymorphism on Survival to ESRF in PKD1 and PKD2 Individuals. *No difference in renal survival (ESRF) between the II, ID and DD genotypes was found in PKD1 ( $p=0.45$ ) and PKD2 ( $p=0.16$ ).*

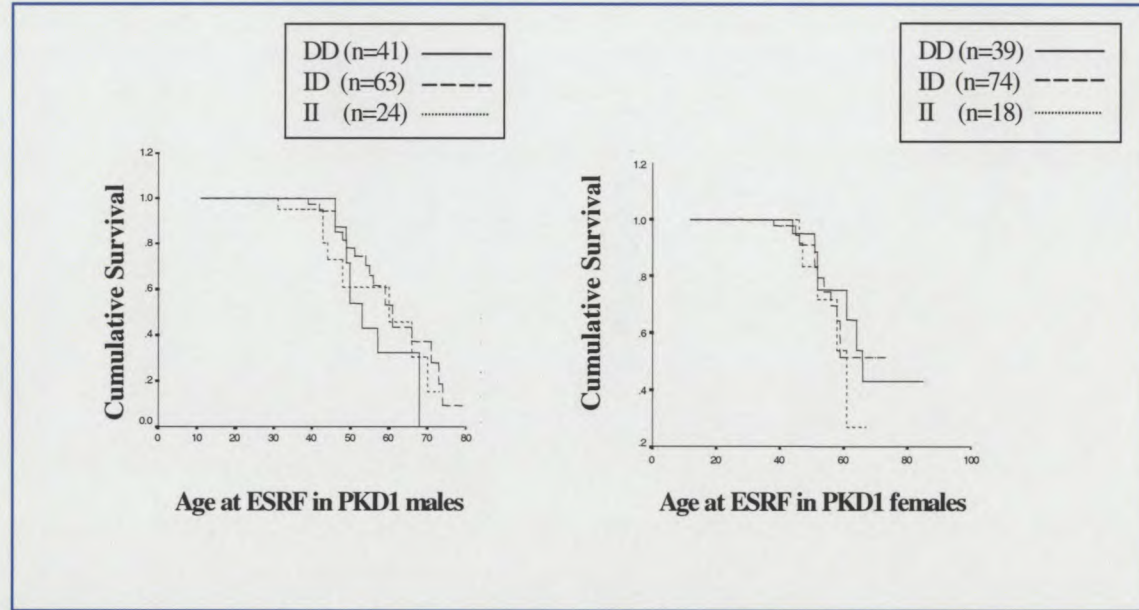


**Figure 5.7:** Analysis of the Effect of the ACE I/D Polymorphism on Survival to CRF in PKD1 Male and Female Subjects. *No difference in renal survival (CRF) between the ACE genotypes in males ( $p=NS$ ) and females ( $p=NS$ ) with PKD1 was found.*

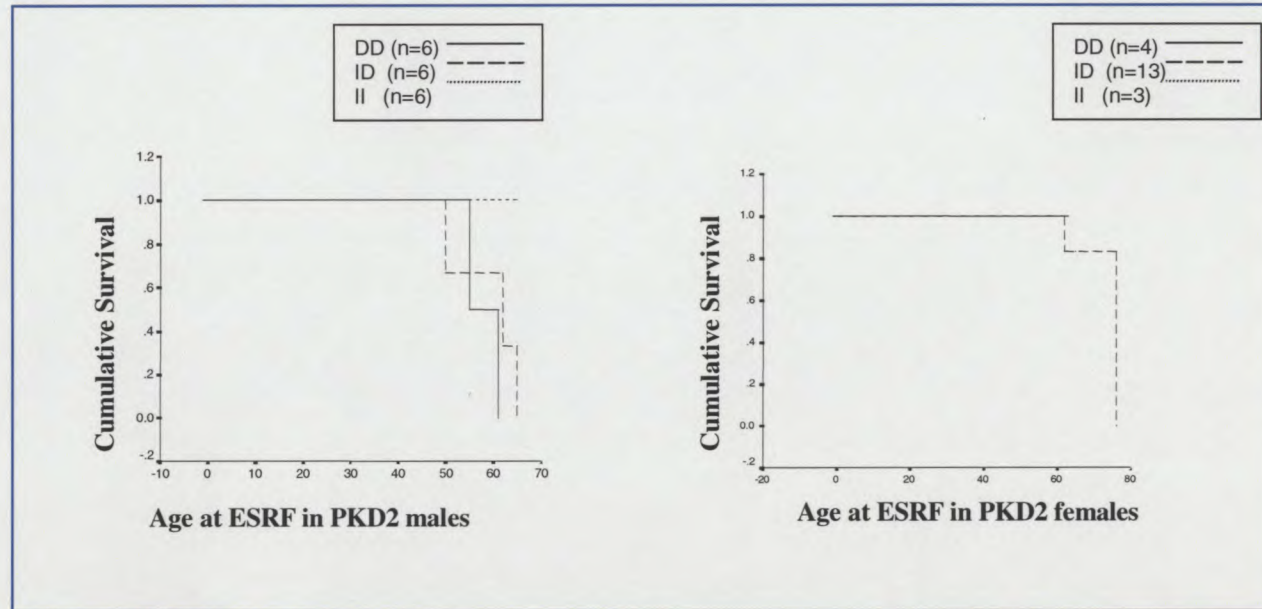




**Figure 5.8:** Analysis of the Effect of the ACE I/D Polymorphism on Survival to CRF in PKD2 Male and Female Individuals. *No difference in renal survival (CRF) between the ACE genotypes in males ( $p=NS$ ) and females ( $p=NS$ ) in PKD2 was found; note small sample size.*



**Figure 5.9:** Analysis of the Effect of the ACE I/D Polymorphism on Survival to ESRF in PKD1 Male and Female Individuals. *No difference in renal survival (ESRF) between ACE genotypes in males ( $p=NS$ ) and females ( $p=NS$ ) with PKD1 was found.*



**Figure 5.10:** Analysis of the Effect of the ACE I/D Polymorphism on Survival to ESRF in PKD2 Male and Female Individuals. *No difference in renal survival (ESRF) between ACE genotypes in males ( $p=NS$ ) and females ( $p=NS$ ) with PKD2 was found; note small sample size.*



5.3.4                    *Association between I/D Genotypes in the ACE Gene and Hypertension*

The distribution of I/D genotypes was compared between 97 normotensive and 192 hypertensive ADPKD subjects (Table 5.13). The analysis revealed no significant differences ( $p = 0.38$ ).

**Table 5.13: Relationship between the ACE I/D Polymorphism and Hypertension in ADPKD**

Patients				
	ACE genotype	n	Percentage	p value
Hypertension	I/I	35	18	0.38
	I/D	98	51	
	D/D	59	31	
	Total	192	100	
Normotension	I/I	12	12	
	I/D	56	58	
	D/D	29	30	
	Total	97	100	

5.3.5                    *I/D Genotypes in the ACE Gene and Left Ventricular Mass*

The relationship between the ACE I/D polymorphism and echocardiographically determined left ventricular mass (LVM) was examined in 63 Australian ADPKD individuals. This analysis identified no significant differences ( $p = 0.78$ ).

The comparison was repeated on 59 ADPKD subjects adjusted for the covariate hypertension (4 patients were excluded because of missing data on presence or absence of hypertension). In this case, a significant difference in LVM was found between *ACE* genotypes ( $p < 0.01$ ; Table 5.14).

**Table 5.14 The Relationship between the ACE I/D Polymorphism and Left Ventricular Mass in**

ADPKD Patients				
Variable	ACE genotype	Number	LVM (gm)	p value
LVM (gm)	I/I	11	124.7±8.2	0.78
	I/D	30	128.7±7.3	
	D/D	22	134.0±8.2	
	Total	63		
*LVM (gm)	I/I	9	126.9±12.0	<0.01*
	I/D	28	131.2±6.8	
	D/D	22	134.8±7.8	
	Total	59		

5.3.6 I/D Genotypes in the ACE Gene and Renal Cystogenesis

The effect of I/D genotypes on the development of kidney cysts was examined in 175 ADPKD subjects. The three ACE genotypes were compared in terms of kidney length and size of the largest cyst. No significant differences were detected. The *p* values were 0.34 and 0.77 for the length of the left and right kidney respectively and 0.87 and 0.99 for the size of the largest cyst in left and right kidney (Table 5.15).

Table 5.15: The Effect of the ACE I/D Polymorphism on Renal Structure in ADPKD Patients

Kidney/Cyst size (mm)	ACE Genotype	Number of subjects	Length (mm)	<i>p</i> value
Length L	DD	52	141±5.6	0.34
	ID	89	141±4.1	
	II	34	130±7.1	
	Total	175	139±3.0	
Length R	DD	52	138±5.4	0.77
	ID	89	135±4.7	
	II	34	132±7.8	
	Total	175	135±3.3	
LgDiamL	DD	52	28±2.6	0.87
	ID	89	29±2.1	
	II	34	271±3.6	
	Total	175	28±1.5	
LgDiamR	DD	52	28±2.8	0.99
	ID	89	28±2.3	
	II	34	27.65±3.12	
	Total	175	28.10±1.55	

*L* – left; *R* – right; *LgDiamL* – length of the largest cyst in the left kidney;  
*LgDiamR* – length of the largest cyst in the right kidney (mean±SE)

5.4 ANGIOTENSINOGEN (AGT) RESULTS

5.4.1 Frequency of the AGT Dinucleotide Repeat Alleles

In the overall group studied (*n* = 316), the frequencies of the AGT repeat alleles were similar to those seen in earlier studies of Caucasian populations (Kotelevtsev *et al.*, 1991) (Table 5.16). A difference between the three populations was observed, with a lower frequency of alleles 2, 4 and 9 in the Polish and of alleles 1 and 3 in the Australian population (*p* = 0.04) (Table 5.17). The genotype frequencies were not in Hardy-Weinberg equilibrium in the overall population (*p* = 0.01) or in the Bulgarian group (*p* = 0.01).

**Table 5.16: Frequency of the AGT Dinucleotide Repeat Alleles in this Study vs Other Studies**

This study		Kotelevtsev <i>et al.</i> 1991*	Brand <i>et al.</i> 1998
Allele	Frequency	Frequency	Frequency
1	0.00	0.01	0.00
2	0.07	0.02	0.05
3	0.03	0.03	0.03
4	0.00	0.03	0.04
5	0.12	0.09	0.10
6	0.09	0.17	0.12
7	0.34	0.40	0.37
8	0.09	0.07	0.06
9	0.17	0.13	0.16
10	0.04	0.04	0.01
11	0.04	0.03	0.03

\* Kotelevtsev *et al.* (1991) and Brand *et al.* (1998) (PCR product was 50 bp shorter than the product in the current study, alleles correspond)

**Table 5.17: Frequency of the AGT Dinucleotide Repeat Alleles between the Three Countries**

Included in this Study			
AGT Alleles	Australia (n = 116)	Country Bulgaria (n = 133)	Poland (n = 67)
1	0.00	0.02	0.01
2	0.06	0.05	0.01
3	0.02	0.16	0.26
4	0.05	0.09	0.01
5	0.11	0.32	0.28
6	0.06	0.09	0.13
7	0.33	0.11	0.15
8	0.14	0.03	0.05
9	0.13	0.06	0.01
10	0.05	0.06	0.09
11	0.06	0.01	0.00

#### 5.4.2 AGT Dinucleotide Repeat and Renal Function

##### 5.4.2.1 AGT genotype and overall risk of renal failure

The distribution of AGT repeat alleles among the 316 ADPKD subjects was examined by comparing subjects with normal renal function to those with CRF, and those with ESRF versus lack of ESRF (Table 5.18). No significant differences were observed in the distribution of the AGT repeat alleles between groups.

**Table 5.18: Comparison in the Frequency of AGT Repeat Alleles between ADPKD Patients with Normal Renal Function (NRF) vs those with Renal Impairment (CRF or ESRF)**

Allele	NRF (n=199)	CRF (n=117)	ESRF (n=67)	<i>p</i> value * (CRF vs NRF)	<i>p</i> value ** (ESRF vs no ESRF)
1	1 (0.5%)	0 (0%)	0 (0%)	0.63	0.78
2	23 (11.6%)	13 (11.1%)	7 (10.4%)	0.53	0.49
3	11 (5.5%)	9 (7.7%)	6 (9.0%)	0.29	0.23
4	16 (8.0%)	9 (7.7%)	9 (13.4%)	0.55	0.06
5	39 (19.6%)	26 (22.2%)	11 (16.4%)	0.34	0.22
6	35 (17.6%)	16 (13.7%)	8 (11.9%)	0.23	0.19
7	106 (53.3%)	59 (50.4%)	35 (52.2%)	0.35	0.55
8	31 (15.6%)	20 (17.1%)	14 (20.9%)	0.42	0.15
9	65 (32.7%)	32 (27.4%)	17 (25.4%)	0.19	0.18
10	18 (9.0%)	8 (6.8%)	7 (10.4%)	0.53	0.30
11	14 (7.0%)	7 (6.0%)	2 (3.0%)	0.32	0.13

#### 5.4.2.2 AGT dinucleotide repeat genotype and rate of deterioration of renal function

The primary aim of this analysis was to determine if there was an association between a specific allele/s and age at renal failure. Hence, the analysis only required that the allele be absent or present in the 289 individuals.

This analysis was performed using two approaches: a comparison between individuals carrying different polymorphic alleles as regards to age at renal failure and comparisons of the Kaplan-Meier cumulative survival curves for subjects carrying specific “high risk” alleles (defined as alleles that predispose an individual to disease) detected by the first analysis.

The relationship between age at development of renal failure and specific AGT microsatellite alleles was examined in a total of 289 individuals where age at CRF was known or could be extrapolated and in 68 individuals with ESRF. The results are presented in Tables 5.19 and 5.20.

A small but significant association was found between earlier age at renal failure and alleles 6 ( $p = 0.05$  for CRF) and 9 ( $p = 0.04$  for ESRF). Allele 8 was found to be more common among “slow” progressors to CRF ( $p < 0.01$ ).

**Table 5.19: The Relationship between the AGT Dinucleotide Repeat Alleles and Observed and Extrapolated Age at CRF in 289 ADPKD Subjects**

AGT alleles	Absent	Mean age	Present	Mean age	<i>p</i> value
1	0	0	0	0	0
2	257	39.8±0.8	32	36.9±2.4	1.00
3	271	39.4±0.8	18	41.9±2.8	0.44
4	265	39.5±0.8	24	40.0±2.2	0.87
5	232	39.1±0.8	57	41.6±1.8	0.19
6	245	40.2±0.8	44	35.9±1.9	<b>0.05*</b>
7	139	39.3±1.1	150	39.8±1.1	0.76
8	242	38.6±0.8	47	44.5±1.9	<b>&lt;0.01*</b>
9	202	40.2±0.9	87	38.1±1.4	0.24
10	268	39.9±0.8	21	34.6±2.8	0.07
11	271	39.7±0.8	18	36.9±3.2	0.39

**Table 5.20: The Relationship between the AGT Dinucleotide Repeat Alleles and Age at ESRF in 68 ADPKD Patients with ESRF**

AGT alleles	Absent	Mean age	Present	Mean age	<i>p</i> value
1	0	0	0	0	0
2	61	54.9±1.2	7	50.2±2.7	0.2
3	62	54.2±1.1	6	56.5±5.7	0.6
4	59	54.8±1.2	9	52.1±2.7	0.4
5	55	54.5±1.3	13	54.3±2.8	0.9
6	60	54.2±1.0	8	56.5±3.8	0.5
7	33	53.1±1.6	35	55.7±1.6	0.2
8	54	53.8±1.2	14	56.7±2.9	0.3
9	51	55.8±1.3	17	50.5±1.9	<b>0.04*</b>
10	63	54.4±1.2	5	54.8±3.6	0.9
11	65	54.5±1.2	3	54.0±5.0	0.9

#### 5.4.3 *AGT Dinucleotide Repeat and Hypertension*

In the overall group of ADPKD subjects, the distribution of AGT repeat alleles was compared between 103 normotensive and 197 hypertensive individuals (16 patients excluded because of missing data). Although allele 7 is the most common allele found in both normotensive and hypertensive subjects, significant differences in the distribution of alleles 4, 5, 6 and 8 were observed between the two groups. Alleles 4 ( $p = 0.04$ ), 5 ( $p = 0.05$ ) and 8 ( $p = 0.03$ ) were significantly more common among Hypertensive subjects, whereas allele 6 was prevalent among normotensive subjects ( $p = 0.01$ ) (Table 5.21).

**Table 5.21: The Frequency of the AGT Dinucleotide Repeat Alleles in Hypertensive and Normotensive ADPKD Patients**

AGT Allele	Hypertension (n = 197)	Normotension (n = 103)	p value
1	1 (1%)	0	0.66
2	20 (10%)	11 (11%)	0.52
3	9 (5%)	10 (10%)	0.07
4	20 (10%)	4 (4%)	<b>0.04*</b>
5	43 (22%)	14 (14%)	<b>0.05*</b>
6	23 (12%)	24 (23%)	<b>0.01*</b>
7	102 (52%)	50 (49%)	0.34
8	37 (19%)	10 (10%)	<b>0.03*</b>
9	55 (28%)	34 (33%)	0.22
10	15 (8%)	6 (6%)	0.38
11	14 (7%)	7 (7%)	0.56

*Values expressed as number and percentage (%)*

**5.5 RENIN RESULTS**

**5.5.1 Frequency of the Renin Tetranucleotide Repeat Alleles**

In the overall group studied (n = 316), the frequency of the tetranucleotide repeat alleles were in agreement with earlier studies of white Caucasian populations (Edwards *et al.*, 1992)(Table 5.22). No inter-population differences in frequency distributions were found (Table 5.23).

**Table 5.22: Frequency of the Renin Tetranucleotide Repeat Alleles in this Study and in Other Populations**

Allele	This study	Allele*	Edwards <i>et al.</i> 1992			
			Whites	Blacks	Mex-Amer	Asians
1	0.01	12	0.09	0.08	0.03	0.04
2	0.13	11	0.13	0.15	0.15	0.05
3	0.11	10	0.09	0.08	0.05	0.15
4	0.75	8	0.78	0.73	0.77	0.77

*\* Alleles 12, 11, 10 and 8 correspond to alleles 1, 2, 3 and 4 in this study (see Chapter Two, pg 61 for explanation)*

**Table 5.23: Frequency of the Renin Tetranucleotide Repeat Alleles between the Three Countries Included in this Study**

Renin alleles	Country		
	Australia	Bulgaria	Poland
1	0.01	0.00	0.00
2	0.13	0.14	0.15
3	0.11	0.12	0.08
4	0.75	0.74	0.77

### 5.5.2 Renin Tetranucleotide Repeat and Renal Function

The association between the renin polymorphism and renal function was tested by comparing the age at CRF and ESRF between individuals with different genotypes for the renin tetranucleotide repeat. The number of individuals and results are shown in Tables 5.24 and 5.25. No statistically significant association between the renin tetranucleotide repeat alleles and age at development of renal failure was demonstrated.

**Table 5.24: The Relationship between the Renin Tetranucleotide Repeat Alleles and Observed and Extrapolated Age at CRF in 289 ADPKD Patients**

Renin Allele	Absent	Age (yr)	Present	Age (yr)	p value
1	286	39.6±0.8	3	36.3±8.2	0.68
2	219	39.5±0.9	70	39.8±1.7	0.87
3	236	38.9±0.9	53	42.3±1.7	0.11
4	40	40.8±1.9	249	39.4±0.9	0.53

*Values are expressed as means ±SE*

**Table 5.25: The Relationship between the Renin Tetranucleotide Repeat Alleles and Age at ESRF in 68 ADPKD Patients with ESRF**

Renin Allele	Absent	Age (yr)	Present	Age (yr)	p value
1	68	54.4±1.1	0	0	1.0
2	51	55.5±1.3	17	51.4±2.4	0.11
3	52	54.9±1.4	16	52.7±2.0	0.42
4	11	51.5±2.0	57	55.0±1.3	0.25

*Values are expressed as means ±SE*

### 5.5.3 Renin Tetranucleotide Repeat and Hypertension

The relationship between alleles of the renin polymorphism and hypertension was examined in 179 hypertensive and 93 normotensive ADPKD subjects (44 patients excluded because of missing data) by comparing allele frequencies in the two groups. No significant differences were revealed (Table 5.26).

**Table 5.26: The Frequency of the Renin Tetranucleotide Repeat Alleles in Hypertensive and Normotensive ADPKD Patients**

Renin Allele	Hypertension (N = 179)	Normotension (N = 93)	p value
1	1 (0.6%)	2 (2.2%)	0.27
2	46 (25.7%)	23 (24.7%)	0.49
3	36 (20.1%)	14 (15.1%)	0.19
4	167 (93.3%)	87 (93.5%)	0.57

*Values expressed as number and percentage (%). The number indicates the number of individuals within the hypertensive (N=179) or normotensive (N=93) group who carry a specific allele, whereas the percentage value represents the proportion of individuals within each group (hypertensive/ or normotensive) who carry a specific allele.*

## 5.6 DISCUSSION

This part of the project examined the possible role of genetic variants in the renin angiotensin system (RAS) as modifiers of ADPKD phenotype severity. Such a role seems plausible, in view of the complexity of ADPKD pathogenesis, the physiological function of the RAS, and the substantial intra-familial variation in the clinical course of ADPKD, each of which could be explained by the effects of modifying genes. Previous studies of RAS genes in ADPKD have been inconclusive. This could be due to a number of factors, including sample size, study design, or true variation between the different populations investigated.

The design of this study aimed to minimise the pitfalls and confounding variables that may lead to inconsistent findings in association studies. It included 316 patients, representing the largest sample reported to date. The ADPKD subjects were resident in the three different countries: Australia, Bulgaria and Poland. Although the ethnic background of the Australian group was diverse, it comprised mostly patients of British descent. The study thus involved individuals representing ethnic groups from three parts of Europe that have been shown by population genetic research to differ in their present genetic composition, as a result of past population movements and demographic history (Rosser *et al.*, 2000). In the absence of polymorphisms of proven functional relevance, the analysis of association between the renin and angiotensin genes, and ADPKD severity, was based on the investigation of intragenic polymorphic microsatellites. In the case of the angiotensin converting enzyme (ACE) two approaches were used. The first approach was a replication of previous studies investigating the association between different measures of ADPKD severity and the



patients' genotypes for the Alu I/D polymorphisms. The second approach involved a direct investigation of the biological variable, namely ACE enzyme activity, in the Australian subset of patients.

Many of the previous studies of RAS variation in patients with kidney disease have focussed on ACE (Baboolal *et al.*, 1997; Uemasu *et al.*, 1997; Perez-Oller *et al.*, 1999; van Dijk *et al.*, 1999; 2000), while angiotensinogen and renin have received little attention (Baboolal *et al.*, 1997). This preference is probably due to two reasons: the therapeutic implications of such research and the hope that ACE inhibitors could be used to control the progression of renal disease (Maschio *et al.*, 1996; van Essen *et al.*, 1996), and the proposed high correlation between the Alu I/D genotype and ACE activity (Rigat *et al.*, 1990; Tiret *et al.*, 1992). The results of such studies have been controversial.

In this study, we have examined the association between the alleles of the Alu polymorphism in the *ACE* gene and different aspects of the ADPKD phenotype. These included hypertension, the development of left ventricular hypertrophy and renal cystogenesis. The rationale behind the study of hypertension and cystogenesis was the involvement of the RAS in the regulation of blood pressure (Jeunemaitre *et al.*, 1992b), and the growth-promoting effect of the end product, Ang II (Norman *et al.*, 1987). The effect on left ventricular mass (LVM) was studied in view of previous findings that have demonstrated the favourable effect of ACE inhibition on the reversibility of LVH (Cannella *et al.*, 1993; Cannella and Paoletti 1996), and the association between the D allele with left ventricular hypertrophy (LVH) (Schunkert *et al.*, 1994).

This study was not able to demonstrate a relationship between the I/D genotype and hypertension or renal cystogenesis. The effect on hypertension was examined in the entire group of ADPKD subjects, with negative findings that are consistent with the results of other investigations (Baboolal *et al.*, 1997; Uemasu *et al.*, 1997; van Dijk *et al.*, 1999, 2000). The effect of ACE on cystogenesis in ADPKD has not been examined by any of the previously published studies. Our negative findings, based on a sample of 175 affected subjects, suggest that such an effect is unlikely to exist. The study of LVM included 63 Australian patients only. The results from this study initially failed to show

a relationship between the ACE I/D polymorphism and LVM, however when adjusted for hypertension, a significant relationship was observed. Subjects homozygous for the D allele were found to have a significantly larger LVM, followed by heterozygote I/D subjects and homozygote I/I patients ( $p < 0.01$ ). The results of our study are consistent with a previous report, which suggested an association between the ACE I/D polymorphism and cardiovascular pathology (Schunkert *et al.*, 1994).

A decline in kidney function and the development of renal failure is an overall measure of severity, which can be regarded as the end product of all the different factors affecting the clinical course of the disease. Its relationship with the ACE D/D genotype was investigated in 307 ADPKD subjects from the three different populations. Inter-ethnic variation was found to exist both in terms of the frequency of I/D alleles and of the ratio of PKD1 to PKD2 patients. However, no individual population, or the overall group of patients displayed a significant association between I/D genotype and ADPKD phenotype severity. The findings were negative in all types of analysis using different measures of progression to renal failure: *i.e.* overall risk of renal failure, age at development of chronic or end-stage renal failure, or rate of progression from normal function to CRF and to ESRF. The findings were also negative when the analysis was performed after grouping the patients according to linkage status or sex.

In discussing the results of association studies, one has to keep in mind their simplistic design, where one DNA polymorphism is used to represent the overall effect of a whole pathway, which is likely to be under complex control. The approach is oversimplified even with regard to ACE itself. The Alu polymorphism in the ACE gene has no known functional significance but rather is believed to be in linkage disequilibrium with another, biologically relevant polymorphism directly affecting ACE activity levels (Tiret *et al.*, 1992). Strong evidence coming from recent studies suggests that the ACE-linked QTL is probably located in the 3' region of the gene, with the I or D allele of the Alu polymorphism occurring on diverse haplotype backgrounds (Zhu *et al.*, 2000). The diversity of intragenic ACE haplotypes, their complex additive effects on ACE activity, and the observed inter-population differences (Lee, 1994; Mizuiri *et al.*, 1995; Foy *et al.*, 1996; Lindpaintner *et al.*, 1996), could account for the discrepant estimates of the

proportion of variance in ACE activity explained by the Alu polymorphism, ranging from nil to 47% (Rigat *et al.*, 1990; Tiret *et al.*, 1992; Danilov *et al.*, 1996).

In the current study, a direct comparison between plasma ACE activity and Alu genotypes in the *ACE* gene was feasible in the Australian group of 92 ADPKD patients. The results support the reported association between the I/D polymorphism and plasma ACE activity (Rigat *et al.*, 1990; Tiret *et al.*, 1992; Costerousse *et al.*, 1993; Uemasu *et al.*, 1997). The D allele was associated with higher enzyme activity and a clear-cut dosage effect could be displayed. However, the proportion of variance in plasma ACE activity explained by the Alu polymorphism was estimated at only 14%. This modest contribution suggests that, at least in the specific population investigated, a much larger sample size would be needed to reveal an association between I/D genotypes and the complex ADPKD phenotype, if such an association exists.

A study of British and Australian ADPKD subjects (Baboolal *et al.*, 1997), reported a significant association between the Alu D allele and early onset of ESRF, with a 17-fold increase in relative risk of early ESRF in D/D homozygotes. Such a strong effect appears improbable for several reasons. The same study found an association in D/D homozygotes alone: it not only failed to find the expected dosage effect, it actually reported longer renal survival in I/D heterozygotes compared to I/I homozygotes. Similar results were also obtained in a study of Spanish ADPKD patients (Perez-Oller *et al.*, 1999). The ADPKD subjects studied by Baboolal *et al.* (1997) comprised patients of the same ethnic background as this study. Therefore one could assume that the Alu polymorphism in the *ACE* gene accounts for a similarly low proportion of the variance in ACE activity and detecting an association could be expected to require a large sample of patients. A total of 189 subjects were included in the Baboolal *et al.* (1997) study, and were subsequently subdivided according to *ACE* genotype into 64 patients with the DD genotype, 83 with ID and 42 with II. However, the relative risk for developing ESRF was calculated in subjects with the D/D genotype only, grouped by age. The relative risk for ESRF below the age of 40 years was assessed in 10 D/D patients, by age 50 in 9 D/D patients and by age 60 in 6 D/D patients. Given the small numbers, the conclusions of the study seem likely to be the product of statistical artefact rather than of underlying biological phenomena.

Reports in the literature on numerous haplotypes in the 3' region of the *ACE* gene and their variable combinations with the Alu polymorphism in intron 16 (Zhu *et al.*, 2000) also suggest that, unless information on the relationship between the Alu polymorphism I/D genotypes and ACE enzyme activity is available, association studies based on the I/D genotype are both inconclusive and non-comparable.

Our analysis of plasma ACE activity did not reveal any effect on the development of renal failure, in agreement with Uemasu *et al.* (1997) and van Dijk *et al.* (1999). Taken together, the conclusions of these studies, all involving direct measurement of ACE activity as the plausible biological variable, suggest that the ACE is not likely to play a role as a determinant of ADPKD phenotype severity.

This study also examined the possible association between deterioration of kidney function in ADPKD and the alleles of microsatellite polymorphisms in the renin and angiotensinogen genes. The results were entirely negative for renin. In the case of angiotensinogen, a significant association was found between alleles 6 and 9 of the angiotensinogen microsatellite and more rapid progression towards renal failure. The studies of Baboolal *et al.* (1997), Saggar-Malik *et al.* (2000) and Konoshita *et al.* (2001) failed to find an association between the *AGT* M235T polymorphism and ADPKD severity examined in 52, 33 and 76 patients respectively with ESRF. The effect of *AGT* on renal failure deserves further investigation in larger sample sizes.

In conclusion, this investigation of a large group of ADPKD subjects did not demonstrate an association between phenotype severity and ACE activity, the Alu polymorphism in the *ACE* gene or the intragenic renin microsatellite. The positive results on angiotensinogen justify replication, as well as a detailed sequencing analysis aimed at identifying functionally meaningful variants in the coding sequence of the angiotensinogen gene that are associated with the microsatellite marker allele 9.

**CHAPTER SIX: THE RELATIONSHIP BETWEEN  
ENVIRONMENTAL FACTORS AND ADPKD**

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# CHAPTER SIX: THE RELATIONSHIP BETWEEN ENVIRONMENTAL FACTORS AND ADPKD

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## 6.1 INTRODUCTION AND LITERATURE REVIEW

An association between smoking, alcohol consumption, diet and physical activity, and the progression of cardiovascular and renal disease has been demonstrated in several studies (D'Amico and Gentile 1993; Polat *et al.*, 1998; Perneger *et al.*, 1999; Hu *et al.*, 2000; Muntner *et al.*, 2000). In ADPKD, the influence of environmental risk factors has received little attention but several reasons make these factors potential modifiers of ADPKD clinical severity.

### 6.1.1 *Smoking and Renal Failure*

The effect of smoking as a modifiable risk factor and major contributor to cardiovascular disease (Holbrook *et al.*, 1984), cancer (La Vecchia *et al.*, 1991; Newcomb and Carbone 1992; Parkin *et al.*, 1994) and more recently, progression of diabetic nephropathy (Orth *et al.*, 1997) is well recognised. In the kidney, smoking has three major known effects: 1) on renal haemodynamics, 2) on water diuresis and electrolyte excretion, and 3) on the proximal tubule. The relationship between smoking and renal haemodynamics is demonstrated by a significantly higher prevalence of glomerular hyperfiltration in smokers versus non-smokers (41% versus 18%) with IDDM (Ekberg *et al.*, 1990). The Ekberg study also showed a relationship between glomerular filtration rate (GFR) and the amount of smoking (defined as the number of cigarettes smoked per week).

The increase of GFR induced by smoking may play a role in the genesis of hyperfiltration as a potential mediator of accelerated progression of chronic renal disease (Neuringer and Brenner, 1993; Hostetter, 1995). As early as 1945, Burn *et al.* (1945) reported that nicotine had an antidiuretic effect in both rats and humans. In rats of approximately 190 grams body weight the subcutaneous injection of a high dose of nicotine (0.25 mg) induced initial diuresis during the 15 minutes post injection. This was followed by an inhibition of the diuresis for about 45 minutes compared to control rats. Similar observations have also been made in humans (Burn, 1945). Antidiuresis is

probably due to increased vasopressin secretion during smoking (Husain *et al.*, 1975; Rowe *et al.*, 1980), and in this context it has been proposed as a potential factor in the progression of chronic renal failure (Bankir and Bouby, 1991; Bouby *et al.*, 1991). As to the mechanisms involved, it has been proposed that vasopressin increases GFR inappropriately, by modulating the activity of tubuloglomerular feedback. This is thought to result from changes in urea recycling (Bankir and Bouby, 1991).

Smoking is also associated with changes in proximal tubular function, including the increased excretion of N-acetyl-B-glucosaminidase (NAG) (Hultberg *et al.*, 1992) and the impairment of organic cation transport (Wong *et al.*, 1992).

The effects of smoking on systemic haemodynamics have also been reported (Groppelli *et al.*, 1992) and, in view of the paramount importance of blood pressure on the evolution of renal disease, the effect of smoking on blood pressure is also pertinent. Hansen *et al.* (1994) reported that the normal decrease in night-time systolic and diastolic blood pressure was less in healthy smokers ("non-dippers") than in non-smokers. However, in this study no similar effect was found in patients with IDDM who smoked (Hansen *et al.*, 1994). On the other hand, Barna *et al.* (1996) found that the night-time fall in blood pressure was less in smoking healthy volunteers as well as in smoking subjects with IDDM (Barna *et al.*, 1996).

The effect of smoking on the progression of renal disease has been studied extensively in diabetic patients (IDDM as well as NIDDM). The findings of these studies are summarised in Table 6.1. The results show that, in diabetic patients, the risk of developing microalbuminuria, proteinuria and retinopathy is substantially higher in smokers. The concept of "pack years", defined as one pack of 20 cigarettes per day for one year (Paetkau *et al.*, 1977), was used in all studies to provide a quantitative measure of exposure to tobacco over the duration of diabetes. Although there is substantial evidence for the adverse effects of smoking on diabetic complications, negative results have also been demonstrated (Gall *et al.*, 1997).

The role of smoking in other types of kidney disease is less well studied and with more contradictory results. Two large case control studies did not observe any relationship

between smoking and chronic renal failure from various nephropathies (Steenland *et al.*, 1990; Nuyts *et al.*, 1995). Another cohort of 11,912 hypertensive men provided no evidence of a dose-effect relationship between smoking and end-stage renal disease (Perry *et al.*, 1995). On the other hand, smoking has been associated with an accelerated decline in renal function among patients with lupus nephritis (Ward and Studenski, 1992). More recently, a large cohort study investigated the association between smoking and glomerular nephropathy. The sample included 295 patients with biopsy-proven glomerular nephropathy, 80 with membranous nephropathy, 116 IgA nephropathy, 99 nephrotic syndrome and 242 matched hospital controls, with disease unrelated to smoking. The study revealed that smoking is related to the severity of glomerular nephropathy, particularly in the at-risk groups of men older than 40 and/or hypertensive patients (Stengel *et al.*, 2000).

Very few data exist on the effect of smoking on progression of renal disease in ADPKD. Chapman *et al.* (1994) found that ADPKD patients with established proteinuria (as an indicator of adverse renal prognosis) had not only higher mean systemic blood pressure and more severe cystic transformation of the kidney, but also a greater pack-year smoking history as compared to their non-proteinuric counterparts.

More recently, Orth *et al.* (1998) investigated smoking as a risk factor for ESRF in men with primary renal disease. The study included 44 pairs of males with immunoglobulin A-glomerulonephritis (IgA-GN) and 28 pairs with ADPKD. Subjects were divided into cases and controls, where cases included individuals who had progressed to ESRF and controls were individuals who had not reached ESRF (failure to progress to serum creatinine >3 mg/dl). Information about the type and amount of tobacco consumption (cigarettes, cigars, pipe) was collected and smoking was calculated in pack years (PY). Results from the study revealed a significant dose-dependent increase of the risk of progression to ESRF. The baseline risk (in this study it is defined as the value or quantity of PY's with which the smoking groups are compared when measured or assessed) was defined as <5 PY: (1) 5 to 15 PY, odds ratio 3.5,  $p = 0.017$ ; (2) >15 PY odds ratio 5.8,  $p = 0.001$ . Moreover, the risk for ESRF was substantially higher in smokers with no history of ACE inhibition (odds ratio 10.1,  $p = 0.002$ ) versus smokers with a history of ACE inhibition treatment (odds ratio 1.4,  $p = 0.65$ ). The role of ACE



**Table 6.1: Studies that have Investigated the Effect of Smoking on Diabetic Complications**

<b>Group and Year</b>	<b>Sample</b>	<b>Methodology and Laboratory analyses</b>	<b>Statistical Methodology</b>	<b>Results/Evidence for association</b>
Paetkau <i>et al.</i> 1977	* 97 IDDM patients with nonproliferative and 84 with proliferative retinopathy	* Smoking defined as smoking $\geq 1$ cigarette per day at time of interview or within 7 years of study * Non-smoker defined as a person who had not smoked at all in 8 years prior to study * Pipe and cigar smokers were excluded from the study * Smoking calculated as 'pack years'	* Data analysed by $\chi^2$	* Patients with proliferative retinopathy increased with tobacco consumption * Severity of DR associated with smoking and not with duration, age
Norden & Nyberg 1984	* 108 patients with IDDM and DN * 140 patients with IDDM and without DN	* Cases and controls matched for sex, age at diagnosis, current age, and duration of diabetes * A smoking index was calculated based on the number smoked per day * Onset of diabetes was <25 years	* Wilcoxon's matched pair rank test and t-test used to compare differences between matched pairs * $\chi^2$ for differences between groups of patients	* Significantly increased smoking index found in patients with DN
Telmer <i>et al.</i> 1984	* 668 patients with IDDM diagnosed <30 years	* Clinical data collected on: sex, age, IDDM duration, weight, height, insulin requirement, presence of DR and DN * Patients divided into: (1) average daily tobacco consumption; heavy smokers (>10 cigarettes p/day >1 yr) vs non-heavy smokers	* t-test, $\chi^2$ test, Fisher's exact test and Mann-Whitney test	* DN significantly higher in heavy smokers vs non-heavy smokers, 19.2% vs 12.1% ( $p < 0.02$ ). * High frequency of DN with increasing cigarette consumption ( $p < 0.01$ ) * No difference in prevalence of DR between smokers vs non-smokers
Muhlhauser <i>et al.</i> 1986	* 192 cigarette smoking patients with IDDM * 192 non- smoking patients with IDDM	* Clinical data collected:: 24 hr urine for determination of proteinuria and Ccr, serum triglyceride, cholesterol, HDL-cholesterol and Cr levels, BP, BP treatment and DR	* $\chi^2$ used for statistical analysis	* Macroproteinuria found in 19.3% of smoking vs 8.3% of non-smoking group ( $p < 0.001$ ) * DR present in 12.5% of smoking vs 6.8% of non-smoking group ( $p = 0.025$ )
Stegmayr & Lithner, 1987	* 22 IDDM DN patients * 22 IDDM patients without DN	* Groups matched for age, sex and diabetes duration * Smoking calculated as 'pack years' * DN cases reviewed for onset of albuminuria; * Present kidney function measured in non-DN patients		* Significantly more tobacco users in the uraemic group vs controls * Lifetime tobacco consumption was significantly less in controls vs uraemic group

Stegmayr, 1990	* 34 IDDM patients with DN and DR	* Tobacco calculated as the daily mean and the total consumption (expressed in pack years)	* $\chi^2$ for statistical analysis	* 90% patients used tobacco >5 yrs * tobacco consumption higher in DN patients ( $p < 0.001$ ) vs without DN * Increase in early death ( $p < 0.01$ ) in current uraemic smokers vs never or ex-smokers
Chase <i>et al.</i> 1991	* 359 young IDDM patients	* IDDM patients approximately 14 yrs and IDDM >5 yrs duration * Smoking habits classified into nonsmoker, ex-smoker, current smoker, tobacco chewers * Smokers and non-smokers divided into 3 groups based on AER	* $\chi^2$ , t-test, ANOVA. LRA for influence of independent factors	* smoking associated with progression of albuminuria and DR
Sawicki <i>et al.</i> 1994	* 96 IDDM patients with DN and HBP	* Clinical data collected on: weight, total cholesterol, HDL cholesterol, total serum protein, serum K, Na, BP, BP treatment, HbA <sub>1c</sub> * Smoking calculated as 'pack years' * Smoking habits classified into: nonsmoker; ex-smoker; and current smoker	* Fisher's exact test for proportions; unpaired & paired Students t-test & Wilcoxon's rank-sum test for comparison of means * LRA to calculate influence of prognostic factors & DN	* Progression of DN over 1 year less common in non-smokers 11%, vs smokers 53% ( $p < 0.01$ ), and ex-smokers 33%
Bruno <i>et al.</i> 1996	* 1574 NIDDM patients	* Clinical collected on: smoking habits, medical history, family history of diabetes, BP, BP treatment, BMI, UAE, plasma glucose, triglycerides, total cholesterol, uric acid, Cr, HDL cholesterol, apo A1 and apo B and HbA <sub>1c</sub> * Smoking habits classified into: nonsmoker; ex-smoker; and current smoker	* t-test and $\chi^2$ test used for comparisons, ORs, LRA performed to control confounding and to assess the effect modification	* variables independently related to micro- & macroalbuminuria: age, HbA <sub>1c</sub> , smoking, plasma uric acid, and DBP

DN – diabetic nephropathy; DR – diabetic retinopathy; IDDM – insulin dependent diabetes mellitus; NIDDM – non-insulin dependent diabetes mellitus; BP – blood pressure; Cr – creatinine; Ccr – creatinine clearance; UAE – urinary albumin excretion; BMI – body mass index; HDL – high density lipoprotein; LRA – logistic regression analysis; HbA<sub>1c</sub> – hemoglobin A<sub>1c</sub>; ANOVA – analysis of variance

inhibitors in delaying renal disease progression is recognised in diabetes and ADPKD (Orth *et al.*, 1998). The power of the study was, however, not sufficient to go beyond the statement that the effect of smoking is dose-dependent. In addition, the study could not permit a differentiation of whether the onset of overt renal disease occurred at an earlier age or the rate of progression was accelerated in smokers.

In summary, the deleterious effect of smoking in renal disease progression in diabetes is well recognised. However, there is little information about the effects of smoking and renal survival in autosomal dominant polycystic kidney disease.

### **6.1.2      *The carcinogenic and mutagenic effects of smoking***

Apart from its effect on renal hemodynamics and the cardiovascular system, smoking is also recognised as a carcinogen (Burns, 1991; Newcomb and Carbone, 1992). Organs in direct contact with smoke, such as the oral cavity, esophagus, lung and bronchus are at the greatest risk of malignancy among smokers. Organs and tissues more distant from smoke (such as the cervix, pancreas, bladder, kidney, stomach and haematopoietic tissues) are also at some increased risk. The risk of cancer at all sites increases with increasing exposure to cigarette smoke, which contains potent carcinogens that influence carcinogenesis at early and late stages. The carcinogens can interact with other exposures, such as alcohol, to synergistically increase the risk of cancer (Newcomb and Carbone, 1992).

Carcinogens are known to cause direct damage to DNA and hence are mutagenic. Exogenous and endogenous carcinogens can also act by altering gene expression, cell proliferation and differentiation. The mechanisms include aberrant DNA methylation, oxidative damage, activation of receptors and transcription factors, cyclins and other cell cycle proteins (Perera and Weinstein, 2000).

The effect of smoking on mutations in the *p53* tumor suppressor gene has been studied since it is the most frequently mutated gene in human cancer (Greenblatt *et al.*, 1994). Studies have shown that the *p53* mutational spectrum of smoking-related neoplasms is distinctly different from that present in non-smoking-related cancers. More recently, benzo( $\alpha$ )pyrene diol epoxide, the active form of benzopyrene and a potent carcinogen

in cigarette smoke has been shown to bind preferentially to select regions of the *p53* gene (Puisieux *et al.*, 1991; Denissenko *et al.*, 1996). Formation of these benzopyrene adducts is likely to reflect the high frequency of certain *p53* mutations in smoking-associated tumors and correlates with several known mutational hotspots in the *p53* gene (Denissenko *et al.*, 1996).

Studies in the evolution of bowel cancer have shown that cancers develop as a result of serial genetic mutations accumulating over a lengthy time span (Nowell, 1976; Fearon and Vogelstein, 1990). Commonly occurring somatic mutations have now been found in the evolution of lung cancer. The carcinogens in smoking accelerate somatic mutations, causing a field effect in the respiratory mucosa whereby areas of loss of heterozygosity may be demonstrated throughout the mucosa of the lung, even when no preneoplastic histological abnormality exists. However, the mutations must occur at specific genetic loci, which are involved in the regulation of cellular proliferation or repair (Roland and Rudd, 1998).

Studies of chromosomal changes in cells provide an index of the mutagenic damage caused by exogenous agents such as smoking. A study to examine the effects of smoking on immature cells in the bone marrow and peripheral blood was performed in 18 smokers with an average cigarette use of 6 pack-years. The results showed a higher frequency of sister chromatid exchanges in peripheral blood lymphocytes than in bone marrow cells. The peripheral blood lymphocytes demonstrated significantly higher frequency of fragile sites, an increased number of metaphases with extensive breakage and elevated expression of fragile sites at cancer breakpoints 3p14.2, 11q13.3, 22q12.2 and 11p13-p14.2 and at the oncogene sites *bcl 1*, *erb B*, *erb A*, and *sis* (Kao-Shan *et al.*, 1987).

In ADPKD, the carcinogenic and mutagenic effects of smoking may play a role in cystogenesis by providing the second hit in the normal PKD cell, hence contributing to the development of cystogenesis.

### 6.1.3 *Alcohol and Renal Failure*

Another potential and modifiable risk factor that may influence renal disease progression in ADPKD is alcohol consumption. Epidemiological studies suggest that light to moderate alcohol consumption reduces the risks of cardiovascular complications (Ajani *et al.*, 2000; Imhof *et al.*, 2001), while excessive alcohol intake is associated with a higher risk of hypertension, coronary occlusion, arrhythmias, hepatic cirrhosis, and upper gastrointestinal cancers (Da Luz and Coimbra 2001). Alcohol ingestion may thus indirectly affect the kidneys by causing an increase in blood pressure, while stimulating the renin-angiotensin system and subsequently affecting renal haemodynamics. Alcohol ingestion also increases visceral obesity, insulin resistance and hyperlipidaemia, which may promote sclerosis in renal mesangial cells (Puddey *et al.*, 1985; Marmot *et al.*, 1994).

Several pathogenic pathways may relate alcohol consumption to chronic renal failure by initiating and/or promoting atherogenic risk factors including hypertension (Puddey *et al.*, 1985; Marmot *et al.*, 1994), hyperuricemia (Nishimura *et al.*, 1994) and diabetes (Holbrook *et al.*, 1990). Regular alcohol consumption raises blood pressure and is estimated to be responsible for approximately 10% of all cases of hypertension (Victor and Hansen, 1995), which *per se* is a risk factor for renal damage (Heidland *et al.*, 1985). A statistical association between alcohol and renal disease has been found in immunoglobulin-A nephropathy (IgAN) (Smith *et al.*, 1993) and in patients with renal papillary necrosis, a marker for other excessive behaviour including smoking and analgesics (Pablo *et al.*, 1986).

The relationship between alcohol consumption and risk of developing ESRF was examined in a large case-control study (Perneger *et al.*, 1999). The sample comprised 716 patients from the USA who had commenced treatment for ESRF, and 361 controls selected by random digit dialling. The results from the study showed that >2 glasses per day of any type of alcoholic beverage increased the risk of developing renal failure fourfold. A lower intake of alcohol did not appear to be harmful (Perneger *et al.*, 1999). The study also showed that adjustment for hypertension and diabetes strengthened, rather than weakened the odds ratio for ESRD associated with current alcohol consumption, a finding which may reflect a secondary reduction of alcohol intake by

many patients who had received a diagnosis of hypertension or diabetes. Associations between alcohol consumption and the odds of ESRD were also estimated for subtypes of ESRD. The relationship was strongest for ESRD of unknown cause and was absent for ESRD attributed to diabetes (Perneger *et al.*, 1999). Furthermore, alcohol may possibly exercise an additive toxic effect on the kidney, in the presence of other recognised nephrotoxic exposures such as lead (Pablo *et al.*, 1986; Steenland *et al.*, 1990). In contrast, a low intake of alcohol (1-2 glasses per day; 10-20 g per day) is known to protect against the risk of cardiovascular disease compared to non-drinkers or heavy drinkers (Perneger *et al.*, 1999).

To date, no study has investigated the relationship between alcohol consumption and the risk of developing renal failure and rate of progression in ADPKD. Variation in ethanol content of beverages and national patterns make this a more difficult parameter to study than smoking.

#### **6.1.4            *Physical Activity***

Very few studies have examined the relationship between physical activity and renal disease. Kriska *et al.* (1991) assessed the relationship between leisure-time physical activity and diabetic complications (neuropathy, retinopathy and nephropathy) in 268 individuals with IDDM. Past week physical activity was found to be inversely related to complication status. Males reporting higher levels of historical physical activity had a significantly lower prevalence of nephropathy and neuropathy but not retinopathy. The lack of similar findings in women may be attributed to their generally lower levels of reported physical activity (Kriska *et al.*, 1991).

Patients on dialysis have extremely limited exercise capacity, and poor physical functioning. The reason for the debility of patients on dialysis is far from clear, however, anaemia and uraemia from chronic renal disease are contributing factors (Johansen, 1999). More recently, Johansen *et al.* (2000) assessed whether haemodialysis patients are less active than healthy sedentary controls and examined predictors of physical activity levels in 34 haemodialysis patients and 80 healthy sedentary individuals. Physical activity was measured for seven days with a three dimensional accelerometer, and with an activity questionnaire. The results of the study showed a

difference in physical activity between dialysis patients and controls. Patients on dialysis were found to be approximately 35% less active than sedentary healthy individuals. Among the dialysis subjects, some measures of nutritional status correlated with physical activity level, including low serum albumin concentration ( $p = 0.003$ ) and low serum creatinine concentration ( $p = 0.03$ ). The association between activity levels and these markers of nutritional status is especially interesting since low albumin and low creatinine have been associated with increased mortality in the dialysis population (Johansen *et al.*, 2000), and low physical activity is associated with increased mortality in the general population (Johansen *et al.*, 2000). In ADPKD, no study to date has compared the relationship between physical activity and renal failure.

#### **6.1.5            *Dietary Lipids***

Dietary lipids and hyperlipidaemia are also recognised as risk factors for cardiovascular disease (Neaton and Wentworth 1992) as well as renal dysfunction (Appel, 1991). The association between hyperlipidaemia and decline in renal function has been demonstrated by a rise in serum creatinine and intrarenal arteriosclerosis (Teuscher *et al.*, 2000).

The association between progression of renal disease and hyperlipidaemia has been recognised since the 1860's by Virchow as cited by Wheeler (1995). At the present time, whilst experimental studies have provided a clear link between hyperlipidaemia and kidney damage in animals, there is only limited evidence to suggest that lipids contribute to the progression of renal disease in humans (Wheeler, 1995).

Research using a rat model without glomerular hyperfiltration demonstrated that hyperlipidaemia increased the development of proteinuria and glomerulosclerosis (Joles *et al.*, 1995). Moreover, experimental data support the hypothesis that glomerulosclerosis and atherosclerosis share common mechanisms (Moore, 1985; Ross, 1986; Avram, 1989; D'Amico and Gentile 1993). Similarities in anatomical origin and functional properties of glomerular mesangial cells to those of vascular smooth muscle cells provide clues to analogous cellular responses common to both atherosclerosis and glomerulosclerosis (Diamond and Karnovsky, 1988; Avram, 1989; D'Amico and Gentile, 1993). Many studies have also shown that both glomerular lipid deposits and

the appearance of foam cells accompany renal injury in models of hyperlipidaemia. In several animal models, high cholesterol diets were associated with renal injury such as glomerulosclerosis and tubulointerstitial damage (Kasiske *et al.*, 1990), and the initiation of renal dysfunction (Diamond and Karnovsky, 1988). Antilipaemic agents reduced the amount of renal injury in the obese Zucker rat model of endogenous hyperlipidaemia (Kasiske *et al.*, 1988, 1990).

Several prospective studies in humans have shown an association between dyslipidaemia and renal disease progression among patients with proven renal disease (Mulec *et al.*, 1993; Krolewski *et al.*, 1994). In the Modification of Diet in Renal Disease (MDRD) study, low HDL cholesterol independently predicted a faster decline in GFR among 840 participants, but triglycerides were not examined (Hunsicker *et al.*, 1997). In smaller studies, baseline concentrations of total cholesterol, LDL cholesterol, and apo (B) lipoprotein were significantly associated with the decline in renal function (Samuelsson *et al.*, 1997). A detailed lipoprotein analysis in 44 patients (Samuelsson *et al.*, 1998) showed a strong correlation of the rate of GFR decline with the plasma concentration of triglyceride-rich apoB containing lipoproteins, but no significant association with cholesterol-rich apoB-containing lipoproteins.

In ADPKD, the relationship between lipid abnormalities and renal disease progression has been examined in experimental models, and in humans. The effect of lipid abnormalities on hypertension and cardiovascular changes in ADPKD has also been investigated. Polat *et al.* (1998) conducted a study on 10 healthy subjects (group 1); 11 primary hypertensives (group 2); 12 normotensive ADPKD patients (group 3); and 12 hypertensive ADPKD patients (group 4). Results from the study showed no difference in plasma renin activity (PRA), plasma aldosterone (PA) and angiotensin II levels between groups. Left ventricular hypertrophy, aortic thickening, aortic enlargement, left ventricular dilatation and the incidence of mitral valve prolapse were all prominent in group 4, followed by group 2 and least in group 3. All three lipid classes (total cholesterol, LDL, total lipids), were higher in patients in group 4, particularly the HDL and the triglyceride levels (Polat *et al.*, 1998).



In contrast, another study by Massy *et al.* (1999) found no or minimal association between dyslipidaemia and the progression of renal disease in chronic renal failure patients. The study included patients with chronic glomerulonephritis, interstitial nephritis, hypertensive nephrosclerosis, ADPKD and diabetic nephropathy with and without ESRF. The decline in renal function was based on the rate of decline in creatinine clearance (Ccr) over time. Their study was unable to demonstrate a significant role for dyslipidaemia in the rate of progression of CRF to dialysis, and only a weak association was observed between triglycerides and CRF progression (Massy *et al.*, 1999).

The relation between lipids and cystogenesis has also been examined in Han:SPRD-cy rats with cystic disease (Jayapalan *et al.*, 2000). In that study, both control and cystic rats at 4 weeks of age were fed either a low or a high fat diet for 6 weeks. Rats with cystic disease on a high fat diet had 17% larger kidneys, 19% more renal fluid, and cyst scores were 30% higher, indicating increased cystogenesis.

Thus some reports from both animal models and humans suggest that dyslipidaemia may influence renal disease progression, by promoting nephroangiosclerosis and by directly affecting renal hemodynamics and cyst formation.

#### **6.1.6      *Summary of the literature review and aims of the study***

Over the last decade, knowledge of the genetics and mechanisms of cystogenesis in ADPKD has grown rapidly. It is expected that environmental factors also play a role in the progression of ADPKD. Several studies have provided evidence that smoking, alcohol and diet represent major modifiable risk factors in renal disease progression, especially in the diabetic population. Little is known about their role in ADPKD. Therefore this part of the study aimed to:

- 1) To examine the effect of smoking on ADPKD phenotype severity:
  - a) on age and rate of progression to renal failure
  - b) renal cystogenesis
  - c) hypertension
- 2) To examine the effect of alcohol consumption on phenotype severity:
  - a) on age and rate of progression to renal failure

- b) renal cystogenesis
  - c) hypertension
- 3) To examine the relationship between current physical activity and age and rate of progression to renal failure
- 4) To examine the relationship between dietary and plasma lipids on ADPKD phenotype severity:
  - a) on age and rate of progression to renal failure
  - b) renal cystogenesis
  - c) hypertension
- 5) Given the interactions between the time-dependent effects of age, renal failure and blood pressure, and the additive effects of smoking, hypertension and hyperlipidaemia, multivariate analysis and Kaplan-Meier analysis were performed.

## 6.2 SUBJECTS AND METHODS

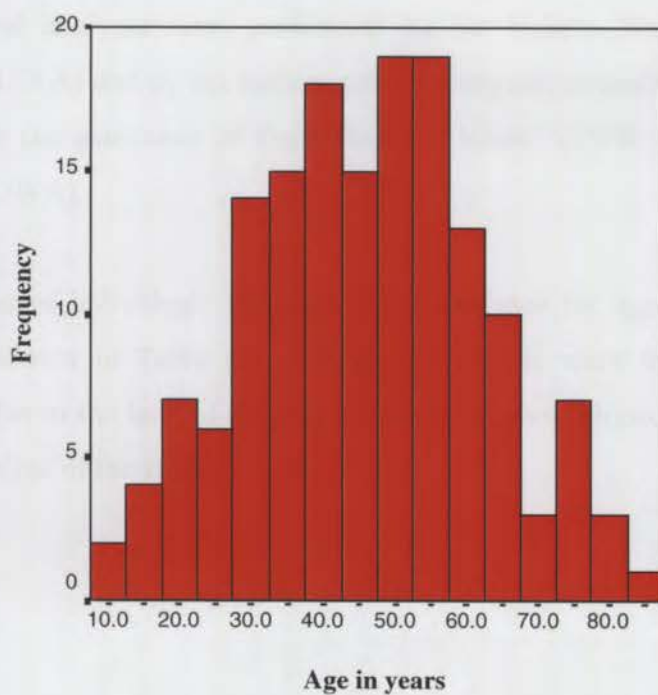
### 6.2.1 Subjects

This part of the study included 156 patients from Australia and Poland. The mean age of the subjects was 46.1 years (Table 6.2). A histogram showing the age distribution is shown in Figure 6.1. Details on the diagnostic and clinical assessment are provided in Chapter Two.

**Table 6.2: Characteristics of 156 ADPKD Patients included in the Environmental Analyses**

	Country of residence		Overall
	Australia	Poland	
Total number of Affected Individuals	89	67	156
Male/female	40:49	38:29	78:78
Mean current age	50.0±1.6	40.9±1.9	45.5±1.2
Hypertension	68(81%)	43(64.2%)	111(73.5%)
CRF	37(41.6%)	30(44.8%)	67(42.9%)
ESRF	24(27.3%)	9(13.4%)	33(21.3%)

*CRF – chronic renal failure; ESRF – end-stage renal failure*



**Figure 6.1:** Age Distribution of 156 ADPKD Australian and Polish Subjects

### **6.2.2        *Methods***

The analysis for serum cholesterol was performed on 67 Australian and 68 Polish subjects, and 45 Australian and 67 Polish subjects were tested for serum triglycerides. Details of the biochemical analyses and data on smoking, alcohol consumption, physical activity and diet collected from the questionnaires are described in Chapter Two, Sections 2.3.2 and 2.3.7.

### **6.2.3        *Statistical Analysis***

Statistical procedures are discussed in Chapter Two, Section 2.7. Frequency, ANOVA and survival analyses were performed by Dr Valerie Burke (University Dept. of Medicine, UWA) and by the author, and Cox regression analysis was performed by the author with the assistance of Prof. Nick De Klerk (TVW Institute for Child Health Research, UWA).

The number of individuals included in the analyses for age and progression to renal failure is shown in Table 6.3. Not all individuals were included in each analysis, primarily due to the lack of data on smoking, alcohol, physical activity and lipidaemia data at the time of the study.

**Table 6.3: The number of individuals included in the environmental analyses for age at and cumulative renal survival**

Variable	Smoking history			Alcohol	Physical activity	Lipidaemia	
	Status	Duration	Number of cigarettes			Total Cholesterol	Total triglycerides
Age at renal failure							
CRF observed	50	51	49	51	47	38	32
CRF observed & extrapolated	125	123	111	122	125	90	73
ESRF	32	32	27	32	32	25	21
Cumulative survival							
To CRF observed & extrapolated	125	123	111	122	-	90	73
To ESRF	151	151	132	147	-	109	89

**6.3 RESULTS: RELATIONSHIP BETWEEN SMOKING AND RENAL FAILURE**

**6.3.1 Smoking and overall risk of renal failure**

The relationship between smoking and development of renal failure (CRF and ESRF) was examined in 151 Australian and Polish patients based on three variables: (1) smoking status, (2) smoking duration and (3) daily number of cigarettes. The risk of developing renal failure was assessed by comparing the frequency of renal failure between groups based on the above three variables and by Cox regression analysis (section 6.8).

A lower frequency of renal failure (CRF and ESRF) was observed in non-smokers than smokers, although the difference was not statistically significant (Table 6.4). This pattern was also found between groups based on number of cigarettes per day. A statistically significant difference in the frequency of renal failure was found between groups based on smoking duration. The highest frequency of renal failure was found in smokers >20 years for both CRF ( $p = 0.01$ ) and ESRF ( $p = 0.04$ ; Table 6.4), followed by smokers  $\leq 20$  years and lowest in non-smokers.

**Table 6.4: Overall Risk of Renal Failure Based on Smoking History, Duration and Number of Cigarettes Smoked per Day in ADPKD Patients**

	Smoking Status			<i>P</i> value
	Non-smoker (n=64)		Smoker (n=92)	
CRF	22 (34%)		41 (45%)	0.13
ESRF	11 (17%)		23 (25%)	0.17
Smoking Duration				
	Non-smoker (n=64)	≤20 years (n=62)	>20 years (n=30)	<i>p</i> value
CRF	22 (34%)	22 (36%)	19 (63%)	<b>0.01</b>
ESRF	11 (17%)	12 (20%)	11 (38%)	<b>0.04</b>
Number of Cigarettes Per Day				
	Non-smoker (n=64)	≤20 (n=57)	>20 (n=16)	<i>p</i> value
CRF	22 (34%)	27 (47%)	8 (50%)	0.13
ESRF	11 (17%)	14 (25%)	4 (25%)	0.33

### 6.3.2 *The relationship between smoking and deterioration of renal function*

The effect of smoking on progression to renal failure was examined on the following three variables: (1) smoking status, (2) smoking duration and (3) daily number of cigarettes with regard to mean age at renal failure and in terms of Kaplan-Meier cumulative survival curves.

Age at CRF was analysed by comparing the observed, as well as the extrapolated age (based on the individual regression lines of time versus 1/creatinine values described in Chapter Two, Section 2.7).

#### *Observed age at renal failure*

The initial analysis compared the observed mean age at renal failure in the different groups based on smoking history, duration of smoking and number of cigarettes. The results are shown in Table 6.5. No significant differences in age at CRF or ESRF were found in any of the comparisons. A later age at ESRF was observed in patients smoking >20 years, but this were not statistically significant and may be due to the small sample size. CRF and ESRF were found to develop approximately 7 years earlier in subjects smoking >20 cigarettes per day, as compared to non-smokers and subjects smoking ≤20 cigarettes per day. Again, the results did not reach statistical significance.

#### *Extrapolated age at renal failure*

Differences in the rate of progression to renal failure were further examined by comparing age at development of CRF using both extrapolated and observed age at CRF in the same groups based on smoking history.

No significant difference in age at CRF was found (Table 6.6), although, CRF in patients smoking >20 years occurred approximately 5 years later than in non-smokers and in those smoking ≤20 years. Again, the results were statistically non-significant.

**Table 6.5: Comparison of Observed Mean Age at Renal Failure between Smoking Groups Based on Status, Duration and Number Smoked per day**

	Smoking Status			<i>p</i> value
	Non-smoker		Smoker	
CRF (observed age)	42.1±4.1 (n=18)		40.9±3.1 (n=32)	0.84
ESRF	53.3±2.9 (n=11)		52.7±2.1 (n=21)	0.81

	Smoking Duration			<i>p</i> value
	Non-smoker	≤20 years	>20 years	
CRF (observed age)	42.1±4.1 (n=18)	37.8±3.9 (n=19)	44.6±4.5 (n=14)	0.51
ESRF	53.3±2.9 (n=11)	48.0±2.7 (n=11)	57.3±2.8 (n=10)	0.08

	Number of Cigarettes Per Day			<i>p</i> value
	Non-smoker	≤20	>20	
CRF (observed age)	42.1±4.1 (n=18)	41.8±3.4 (n=25)	35.2±7.1 (n=6)	0.67
ESRF	53.3±2.9 (n=11)	52.8±2.7 (n=13)	44.7±5.6 (n=3)	0.38

**Table 6.6: Comparison of Mean Age (observed and extrapolated) at Renal Failure between Smoking Groups Based on Status, Duration and Number Smoked per Day**

	Smoking Status			<i>p</i> value
	Non-smoker	Smoker		
CRF (observed & extrapolated age)	40.1±1.9 (n=46)	40.3±1.4 (n=79)		0.84

	Smoking Duration			<i>p</i> value
	Non-smoker	≤20 years	>20 years	
CRF (observed & extrapolated age)	40.1±1.9 (n=46)	39.5±1.9 (n=48)	44.7±2.5 (n=29)	0.21

	Number of Cigarettes Per Day			<i>p</i> value
	Non-smoker	≤20	>20	
CRF (observed & extrapolated age)	40.1±1.9 (n=46)	42.1±1.8 (n=50)	37.8±3.3 (n=15)	0.65



### *Cumulative survival to renal failure*

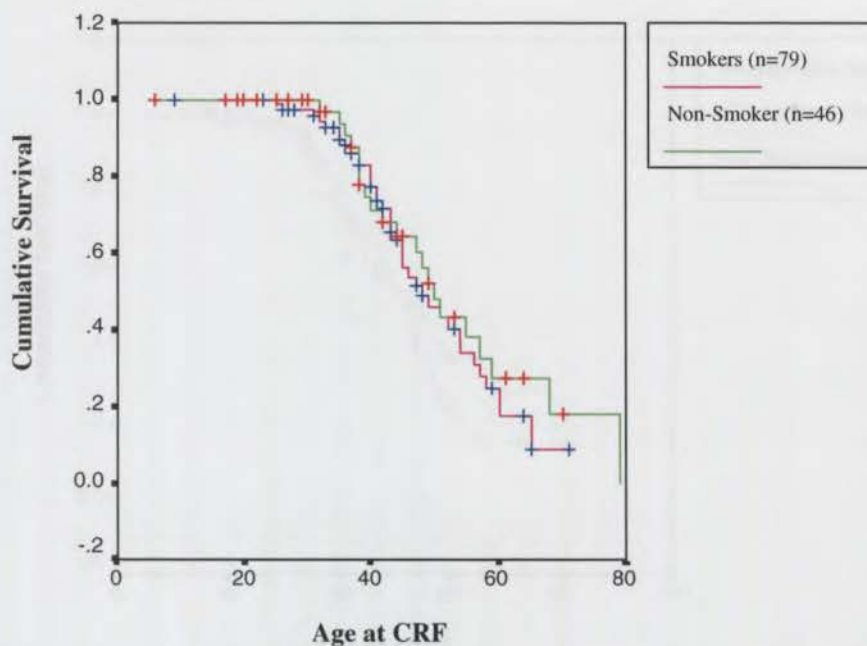
The second approach to assess the effect of smoking on deterioration of renal function was based on a comparison of Kaplan-Meier cumulative survival curves. No statistical difference in cumulative renal survival to CRF was found between patients according to smoking history ( $p = 0.67$ ; Figure 6.2) and smoking duration ( $p = 0.45$ ; Figure 6.3). The difference in renal survival to CRF according to daily number of cigarettes was found to approach significance, showing faster progression to CRF in subjects smoking  $>20$ , followed by those smoking  $\leq 20$  and non-smokers ( $p = 0.08$ ; Figure 6.4).

For ESRF, the number of individuals and the results are shown in Figures 6.5-6.7. No statistically significant difference in cumulative renal survival to ESRF was found between ADPKD patients according to smoking history ( $p = 0.19$ ; Figure 6.5), smoking duration ( $p = 0.38$ ; Figure 6.6) and amount of cigarettes consumed ( $p = 0.09$ ; Figure 6.7). Similar to CRF, a trend to better renal survival was observed among non-smokers.

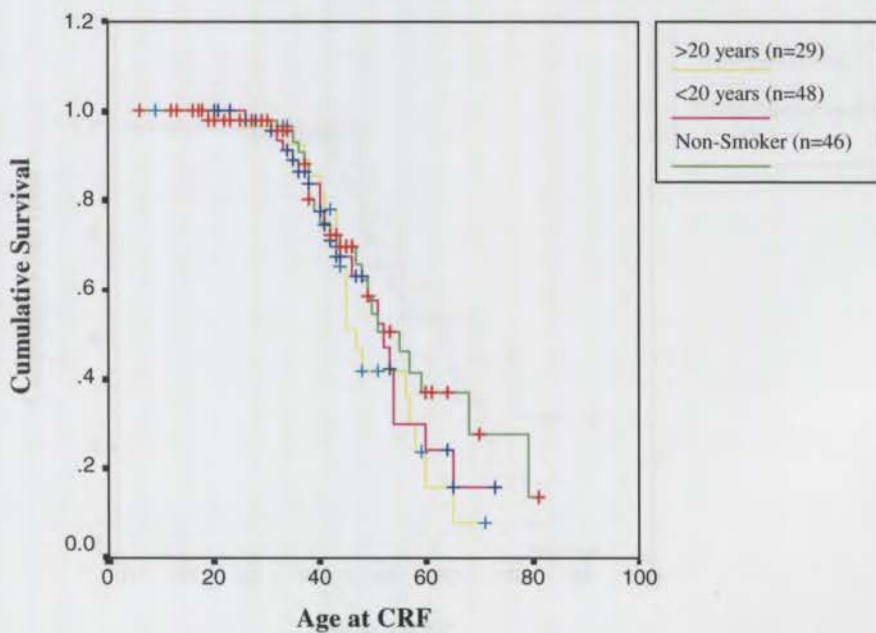
### **6.3.3      *The Relationship between Smoking and Hypertension***

The relationship between smoking and hypertension was examined in two ways. The first analysis compared the frequency of hypertension and the second analysis compared age at development of hypertension between smoking groups. A significantly higher frequency of hypertension was observed in smokers as compared to non-smokers ( $p = 0.004$ ; Table 6.7). A dose-effect relationship showing an increase in the frequency of hypertension was observed in the groups based on smoking duration and number of cigarettes smoked per day. The lowest frequency of hypertension was observed in patients who had not smoked, followed by patients who had smoked  $\leq 20$  years or smoked  $\leq 20$  cigarettes per day, and highest in patients who had smoked  $>20$  years and  $>20$  cigarettes per day (Table 6.7).

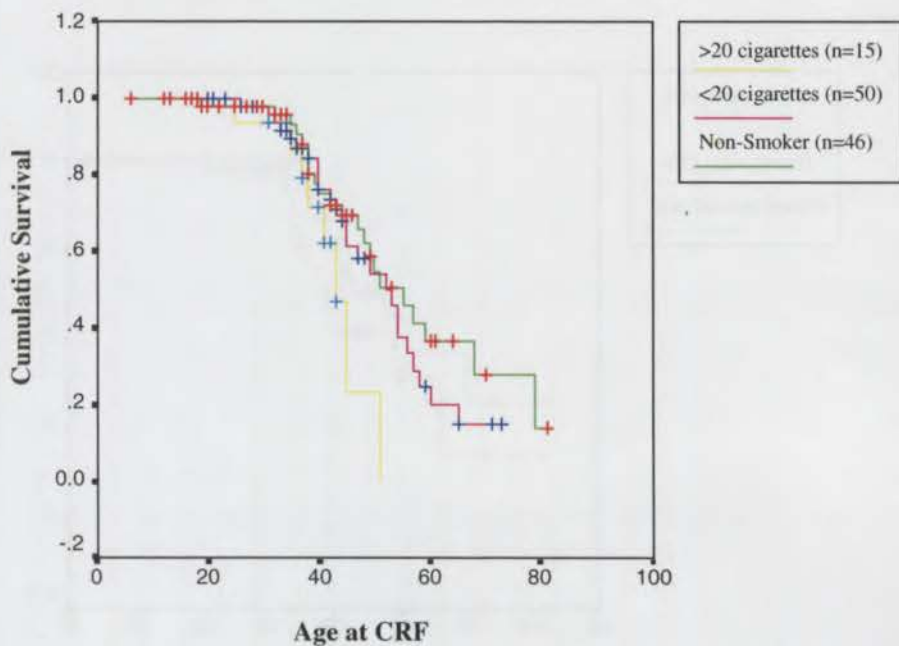
The second method compared the age at development of hypertension within each of the three smoking groups. No significant difference in age at hypertension was observed between groups (Table 6.7), however, smokers tended to develop hypertension at a slightly younger age than non-smokers.



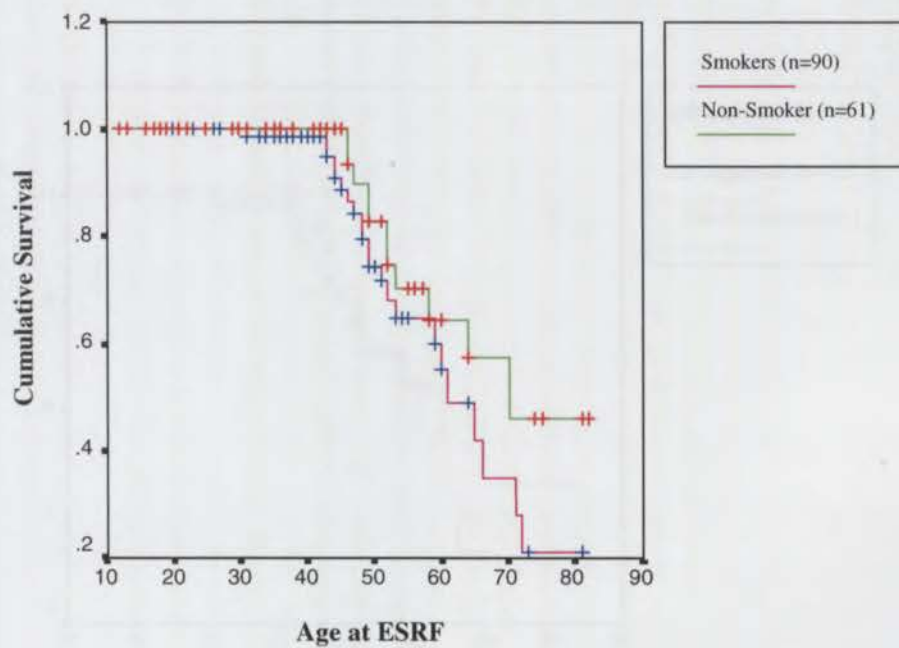
**Figure 6.2:** Cumulative Survival to CRF in ADPKD Patients According to Smoking Status. *No significant difference in renal survival was found between smokers and non-smokers ( $p=0.67$ )*



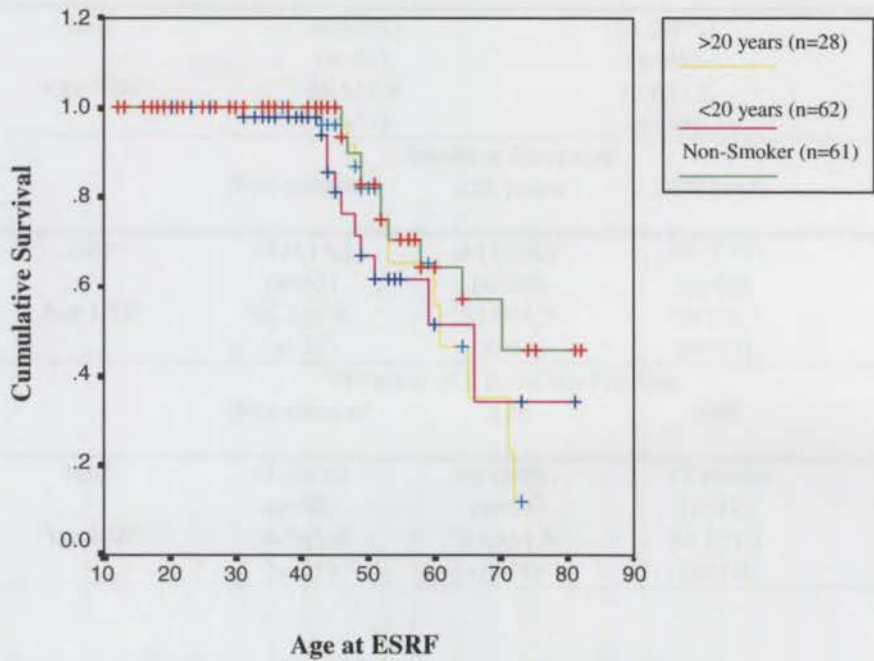
**Figure 6.3:** Cumulative Survival to CRF in ADPKD Patients Based on Smoking Duration. *No significant difference in renal survival was found between smoking duration groups ( $p=0.45$ )*



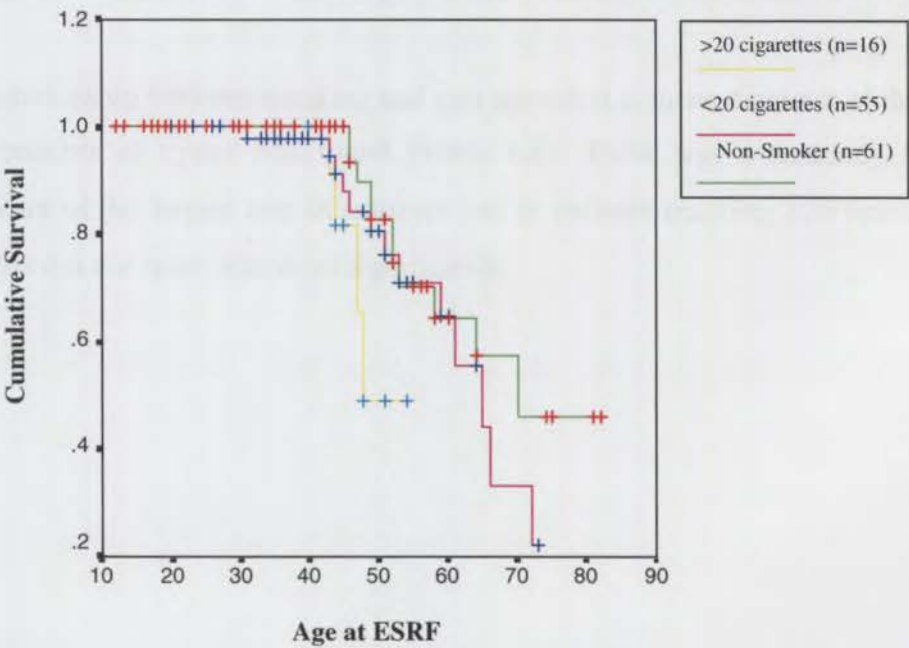
**Figure 6.4:** Cumulative Survival to CRF in ADPKD Patients Based on Daily Number of Cigarettes. *No significant difference in renal survival according to smoking number ( $p=0.08$ )*



**Figure 6.5:** Cumulative Survival to ESRF in ADPKD Patients Based on Smoking Status. *No significant difference in renal survival found according to smoking status ( $p=0.19$ )*



**Figure 6.6:** Cumulative Survival to ESRF According to Smoking Duration. No significant difference in renal survival according to smoking duration was found ( $p=0.38$ )



**Figure 6.7:** Cumulative Survival to ESRF in ADPKD Patients Based on Daily Number of Cigarettes. No significant difference in renal survival according to smoking number was found ( $p=0.09$ )

**Table 6.7: The Effect of Smoking on the Development of Hypertension in ADPKD Patients**

Smoking Status				
	Non-smoker	Smoker		<i>p</i> value
HBP	38(61%) (n=63)	73 (82%) (n=89)		<b>0.004</b>
Age HBP	36.5±1.9 (n=31)	32.6±1.5 (n=49)		0.12
Smoking Duration				
	Non-smoker	≤20 years	>20 years	<i>p</i> value
HBP	39 (61%) (n=63)	44 (75%) (n=59)	28 (97%) (n=29)	<b>0.001</b>
Age HBP	36.5±1.9 (n=31)	31.0±1.9 (n=32)	36.7±2.7 (n=27)	0.11
Number of Cigarettes Per Day				
	Non-smoker	≤20	>20	<i>p</i> value
HBP	38 (61%) (n=62)	45 (80%) (n=56)	15 (94%) (n=16)	<b>0.002</b>
Age HBP	36.5±1.9 (n=31)	33.5±1.9 (n=30)	34.1±3.4 (n=10)	0.62

#### **6.3.4      *The Effect of Smoking on Renal Cystogenesis***

The relationship between smoking and cystogenesis was examined by comparing the mean diameter of the largest cyst and number of cysts within each of the three groups (adjusted for age). The number of patients and results are shown in Table 6.8.

No relationship between smoking and cyst growth (i.e. mean diameter of the largest cyst and number of cysts) was found (Table 6.8). There was a tendency for a greater diameter of the largest cyst in smokers and in patients smoking ≥20 years (Table 6.8), but this did not reach statistical significance.

**Table 6.8: The Effect of Smoking on Renal Cystogenesis**

	Smoking History		<i>P</i> value
	Non-smoker	Smoker	
Number cysts	7.9±0.4 (n=44)	8.7±0.3 (n=72)	0.20
Diam lrg cyst (mm)	31.5±2.4 (n=44)	37.5±1.8 (n=72)	0.24
	Smoking Duration		
	Non-smoker	≤20 years	>20 years
Number cysts	7.9±0.4 (n=44)	8.7±0.4 (n=45)	8.5±0.5 (n=26)
Diam lrg cyst (mm)	32.1±2.4 (n=44)	32.6±2.3 (n=45)	40.1± (n=26)

\* *Diam* – diameter; *lrg* – largest

## 6.4 RESULTS: THE RELATIONSHIP BETWEEN ALCOHOL CONSUMPTION AND RENAL FAILURE

### 6.4.1 Alcohol and overall risk of renal failure

The relationship between alcohol and development of renal failure was examined in 152 Australian and Polish patients, grouped on the basis of alcohol consumption into non-drinkers, light/moderate drinkers and heavy drinkers. The risk of developing renal failure was assessed by comparing the frequency of renal failure between groups based on the above three variables and by Cox regression analysis (section 6.8).

The percentage of individuals with CRF ( $p = 0.81$ ) and ESRF ( $p = 0.52$ ) did not differ significantly between non-drinkers, light/moderate drinkers and heavy drinkers (Table 6.9).

**Table 6.9: Overall Risk of Renal Failure in ADPKD Subjects Based on Alcohol Consumption**

	Non-drinker	Light/moderate drinker	Heavy drinker	<i>p</i> value
CRF	17 (40%)	25 (42%)	21 (42%)	0.81
ESRF	10 (23%)	25 (25%)	9 (18%)	0.52

6.4.2            *The relationship between alcohol consumption and deterioration of renal function*

The effect of alcohol consumption on progression to renal failure was examined between non-drinkers, light/moderate and heavy drinkers with regard to mean age to renal failure and in terms of Kaplan-Meier cumulative survival curves.

*Observed age at renal failure*

The initial analysis compared the observed mean age at renal failure between the three groups according to alcohol consumption. The numbers and results are shown in Table 6.10. No significant difference in age at CRF and ESRF was found between alcohol consumption groups, although an earlier age at CRF was observed in heavy drinkers, followed by light/moderate drinkers and non-drinkers (Table 6.10).

**Table 6.10: The Relationship between Alcohol Consumption on Observed Age at CRF and ESRF in ADPKD Patients**

	Non-drinker	Light/moderate drinker	Heavy drinker	<i>p</i> value
CRF (observed age)	51.3±5.3 (n=10)	39.6±3.8 (n=19)	37.9±3.6 (n=22)	0.11
ESRF	52.8±1.5 (n=10)	52.5±1.3 (n=14)	52.9±1.7 (n=8)	0.96

*Extrapolated age at renal failure*

Differences in the rate of progression to renal failure were further examined by comparing age at development of CRF extrapolated from the individual regression lines of time versus 1/creatinine between the following three groups: non-drinkers, light/moderate drinkers and heavy drinkers. The analysis included 33 non-drinkers, 44 light/moderate drinkers and 45 heavy drinkers. No significant difference in age at CRF was found between the three groups (Table 6.11).

**Table 6.11: The Relationship between Alcohol Consumption and Observed and Extrapolated Age at CRF in ADPKD Patients**

	Non-drinker	Light/moderate drinker	Heavy drinker	<i>p</i> value
CRF (observed & extrapolated age)	41.7±2.3 (n=33)	41.5±2.0 (n=44)	40.6±1.9 (n=45)	0.92

#### *Cumulative survival to renal failure*

The second approach to assess the effect of alcohol on deterioration of renal function was based on a comparison of Kaplan-Meier cumulative survival curves. The numbers and results are shown in Figures 6.8 and 6.9.

No significant difference in cumulative renal survival to CRF was found between the three groups based on alcohol consumption ( $p = 0.64$ ; Figure 6.8). However a trend was observed showing shortest renal survival in heavy drinkers, intermediate in light/moderate drinkers and longest in non-drinkers.

No significant difference in cumulative survival to ESRF was found between the three groups ( $p = 0.82$ ; Figure 6.9). Similarly, a trend was observed revealing shortest renal survival in heavy drinkers followed by light/moderate and longest in non-drinkers.

#### **6.4.3      *The Relationship between Alcohol Consumption and Hypertension***

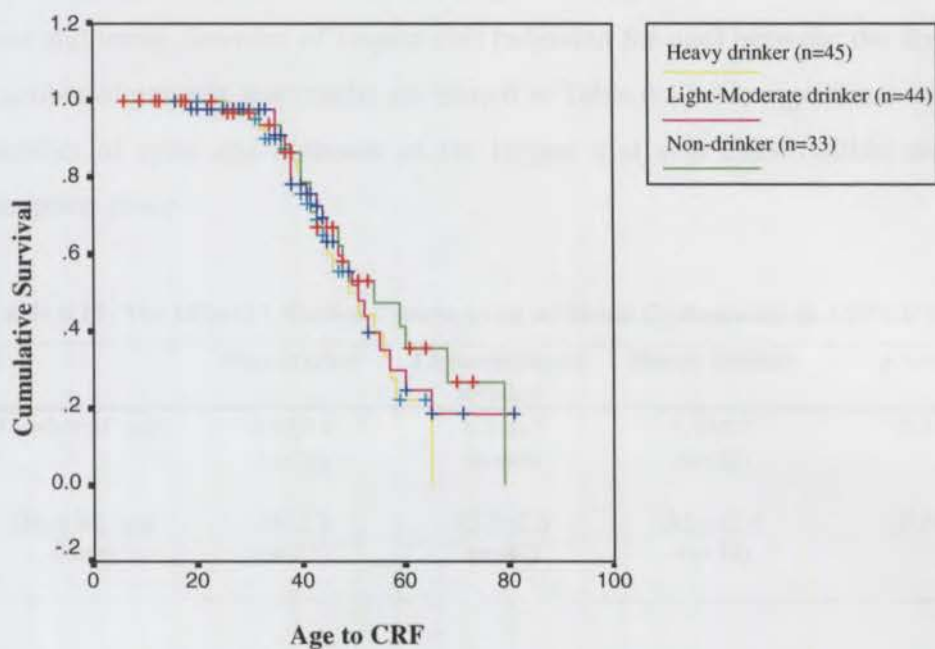
The relationship between alcohol consumption and hypertension in ADPKD was examined in two ways. The initial analysis compared the prevalence of hypertension between the three alcohol groups: non-drinkers, light/moderate and heavy drinkers. No significant difference in the frequency of hypertension was observed between the three groups ( $p = 0.93$ ; Table 6.12).

The second method compared the age at development of hypertension between the three groups. The analysis showed no difference in the age at development of hypertension between the three groups, Table 6.12.

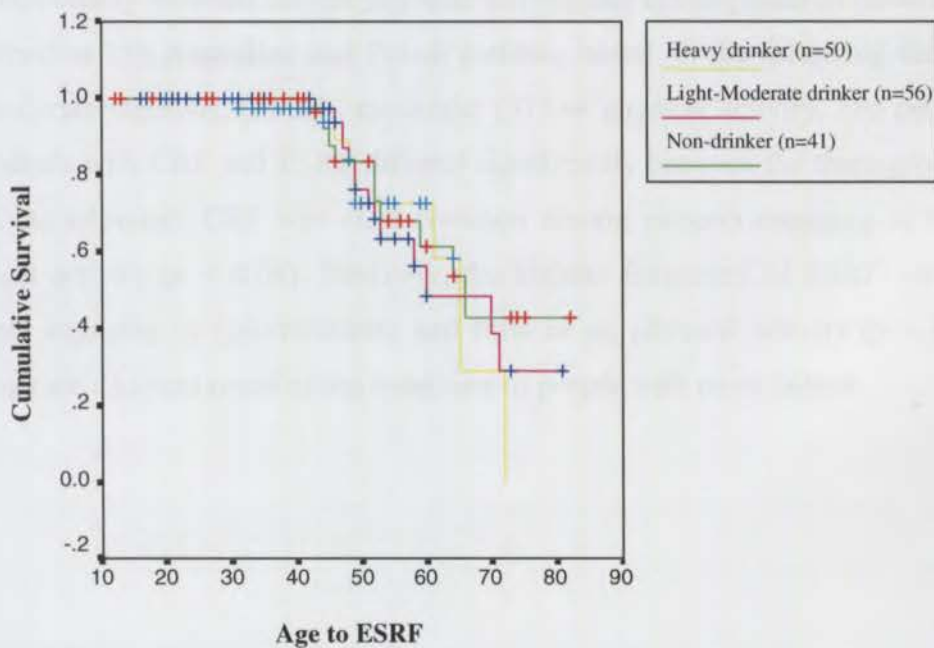
**Table 6.12: The Relationship between Hypertension and Alcohol Consumption in ADPKD Patients**

	Non-drinker (n=41)	Light/moderate drinker (n=56)	Heavy drinker (n=50)	<i>p</i> value
HBP	32 (78%)	38 (68%)	39 (78%)	0.92
Age HBP	34.0±2.2 (n=25)	34.9±2.0 (n=29)	34.0±2.2 (n=25)	0.93





**Figure 6.8:** Cumulative Survival to CRF in ADPKD Patients According to Alcohol Consumption. *No significant difference in renal survival between alcohol groups was found ( $p=0.64$ )*



**Figure 6.9:** Cumulative Survival to ESRF in ADPKD Patients According to Alcohol Consumption. *No significant difference in renal survival between alcohol groups was found ( $p=0.82$ )*

#### 6.4.4 *The Effect of Alcohol Consumption on Renal Cystogenesis*

The relationship between alcohol and cystogenesis was examined by comparing number of cysts and mean diameter of largest cyst (adjusted for age) between the three groups. The number of patients and results are shown in Table 6.13. No significant difference in the number of cysts and diameter of the largest cyst was found within each alcohol consumption group.

**Table 6.13: The Effect of Alcohol Consumption on Renal Cystogenesis in ADPKD Patients**

	Non-drinker	Light/moderate drinker	Heavy drinker	<i>p</i> value
Number of cysts	7.6±0.4 (n=32)	8.7±0.4 (n=44)	8.5±0.9 (n=39)	0.22
Diam lrg cyst (mm)	34±2.7 (n=32)	35.8±2.3 (n=44)	32.6±2.5 (n=39)	0.64

## 6.5 RESULTS: RELATIONSHIP BETWEEN CURRENT PHYSICAL ACTIVITY AND RENAL FAILURE

### 6.5.1 *Renal Failure and Current Physical Activity Patterns*

The relationship between current physical activity and development of renal failure was examined in 156 Australian and Polish patients, based on the following three groups: (1) moderate-vigorous; (2) light-moderate; (3) low physical activity. The percentage of individuals with CRF and ESRF differed significantly between the three groups (Table 6.14). As expected, CRF was most common among patients engaging in little or no physical activity ( $p < 0.01$ ). Similarly, the highest frequency of ESRF was found in patients engaging in light/moderate and little or no physical activity ( $p < 0.01$ ). The findings are a logical result of the weakness of people with renal failure.

**Table 6.14: The Frequency of Renal Failure in ADPKD Patients According to Amount of Current Physical Activity**

Renal failure	Physical activity			<i>p</i> value
	Moderate/ vigorous (n=77)	Light/ moderate (n=49)	Low (n=30)	
CRF	17 (22%)	27 (55%)	19 (63%)	<0.01
ESRF	6 (8%)	18 (37%)	10 (33%)	<0.01

**6.5.2      *Current physical activity and rate of deterioration of renal function***

The relationship between current physical activity and progression to renal failure was also examined, however, the analysis is based on the assumption that current physical activity is likely to be an indicator of past activity patterns (prior to renal failure).

*Observed age at renal failure*

The initial analysis compared the observed mean age at renal failure between the three groups. The number and results are shown in Table 6.15.

No significant difference in the observed age at CRF was found between the three groups based on current physical activity (*p* = 0.85; Table 6.15). In terms of age at ESRF, no difference was detected between the three groups either.

**Table 6.15: Comparison of Observed Mean Age at Renal Failure between ADPKD Patients Based on Current Physical Activity**

Renal failure	Physical activity			<i>p</i> value
	Moderate/ vigorous	Light/ moderate	Low	
CRF (observed age)	34.6±3.7 (n=16)	44.8±3.9 (n=18)	46.2±4.6 (n=13)	0.09
ESRF	50.7±3.7 (n=6)	50.2±2.2 (n=17)	58.8±3.0 (n=9)	0.08

*Extrapolated age at renal failure*

Differences in the rate of progression to renal failure were further examined by comparing age at development of CRF extrapolated from the individual regression lines of time versus 1/creatinine. The number and results are shown in Table 6.16.

A significant difference in extrapolated age at CRF was detected between the three groups ( $p = 0.02$ ; Table 6.16). Patients engaging in moderate/vigorous physical activity were found to reach CRF approximately 5-8 years earlier than patients performing light/moderate and low physical activity.

**Table 6.16: Comparison of Mean Age (observed and extrapolated) at Renal Failure in ADPKD Patients Based on Current Physical Activity**

Renal failure	Physical activity			<i>p</i> value
	Moderate/ vigorous	Light/ moderate	Low	
CRF (observed & extrapolated age)	37.6±1.7 (n=60)	42.6±2.0 (n=41)	46.1±2.7 (n=24)	<b>0.02</b>

**6.5.3      *The Relationship between Current Physical Activity and Hypertension***

The relationship between hypertension and physical activity in ADPKD patients was examined by comparing the prevalence of hypertension between physical activity groups (Table 6.17). A significant difference in the prevalence of hypertension was found between the three groups ( $p = 0.004$ ), with the highest frequency of hypertension observed in patients performing no exercise or light/moderate exercise. The lowest frequency of hypertension was observed in patients engaged in more vigorous physical activity.

**Table 6.17: The Relationship between Hypertension and Physical Activity in ADPKD Patients**

	Physical activity			<i>p</i> value
	Moderate/ vigorous (n=77)	Light/ moderate (n=49)	Low (n=30)	
HBP	47 (61%)	38 (78%)	26 (87%)	<b>0.004</b>

## 6.6 RESULTS: RELATIONSHIP BETWEEN LIPIDAEMIA AND RENAL FAILURE

### 6.6.1 *Lipidaemia and overall risk of renal failure*

The relationship between lipidaemia (total cholesterol and triglycerides) and development of renal failure (CRF and ESRF) was examined in a total of 113 Australian and Polish patients based on two variables: (1) cholesterol; <5.5 and >5.5 mmol/L and (2) triglycerides; <1.8 and >1.8 mmol/L. A significant difference in the frequency of CRF was found between the two cholesterol groups, revealing a higher frequency of CRF in patients with high cholesterol (>5.5 mmol/L). No significant difference in the prevalence of CRF was found between the two triglyceride groups nor ESRF between cholesterol and triglyceride groups (Table 6.18).

Table 6.18: Overall Risk of Renal Failure

	Serum total cholesterol		<i>p</i> value	Serum total triglycerides		<i>p</i> value
	<5.5 (n=68)	>5.5 (n=45)		<1.8 (n=48)	>1.8 (n=43)	
CRF	22 (33%)	25 (56%)	0.01	19 (40%)	22 (51%)	0.18
ESRF	15 (22%)	12 (27%)	0.34	11 (23%)	12 (28%)	0.38

### 6.6.2 *The relationship between lipidaemia and rate of deterioration of renal function*

The relationship between lipidaemia and renal failure was also examined between patients with low and high cholesterol values and between those with low and high triglyceride values with regard to mean age to renal failure using both observed age and extrapolated age at CRF and in terms of Kaplan-Meier cumulative survival curves.

The relationship between lipids and development of renal failure (CRF and ESRF) was examined in two ways. The first analysis included 116 Australian and Polish patients, grouped on the basis of total saturated fat score into <4 and >4. Age at renal failure (CRF and ESRF) was compared between each of the two groups (Table 6.19). No difference in age at renal failure was observed between patients with a saturated fat score of <4 versus >4 for either CRF ( $p = 0.18$ ) or ESRF ( $p = 0.69$ ).

**Table 6.19: The Relationship between Saturated Fat and Age at Renal Failure in ADPKD Patients**

	Saturated Fat Score		<i>p</i> value
	<4	>4	
Age at CRF (observed & extrapolated age)	42.4 (n=63)	38.9 (n=53)	0.18
Age at ESRF	54.1 (n=15)	52.5 (n=13)	0.69

*Observed age at renal failure*

The second method examined the relationship between serum cholesterol and triglyceride levels and renal failure (CRF and ESRF). The patients were divided into groups as shown in 6.6.1. In regard to serum cholesterol, no significant difference in observed age at CRF was found between patients with <5.5 and >5.5 mmol/L. However, patients with a cholesterol value >5.5 mmol/L were found to reach ESRF 6 years later.

The results for serum triglycerides showed no significant differences in observed age at CRF and ESRF between patients with <1.8 and >1.8 mmol/L. The results are shown in (Table 6.20).

*Extrapolated age at renal failure*

Differences in the rate of progression to renal failure were further examined by comparing extrapolated age at CRF between groups based on cholesterol and triglyceride values.

**Table 6.20: The Relationship between Lipidaemia and Observed Age at CRF and ESRF in ADPKD Patients**

Renal failure	Serum total cholesterol		<i>p</i> value
	<5.5 mmol/L	>5.5 mmol/L	
Age at CRF (observed age)	44.1±3.1 (n=18)	44.5±2.9 (n=20)	0.91
Age at ESRF	47.8±2.1 (n=14)	54.4±2.4 (n=11)	0.05
	Serum total triglycerides		<i>p</i> value
	<1.8 mmol/L	>1.8 mmol/L	
Age at CRF (observed age)	44.7±3.7 (n=14)	43.3±3.2 (n=18)	0.78
Age at ESRF	51.5±2.7 (n=11)	49.5±2.8 (n=10)	0.64

A significantly later age at CRF was found in patients with a cholesterol value  $>5.5\text{mmol/L}$  ( $p = 0.002$ ). No difference in age at CRF was found between triglyceride groups ( $p = 0.84$ ). The results are shown in Table 6.21.

**Table 6.21: The Relationship between Lipidaemia and Observed and Extrapolated Age at CRF in ADPKD Patients**

Renal failure	Serum total cholesterol		<i>p</i> value
	$<5.5\text{ mmol/L}$	$>5.5\text{ mmol/L}$	
Age at CRF (observed & extrapolated age)	$37.0 \pm 1.7$ ( $n=51$ )	$45.1 \pm 2.0$ ( $n=39$ )	<b>0.002</b>
	Serum total triglycerides		<i>p</i> value
	$<1.8\text{ mmol/L}$	$>1.8\text{ mmol/L}$	
Age at CRF (observed & extrapolated age)	$39.7 \pm 2.1$ ( $n=34$ )	$40.3 \pm 2.0$ ( $n=39$ )	0.84

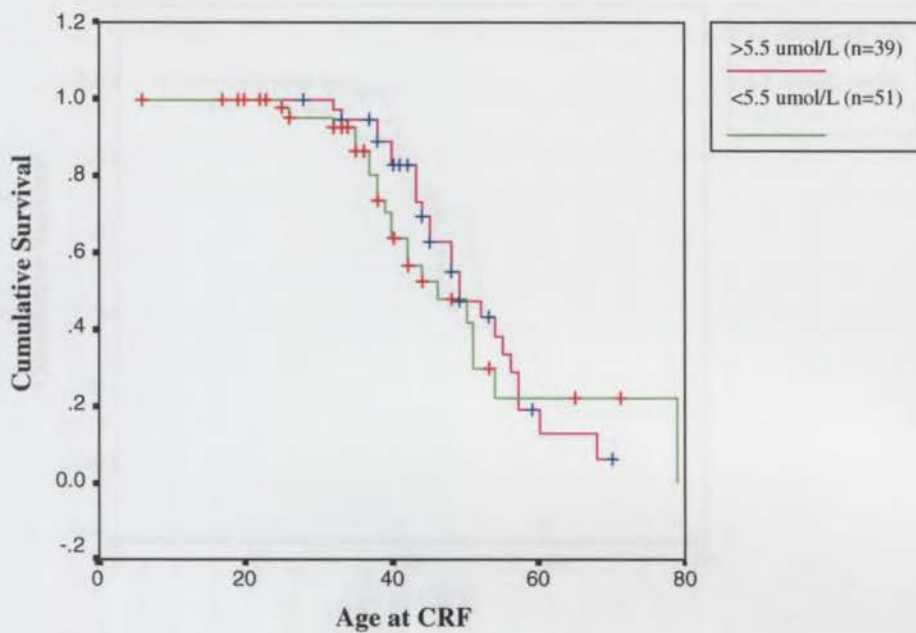
#### *Cumulative survival to renal failure*

The second approach to assess the effect of lipidaemia on deterioration of renal function was based on a comparison of Kaplan-Meier cumulative survival curves. Survival to CRF was compared between 51 patients with serum cholesterol  $<5.5\text{umol/L}$  and 39 with serum cholesterol  $>5.5\text{umol/L}$ , and between 34 patients with a serum triglyceride  $<1.8\text{umol/L}$  and 39 with a serum triglyceride  $>1.8\text{ umol/L}$ .

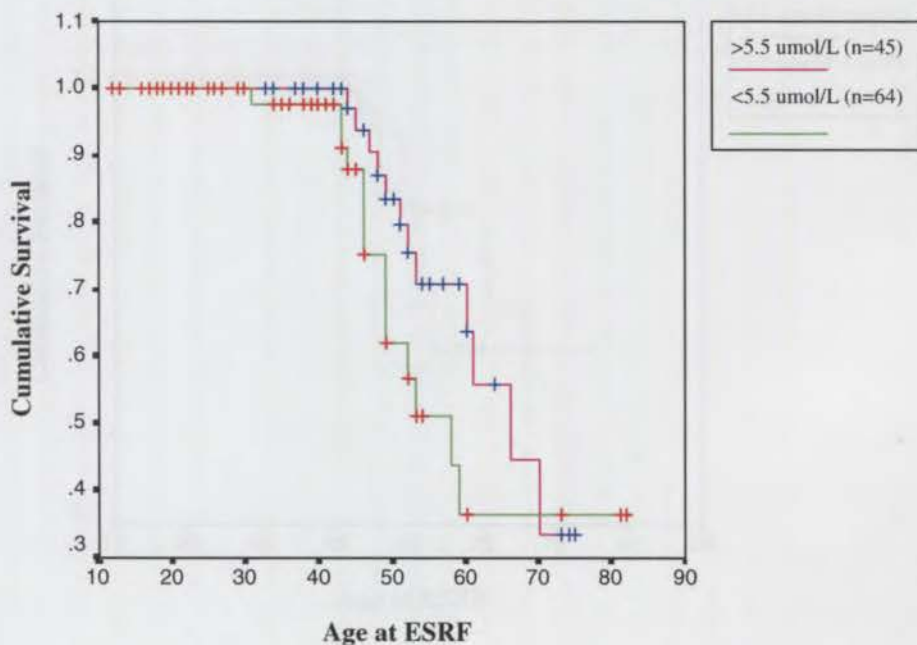
No statistical difference in cumulative renal survival to CRF was found between patients according to serum cholesterol levels ( $p = 0.86$ ; Figure 6.10) and serum triglyceride levels ( $p = 0.81$ ; Figure 6.12).

For ESRF, the comparison included 64 patients with a cholesterol of  $<5.5\text{umol/L}$  vs 45 with a serum cholesterol  $>5.5\text{umol/L}$ , and 47 patients with a triglyceride value  $<1.8\text{umol/L}$  vs 42 with a value  $>1.8\text{umol/L}$ .

No statistical difference in cumulative renal survival to ESRF was found between patients according to serum cholesterol levels ( $p = 0.06$ ; Figure 6.11) and serum triglyceride levels ( $p = 0.69$ ; Figure 6.13).

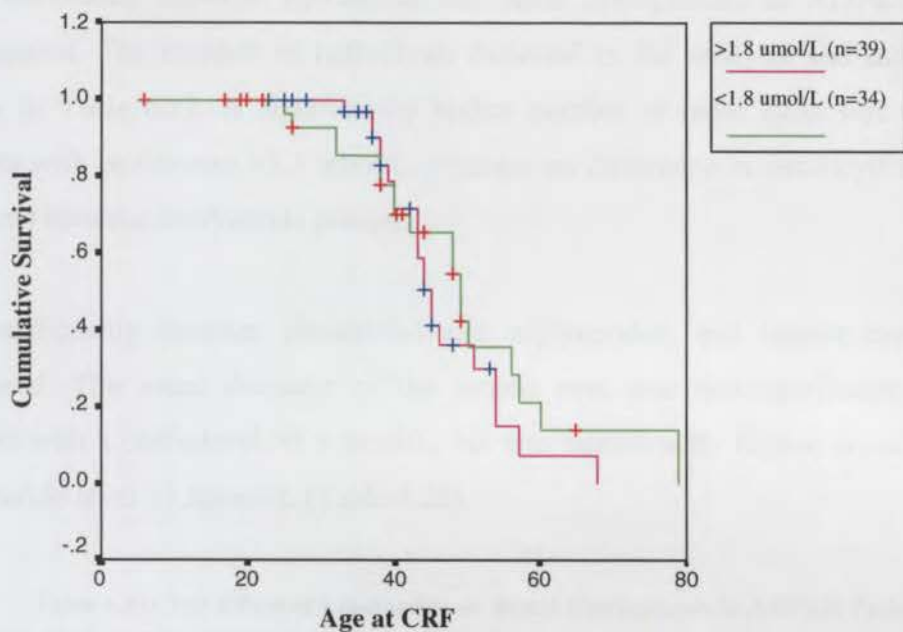


**Figure 6.10:** Cumulative Survival to CRF Based on Cholesterol Levels. *No significant difference in renal survival was found between cholesterol groups ( $p=0.86$ )*

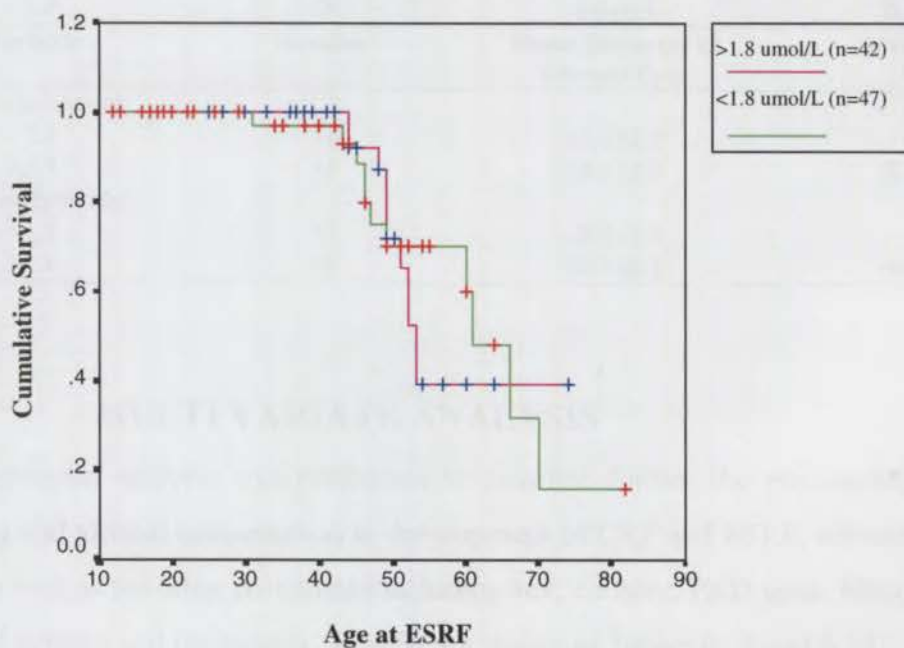


**Figure 6.11:** Cumulative Survival to ESRF Based on Cholesterol Levels. *No significant difference in renal survival was found between cholesterol groups ( $p=0.06$ )*





**Figure 6.12:** Cumulative Survival to CRF Based on Triglyceride Levels.  
*No significant difference in renal survival was found between triglyceride groups ( $p=0.81$ )*



**Figure 6.13:** Cumulative Survival to ESRF Based on Triglyceride Levels.  
*No significant difference in renal survival was found between triglyceride groups ( $p=0.69$ )*

6.6.3                    *The Effect of Lipidaemia on Renal Cystogenesis*

The relationship between lipidaemia and renal cystogenesis in ADPKD was also investigated. The number of individuals included in the analysis and the results are shown in Table 6.22. A significantly higher number of renal cysts was observed in patients with cholesterol >5.5 umol/L, whereas no difference in renal cyst number was observed between triglyceride groups.

The relationship between cholesterol and triglycerides, and largest cyst was also compared. The mean diameter of the largest cyst was non-significantly higher in patients with a cholesterol >5.5 umol/L, but was significantly higher in patients with a triglyceride level >1.8umol/L (Table 6.22).

Table 6.22: The Effect of Lipidaemia on Renal Cystogenesis in ADPKD Patients

Variable	Number	Mean Number of Cysts	p value
Total cholesterol			
<5.5	52	7.8±0.4	
>5.5	36	9.5±0.4	<0.01
Total triglycerides			
<1.8	36	8.2±0.5	
>1.8	38	8.9±0.5	0.29
Variable	Number	Mean Diameter of Largest Cyst	p value
Total cholesterol			
<5.5	52	33.1±2.5	
>5.5	35	38.1±3.0	0.21
Total triglycerides			
<1.8	35	28.9±2.8	
>1.8	38	39.3±2.7	<0.01

6.7                    **MULTI VARIATE ANALYSIS**

Cox regression analysis was performed to examine further the relationship between smoking and alcohol consumption to development of CRF and ESRF, adjusted for each other as well as for other co-variates including sex, country, PKD gene, blood pressure, physical activity and lipidaemia. Results are shown in Tables 6.23 and 6.24.

No significant differences in the relative risk (RR) of developing CRF were found between ADPKD subjects based on smoking status and duration. When adjusted for sex, the RR of a smoker developing CRF decreased, while the RR increased when

adjusted for PKD gene type (Table 6.23). The RR of developing CRF was significantly higher in patients smoking >20 cigarettes per day (RR:2.3[CI:1.0,5.4]) as compared to individuals smoking <20 cigarettes per day (RR:1.2[CI:0.6,2.1]). The RR remained higher in subjects smoking >20 cigarettes per day when adjusted for alcohol consumption, exercise, sex, PKD gene, country, BP and lipidaemia (Table 6.23), although the difference was not statistically significant.

No significant differences in the RR of developing CRF was found between alcohol consumption groups, however when adjusted for sex the RR was reduced to 0.8[0.4,1.8] and 0.8[0.3,1.8] in light/moderate and heavy drinkers (Table 6.23).

For ESRF, the crude RR of developing ESRF was significantly higher in current smokers (2.1[1.0,4.7]) as opposed to ex- (1.3[0.6,3.1]) and non-smokers (Table 6.24). The RR for current smokers was even stronger when adjusted for PKD gene (3.9[1.0,15.9]). According to smoking duration, the RR of developing ESRF including unadjusted and adjusted analyses was non-significantly higher in ADPKD subjects smoking <20 and >20 years as compared to non-smokers. After adjusting for sex, the RR for smokers <20 (1.4[0.6,3.3]) and >20 (1.1[0.4,2.8]) decreased, but remained marginally higher than non-smokers (Table 6.24). In terms of number of cigarettes, smokers <20 (1.4[0.7,3.1]) and >20 (3.6[1.1,11.9]) cigarettes per day had a higher RR of developing ESRF as compared to non-smokers. The RR was significantly higher after adjusting for alcohol (3.9[1.1,14.5]) and serum triglycerides (4.9[1.1,23.1]).

In comparison to alcohol abstainers, both light/moderate and heavy drinkers showed a marginally higher RR of developing ESRF. After adjustment for the influence of sex the RR for both light/moderate and heavy drinkers was reduced.

Table 6.23: Relative Risks of Developing Chronic Renal Failure in ADPKD Patients Based on Smoking History and Alcohol Consumption Groups

Variable	N	Crude	ADJUSTED								
			Alcohol	Smoking	Exercise	Sex	PKD gene	Country	HBP	Triglycerides	Cholesterol
<b>Smoking Status</b>											
Non	46	1.0	1.0	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ex	45	1.2[0.7,2.2]	1.1[0.6,2.3]		1.2[0.6,2.2]	0.7[0.4,1.6]	1.7[0.6,4.4]	1.1[0.6,2.1]	1.1[0.6,2.0]	1.5[0.7,3.3]	1.1[0.5,2.3]
Current	34	1.2[0.7,2.3]	1.1[0.6,2.3]		1.2[0.6,2.2]	0.8[0.4,1.7]	1.8[0.7,5.2]	1.3[0.7,2.4]	1.1[0.6,2.1]	0.9[0.4,2.3]	1.0[0.5,2.1]
<b>Smoking Duration (years)</b>											
Non	47	1.0	1.0	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0
<20	49	1.2[0.7,2.2]	1.1[0.6,2.2]		1.1[0.6,2.2]	0.8[0.4,1.7]	1.5[0.6,4.2]	1.2[0.6,2.3]	1.0[0.6,1.9]	0.9[0.5,2.2]	1.0[0.5,2.1]
>20	29	1.2[0.7,2.3]	1.2[0.5,2.5]		1.2[0.6,2.2]	0.7[0.3,1.6]	1.9[0.7,5.2]	1.2[0.6,2.3]	1.1[0.6,2.1]	1.7[0.7,3.7]	1.1[0.5,2.3]
<b>Smoking Number</b>											
Non	46	1.0	1.0	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0
<20	50	1.2[0.6,2.1]	1.1[0.6,2.1]		1.1[0.6,2.1]	0.7[0.3,1.4]	1.9[0.7,4.7]	1.1[0.6,2.0]	1.0[0.6,1.9]	1.1[0.5,2.2]	0.9[0.5,1.9]
>20	15	<b>2.3[1.0,5.4]§</b>	2.0[0.8,5.3]		2.2[0.9,5.3]	1.5[0.6,3.6]	2.4[0.5,10.3]	2.3[0.9,5.4]	2.3[0.9,5.4]	1.9[0.6,5.6]	1.8[0.6,5.3]
<b>Alcohol</b>											
Non		1.0	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Light/moderate		1.2[0.6,2.4]		1.2[0.6,2.4]	1.3[0.7,2.5]	0.8[0.4,1.8]	0.9[0.4,2.6]	1.3[0.6,2.4]	1.1[0.5,2.1]	1.2[0.5,3.1]	1.0[0.5,2.3]
Heavy		1.3[0.7,2.6]		1.2[0.7,2.6]	1.4[0.7,2.7]	0.8[0.3,1.8]	1.6[0.6,3.7]	1.3[0.6,2.5]	1.2[0.6,2.3]	1.6[0.7,3.6]	1.4[0.6,3.0]

Table 6.24: Relative Risks of Developing End-Stage Renal Failure in ADPKD Patients Based on Smoking History and Alcohol Consumption Groups

Variable	N	Crude	ADJUSTED								
			Alcohol	Smoking	Exercise	Sex	PKD gene	Country	HBP	Triglycerides	Cholesterol
<b>Smoking Status</b>											
Non	61	1.0	1.0	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ex	51	1.3[0.6,3.1]	1.2[0.5,3.1]		1.1[0.5,2.7]	0.9[0.3,2.4]	1.9[0.5,7.9]	1.3[0.6,3.1]	1.3[0.6,3.2]	<b>3.3[1.1,10.2]</b>	1.9[0.7,4.9]
Current	38	<b>2.1[1.0,4.7]§</b>	2.0[0.9,4.7]		1.8[0.8,4.2]	1.6[0.7,3.9]	<b>3.9[1.0,15.9]</b>	2.1[0.9,4.7]	1.7[0.7,3.9]	1.9[0.7,5.9]	1.7[0.7,4.4]
<b>Smoking Duration (years)</b>											
Non	62	1.0	1.0	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0
<20	59	1.7[0.8,4.0]	1.7[0.7,4.0]		1.5[0.7,3.6]	1.4[0.6,3.3]	2.8[0.7,10.9]	1.7[0.7,3.9]	1.7[0.7,4.2]	2.5[0.9,7.5]	1.9[0.8,4.7]
>20	29	1.5[0.7,3.5]	1.5[0.6,3.7]		1.4[0.6,3.2]	1.1[0.4,2.8]	2.6[0.6,10.4]	1.6[0.7,3.7]	1.4[0.6,3.2]	2.3[0.7,7.1]	1.7[0.6,4.6]
<b>Smoking Number</b>											
Non	61	1.0	1.0	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0
<20	55	1.4[0.7,3.1]	1.5[0.6,3.5]		1.3[0.6,2.9]	0.9[0.4,2.4]	2.6[0.7,9.6]	1.4[0.6,3.3]	1.2[0.5,2.7]	2.0[0.7,5.8]	1.5[0.6,3.7]
>20	16	<b>3.6[1.1,11.9]§</b>	<b>3.9[1.1,14.5]</b>		2.9[0.9,9.8]	2.7[0.8,9.3]	3.7[0.4,42.3]	<b>3.6[1.1,11.9]</b>	<b>3.6[1.1,11.9]</b>	<b>4.9[1.1,23.1]</b>	3.3[0.8,11.9]
<b>Alcohol</b>											
Non	41	1.0	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Light/moderate	56	1.3[0.6,2.8]		1.2[0.5,2.7]	1.1[0.5,2.5]	0.9[0.4,2.2]	1.0[0.3,3.5]	1.2[0.5,2.8]	1.0[0.5,2.4]	1.3[0.5,3.8]	1.0[0.4,2.7]
Heavy	50	1.3[0.5,3.2]		1.2[0.4,3.1]	1.3[0.5,3.2]	0.8[0.3,2.3]	2.1[0.6,7.2]	1.4[0.5,3.6]	1.1[0.4,2.8]	1.1[0.4,3.3]	1.1[0.4,3.1]

## 6.8 DISCUSSION

This part of the study was designed to investigate whether environmental risk factors, namely smoking, alcohol consumption and lack of physical activity as well as lipidaemia influence the clinical severity of ADPKD. Such a role seems plausible in view of the physiological effects on the kidney mediated by these factors and the observed intra-familial variation in ADPKD. It has been proposed that the focal nature of cyst formation in ADPKD may be explained by a second hit occurring in somatic cells (Qian *et al.*, 1996; Brasier and Henske, 1997). Environmental factors, therefore may initiate the second hit thus promoting cystogenesis and influencing disease severity. To date, there is only limited literature available on the relationship(s) between environmental factors and ADPKD disease severity. Furthermore, no study has investigated the joint effects of these factors in ADPKD, which seems important since smoking, high alcohol consumption, limited physical activity and lipidaemia are likely to be associated with each other.

The design of this study included patients from Australia and Poland. The relationship between environmental risk factors and hypertension, renal cystogenesis and the deterioration of renal function were investigated. Because many of these factors tend to be inter-related *i.e.* smokers tend to drink more than non-smokers and are most likely to exercise less, the combined effects may be more detrimental to renal function. Therefore, we performed Cox regression analysis to adjust for each variable and other factors including sex, blood pressure, PKD gene and country.

Similar to the other parts of the study we examined two renal outcomes: chronic renal failure (CRF) and end-stage renal failure (ESRF), using two approaches namely age at onset to renal failure (CRF and ESRF) and cumulative renal survival. For CRF, the analyses were performed twice. The initial analysis included observed age, and the subsequent analysis included extrapolated age obtained from the individual curves versus 1/Cr values.

Our major finding is the higher frequency of renal failure and faster rate of progression to renal failure in smokers compared to non-smokers. Our findings can only be compared to a limited number of ADPKD studies. In one study, an increase in renal

failure cases among smokers with ADPKD and immunoglobulin-A-glomerular nephropathy (IgA-GN) was observed (Orth *et al.*, 1998). In another study, Chapman *et al.* (1994) found that patients with established proteinuria as an indicator of adverse prognosis had a greater pack-year smoking history than their non-proteinuric counterparts.

In agreement with Orth *et al.* (1998), this study also showed that smokers have a higher risk of developing renal failure compared to non-smokers. A significantly higher RR (RR: 2.1[1.0,4.7]) was found in current smokers compared to ex-smokers (RR: 1.3[0.5,3.1]) and non-smokers and the risk was increased to 3.9[1.0,15.9] after adjusting for PKD genotype. According to number of cigarettes, individuals smoking >20 cigarettes per day were found to have a RR of 3.6[1.1,11.9] compared to individuals smoking <20 cigarettes per day with a RR of 1.4[0.6,3.1], and this became even greater when adjusted for alcohol consumption 3.9[1.1,14.5]. The relationship between the number of cigarettes smoked per day and progression from microalbuminuria to persistent proteinuria, *i.e.* overt diabetic nephropathy, has also been observed in patients with IDDM and NIDDM. Chase *et al.* (1991) examined the risk to develop microalbuminuria or proteinuria in patients with IDDM and according to smoking status. Their group showed that the prevalence of borderline (>7.6 µg/min) and abnormal (>30 µg/min) albumin excretion rates was 2.8 fold in smokers compared to non-smokers. Klein *et al.* (1993) also showed that smoking is related to progression from the stages of microalbuminuria to proteinuria (>30 µg/min). Their study showed that the relative risk of developing gross proteinuria during four years of observation was 2 to 2.5 fold higher in smokers compared to subjects who had never smoked. The results imply that smoking contributes to renal disease progression and hence merits further investigation.

The combined effects of smoking and hypertension may also exacerbate the rate of deterioration of renal failure in ADPKD. Smoking causes alterations in diurnal blood pressure rhythm, which may subsequently contribute to renal disease progression (Orth *et al.*, 1998). In this study the relationship between smoking and hypertension was investigated. The results revealed a higher frequency of hypertension in smokers compared to non-smokers and it is possible that the combined effect of these factors

may have contributed to the observed faster rate of renal disease progression in smokers.

The effect of smoking on cystogenesis was also examined in view of the mutagenic effects of smoking. Compared to non-smokers, a tendency towards a higher number of cysts and greater cyst size was observed in smokers and in individuals smoking >20 years. Chapman *et al.* (1994) has previously reported the relationship between smoking and cystogenesis. Their study showed that patients with established proteinuria had more severe cystic transformation of the kidney and also a greater pack-year smoking history than did their non-proteinuric counterparts. The relationship between smoking and cystogenesis deserves further investigation.

Other factors are also recognised as possible modifiers of renal disease progression. The effect of alcohol consumption was studied because of the known relationship between heavy alcohol consumption and increasing blood pressure (Perneger *et al.*, 1999). Moreover, alcohol may also exercise a direct toxic effect on the kidney, particularly in the presence of other nephrotoxic exposures (Morgan and Hartley, 1976; Pablo *et al.*, 1986; Perneger *et al.*, 1999). However, the protective effect of light alcohol intake on cardiovascular disease must also be considered.

No other study of ADPKD has examined the influence of alcohol consumption, however, our negative findings for renal failure based on age at onset, rate of progression and the risk of developing renal failure (adjusted and unadjusted) suggest that alcohol does not affect renal failure in ADPKD patients. In addition, we found no relationship between alcohol consumption and hypertension and no effect on cystogenesis. In discussing these findings one must take into account the small numbers, and the fact that this study like others, relied on patient recall and may have produced spurious results.

The effect of lipidaemia was also studied in view of previous findings that have demonstrated the favourable effect of lipid-lowering agents on slower rate of progression to renal failure (Grundy, 1990; Appel, 1991). The results from our study failed to show any negative effect of high triglyceride and cholesterol levels in patients



with ADPKD. In fact, an unexpected finding was the positive effect of cholesterol  $>5.5$  mmol/L on age at renal failure. However, any possible effects of lipidaemia on renal disease progression may have been masked by the younger age of the Polish group, as the true effects of cholesterol and triglycerides may not have yet manifested. Moreover, our study did not evaluate the low density/high density lipoprotein (LDL/HDL) ratio and the apo-lipoprotein profiles, which have previously been reported to be a better predictor of renal disease progression (Samuelsson *et al.*, 1997; Massy *et al.*, 1999). However, our findings are in agreement with a previous study that investigated the relationship between dyslipidaemia and renal disease progression in CRF patients with chronic interstitial nephritis, hypertensive nephrosclerosis, ADPKD and diabetic nephropathy. In that study, no significant role for dyslipidaemia and a faster rate of progression from CRF to ESRF was found, and only a weak association was observed between triglycerides and progression from CRF to ESRF (Massy *et al.*, 1999). Similar to our study, apo-B-containing lipoproteins were not evaluated. Therefore to further investigate the role of lipidaemia in ADPKD disease progression, it is necessary to evaluate the low density/high density lipoprotein (LDL/HDL) ratio and the apo-lipoprotein profiles in a follow-up study.

The relationship between lipidaemia and cystogenesis was also studied. Patients with high cholesterol ( $>5.5$  mmol/L) and triglyceride ( $>1.8$  mmol/L) levels showed a tendency for greater cystic involvement. This finding however, contradicts the better renal survival observed in patients with high cholesterol. Again this finding may be explained by the younger age of the Polish group but warrants further follow-up in our sample. The association between lipidaemia and cystogenesis has been previously reported in Han:SPRD-cy rats with cystic disease (Jayapalan *et al.*, 2000). In that study, an association between high dietary fat and cystogenesis was found suggesting a role for lipidaemia in cystogenesis.

As expected, less physical activity was observed in patients with renal failure (see Table 6.14). This finding is in agreement with another study, which reported that patients on dialysis participate in little or no activity (Johansen, 2000). However, another observation is the fact that patients engaging in vigorous physical activity reach renal failure earlier than those individuals performing low to moderate physical exercise. It

can be speculated from this observation that perhaps vigorous exercise aggravates cysts resulting in subsequent renal damage, therefore patients should perhaps be advised to engage in low-moderate physical activity to find a balance between keeping fit in terms of cardiovascular function and simultaneously protecting their kidneys. The results from this study show that it is difficult to separate cause and effect in examining renal impairment and physical activity. Therefore, a large longitudinal study collecting information on physical activity prior to reaching renal impairment would provide a more reliable and valid assessment of the relationship between physical activity and ADPKD renal disease progression.

Our findings so far suggest a role for smoking as a modifier of ADPKD progression. The effect of smoking as a possible modifier of ADPKD severity may be in keeping with the fact that smoking may initiate the second hit in the normal PKD cell, hence contributing to cystogenesis and modifying the ADPKD phenotype. Smoking may also exert its effect by increasing plasma growth hormone, which is known to enhance basement membrane thickening therefore contributing to cystogenesis (Østerby *et al.*, 1978). The relationship between smoking and hypertension and its overall systemic effects should also be considered, especially in ADPKD whereby hypertension is also an important factor associated with renal disease progression. Due to the small numbers, further study into the investigation of environmental factors is necessary to confirm or refute our findings. Nonetheless, the effect of smoking on the ADPKD phenotype has significant implications in terms of the control, delay and prevention of renal disease progression in ADPKD.

In summary, the findings in this study have important implications on the direction of future ADPKD research. If indeed smoking and lipidaemia modify renal disease progression in ADPKD then it is important that patients modify their lifestyle and behaviours to delay and/or prevent renal disease progression in ADPKD.

# **CHAPTER SEVEN: ASSESSMENT OF THE OVERALL CONTRIBUTION OF GENETIC AND ENVIRONMENTAL FACTORS TO THE ADPKD PHENOTYPE**

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# CHAPTER SEVEN: ASSESSMENT OF THE OVERALL CONTRIBUTION OF GENETIC AND ENVIRONMENTAL FACTORS TO THE ADPKD PHENOTYPE

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## 7.1 INTRODUCTION AND LITERATURE REVIEW

Phenotypic heterogeneity is a term in genetics used to explain variation in disease severity between families and between individuals within the same family, who have inherited the same genetic defect. Such variation suggests complex interactions between genetic and non-genetic factors. Variation in disease severity is observed in most genetic disorders including Neurofibromatosis 1 (NF1) and 2 (NF2) (Szudek *et al.*, 2000), von Hippel-Lindau Disease (Webster *et al.*, 1998), Leri-Weill syndrome (Schiller *et al.*, 2000), Bardet-Biedl syndrome (Riise *et al.*, 1997) among many others and may be explained by many factors as discussed in Chapter One, section 1.7. Phenotypic heterogeneity has important implications for genetic counselling and clinical management. Recognition of heterogeneity within a disease may provide important insights into its pathogenesis (Szudek *et al.*, 2000).

ADPKD is a typical example of a disease that demonstrates phenotypic heterogeneity. Substantial variability exists in terms of most manifestations, and primarily the age of onset and rate of progression to renal failure. While locus heterogeneity, *i.e.* *PKD1* and *PKD2* genes, can explain much of the observed differences between families, other factors are expected to modify the phenotype within families.

In this study, we have examined a small proportion of the potential modifying factors and none of these appear to significantly change or influence the ADPKD phenotype. To determine where future efforts need to be directed, this chapter aims to determine generally the relative contribution of genetics and environment in modifying the disease phenotype. As a novel aspect of this study, we have applied concepts and approaches commonly used in the investigation of 'complex' disorders, to an autosomal dominant disease.

### 7.1.1 *Phenotypic variability*

According to Dipple and McCabe (2000), there are two thresholds relating protein function to phenotype: a level below which the severe phenotype will always be observed, and another level above which the phenotype will be uniformly mild. Between these two thresholds is an indeterminate range, in which mutations do not correlate with the phenotype. Many cases of single gene disorders fall in the indeterminate category, in which there is a considerable range of protein function and a significant number of alleles that will not correlate clearly with clinical phenotype because of the effects of additional, independently inherited, genetic variations and/or environmental influences. Examples of two diseases where mutations do not reliably predict the phenotype include Gaucher disease and sickle cell anaemia. In Gaucher disease the N188S mutation has been associated with a relatively mild disease, which is thought to protect the affected individual against the neuronopathic phenotype (Kim *et al.*, 1996; Choy *et al.*, 1999), however, the same mutation has also been observed in a patient with the more severe type 3 disease (Koprivica *et al.*, 2000). Sickle cell anaemia is a classic example of a simple Mendelian recessive disorder. Yet, individuals carrying identical alleles at the  $\beta$ -globin locus can show markedly different clinical courses, ranging from early childhood mortality to a virtually unrecognised condition at age 50 (Huisman, 1979).

More important is the fact that phenotypic heterogeneity is also demonstrated within families in many disorders. Related individuals who carry the same mutation often express a different phenotype that varies by features such as age at onset and the severity of symptoms. Examples of this include Charcot-Marie-Tooth disease (CMT) and ADPKD. For these simple Mendelian disorders, the phenotypes are, in fact, complex traits.

### 7.1.2 *Phenotypic variability within ADPKD families*

In 1957, Dalgaard reported that the pattern of expression in ADPKD was fairly uniform within families, *i.e.* the age at which the clinical expression of the disease became manifest and the severity of this expression were similar in affected family members. However, this view has since been challenged (Suki, 1982; Gardner, 1988) and many researchers have continued to address the question of intra-familial variation.

Considerable heterogeneity exists with regard to age at ADPKD diagnosis, the development of hypertension, extrarenal manifestations and the rate of progression to renal failure. While some ADPKD patients maintain normal renal function after 65 years of age, others within the same family reach ESRF at a much earlier age (Pochet *et al.*, 1989; Parfrey *et al.*, 1990; Roscoe *et al.*, 1993). This degree of heterogeneity suggests that progression to renal failure in ADPKD probably depends on a complex interplay of genetic and non-genetic factors.

The largest study investigating the extent of intra-familial variation has been conducted by Milutinovic *et al.* (1992). Clinical parameters including age at onset and the type of first symptoms, renal function (SCr concentrations), renal size and age of onset of ESRD were evaluated in 131 patients from 36 unrelated families (Milutinovic *et al.*, 1980) and compared in younger and older affected relatives. Serum creatinine concentrations and kidney size were compared between affected relatives using non-parametric techniques. Pairs were classified as either discordant (defined by less advanced disease in the older affected relative), ties in age (TA) (defined as pairs where both relatives were of similar age but had different clinical findings), ties in clinical findings (TC) (defined by pairs of relatives with similar clinical findings but different ages), double ties (TT) (defined by pairs with similarity in both age and clinical findings), and concordant (defined by more advanced disease in older affected relatives). The total number of pairs examined for SCr, left and right kidney size were 250, 174, and 175 respectively. To examine the magnitude of differences between individuals in respect to SCr levels and kidney size, arbitrary values were selected to classify pairs as discordant or concordant. Analysis of variance was used to incorporate intra-familial information related to age at onset of first symptoms. Non-uniform disease progression was suggested in 38% of affected relatives by SCr and in 53% by kidney sizes. Ages at onset and type of first symptoms were also different in patients from the same families. However, interpretation of the results of this study is difficult because the study did not indicate who formed the pairs, *i.e.* sibs or parent-offspring, or give the number of total pairs and the number of each type of pair.

Of particular interest was the study by Levy *et al.* (1995), who investigated the extent of variation in 32 pairs of twins (20 monozygotic and 12 dizygotic). The difference

between means and the intraclass correlation coefficient ( $r$ ), were used to analyse differences and similarities between members of each twin pair. The parameters investigated included age at discovery of hypertension (defined by a diastolic BP >95mm HG), age at commencement of antihypertensive therapy, age at first dialysis and last plasma creatinine concentrations. Twins were divided into 3 groups based on kidney function: group 1 consisted of both twins with creatinine concentrations <100  $\mu\text{mol/L}$ , group 2 consisted of one or both twins with creatinine concentrations ranging from 100  $\mu\text{mol/L}$  to ESRF, and group 3 comprised both twins in ESRF. Additional information was collected on age at ADPKD diagnosis, history of subarachnoid haemorrhage, myocardial infarction and liver failure, however, these parameters were not analysed in the study. The results for hypertension and renal function are summarised in Table 7.1.

**Table 7.1: A summary of the clinical findings in the study by Levy *et al.*, (1995)**

Twin sets	Age at hypertension		Age at start of antihypertensive therapy		ESRF	
	Mean difference (yr)	$r$	Mean difference (yr)	$r$	Mean difference (yr)	$r$
Monozygotic	5.64 (n=14 pr)	0.57	4.71 (n=14 pr)	0.73	2.1 (n=7 pr)	0.94
Dizygotic	5.2 (n=5 pr)	0.74	5.4 (n=5 pr)	0.74	-	-

*r* – correlation coefficient

The study showed a mean difference of 5 years for the age at development of hypertension and for age at start of antihypertensive therapy for both monozygotic and dizygotic twins. For ESRF, a mean difference of 2 years (range: 1.6-6.0 years) and a high correlation coefficient (0.94) were observed between monozygotic twins. Based on all monozygotic twin pairs and the three renal function groups, the study showed that as renal disease progresses with elevation of creatinine concentration, disease evolution differs between each twin pair and the correlation coefficients become non-significant. Hence, the mean differences between pairs increased significantly from group 1 to group 2 and from group 2 to group 3. The correlation coefficient on all monozygotic twins was 0.50 ( $p = 0.009$ ). Only 1 dizygotic pair in which both patients had reached ESRF were found in the study, therefore, the correlation coefficients calculated on all

dizygotic twins and on groups 1 and 2 were not significant. As a result of these limitations, comparison between monozygotic and dizygotic twins was not achievable.

Another study aimed to evaluate the extent of inter- and intra-familial variation in 38 ADPKD families comprising 300 individuals (Torra *et al.*, 1995). The mean and SD of the mean of age at ESRD in different families was calculated to evaluate inter-familial variation, while the mean of SD of age at ESRD in each family was calculated to evaluate the extent of intra-familial variation. Inter-familial variability based on age of onset of ESRD (SD 9.9 years) was higher than intra-familial variability based on the same variable (SD between 4 and 6.5 years). The number and type of pairs within families was not indicated in this study.

The above results on intrafamilial variation are difficult to interpret because of the small numbers (Levy *et al.*, 1995) and because information on the number and type of pairs of relatives included in the analyses was not provided (Milutinovic *et al.*, 1992; Torra *et al.*, 1995).

### **7.1.3      *Anticipation in ADPKD***

Another parameter, which has been observed in ADPKD families (Dalgaard, 1957; Iglesias *et al.*, 1983; Delaney *et al.*, 1985; Fick *et al.*, 1993, 1994; Torra *et al.*, 1995, 1996), is anticipation. Anticipation is a term used to denote the progressively earlier appearance and increased severity of a disease in successive generations and whose explanation lies in the expansion of unstable triplet repeats from one generation to the next. Examples of genetic disorders due to trinucleotide repeat mutations include Fragile X (Oberle *et al.*, 1991; Yu *et al.*, 1991), Myotonic dystrophy I (Harley *et al.*, 1992; Brook *et al.*, 1992; Aslanidis *et al.*, 1992; Buxton *et al.*, 1992), Huntington's chorea (Huntington's Disease Collaborative Research Group, 1993) to name a few.

Cairns (1925) first suggested anticipation in ADPKD by observing an earlier age at first symptom or at death in affected individuals in the younger generation of seven families (Cairns, 1925). Later, Werner also reported that in 12 of 14 ADPKD families, several members of the second generation died at a significantly younger age than the first (Werner, 1940). The age difference at death was more than 10 years in eight families.



Dalgaard (1957) also observed an earlier age at onset of symptoms or death in the younger generation of 12 families, but dismissed anticipation because no level of significance was achieved. Fick *et al.* (1993) reported on 11 children from 8 ADPKD families who were diagnosed in utero or in their first year of life. A review of the literature revealed 68 children with a diagnosis of ADPKD in utero or in the first year of life. Most of those children were severely affected with greatly enlarged kidneys at birth or on prenatal ultrasound. On follow-up, 34 died within their first year of life. By contrast, 60% of the affected parents did not know of their own disease until the birth of the affected child.

More recently a number of studies have evaluated the presence of anticipation in ADPKD. In these studies, anticipation has been defined as a 10-year earlier onset of ESRD in offspring as compared to the affected parent or as a child diagnosed in the first year of life.

In a review of 86 families (221 parent-offspring pairs) with sufficient data for ESRF, Fick *et al.* (1994) reported anticipation in 42 families (49%) and in 52 (24%) parent-offspring pairs. When including the 8 previously reported families with early onset children with no ESRF (Fick *et al.*, 1993), the total number of families with anticipation in their study was 50 (53% of 94 families).

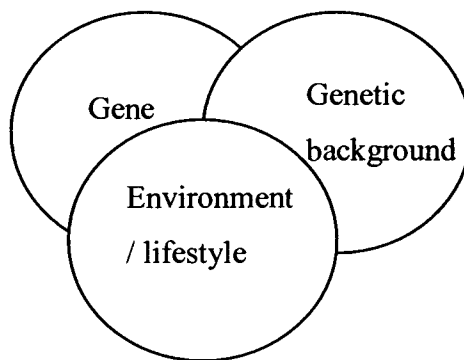
Torra *et al.* (1995) demonstrated anticipation in 45% of 38 families. Of 76 parent-offspring pairs, 44% offspring reached ESRD at least 10 years earlier than the parent. Another 29% offspring reached ESRD 2-9 years earlier than the parent and 28% had ESRD later than the parent. Gonzalo *et al.* (1996) showed that 41% of offspring from 17 parent-offspring pairs had ESRD at least 10 years earlier than the parent. In 41% of parent-offspring pairs, the offspring reached ESRD 2-8 years earlier than the parent and in 17% of the pairs the offspring entered ESRD at a later or equal age to the parent (range from 0 to -5 years).

Geberth *et al.* (1995a) analysed 74 parent-offspring pairs of 148 families from Germany and Austria in whom ADPKD was confirmed by imaging techniques and age at renal death was accurately known. The results from the study showed that the mean

difference in age at renal death between parent and offspring was 0 years, range -26.3 to +27.2 years. Anticipation occurred in approximately 50% of families, however, reverse anticipation occurred in another 50%. There was no deviation from normal (gaussian) distribution according to the Shapiro-Wilk test ( $p = 0.75$ ).

There are only three reports disclosing the molecular defect in cases with anticipation. Peral *et al.* (1996) described a Y3818X mutation in exon 41 in the PKD1 gene in a severely affected child. The same mutation was found in her clinically normal twin brother and in her father who had typical adult onset ADPKD. Torra *et al.* (1997) described a deletion of 28 bp in the PKD1 gene in two members of the same family showing different expression of the disease: a severely affected young man with ADPKD and Caroli's disease diagnosed as a newborn, while his father had typical adult onset ADPKD. Unlike many neurological disorders where anticipation can be explained by the amplification of an unstable DNA repeat in the coding or regulatory region of the relevant genes (Richards and Sutherland, 1992), there are no trinucleotide repeats larger than 5 units in length in the PKD1 gene. Hence, anticipation in ADPKD cannot be explained by a dynamic mutation within the *PKD1* gene.

As discussed in section 7.1, many Mendelian disorders are complicated by the fact that the genotype does not correlate with the phenotype. The wide phenotypic variation involves the interplay between genetic and non-genetic factors and interacting pathways, however, the contribution of each component remains unclear. These disorders can in fact be recognised as 'complex' traits.



## 7.2 COMPLEX GENETIC DISEASES

The distinction between 'simple' monogenic diseases and 'complex' genetic diseases is that the latter do not exhibit classical Mendelian patterns of inheritance and involve the interaction of multiple genes, which interact in complex ways with multiple non-genetic factors (Vogel and Motulsky, 1986). Complex diseases tend to involve greater difficulties in phenotypic definition and are not generally amenable to investigation using techniques that assume monogenic inheritance.

As mentioned in the above sections, many 'simple' Mendelian disorders can be regarded as 'complex diseases' since their pathogenesis involves multiple processes under complex genetic regulation and possible impact of environmental factors.

### 7.2.1 *Measuring Familial Aggregation in complex diseases*

Whether a trait has a genetic basis may be established by demonstrating that it runs in families. Familial occurrence is generally interpreted as evidence for genetic effects. Descriptive genetic epidemiology involves a number of study designs: twin and family-based studies are two of the most common. Twin and family-based studies aim to investigate the degree to which traits aggregate in families, the underlying pattern of inheritance and the number of genes involved in determining a trait (Vogel and Motulsky, 1986).

#### **7.2.1.1 Recurrence risk**

One measure for familial aggregation of a disease is the recurrence risk ratio ( $\lambda_R$ ), which is defined as the recurrence risk for a relative of an affected person divided by the risk of the population. The magnitude of  $\lambda_R$  is related to the degree of concordant inheritance for genetic determinants in affected relative pairs (Lander and Schork, 1994). This concept has been applied in studies of Schizophrenia and insulin dependent diabetes mellitus, among others (Risch, 1990).

#### **7.2.1.2 Segregation analysis**

Segregation analysis aims to identify the genetic mechanisms determining a trait within families. Segregation analysis is useful for estimating the nature of the genetic determinants of a binary or quantitative trait - autosomal/sex linked, recessive/dominant, monogenic/polygenic, or a combination of a single major locus effect plus polygenic effects (the mixed model) (Vogel and Motulsky, 1986).

Segregation analysis involves fitting a general model to the inheritance pattern of a trait in pedigrees. Using a model that assumes the presence of a simple Mendelian factor in a background of multifactorial inheritance, one tries to estimate key parameters such as the allele frequency, penetrance and proportion of cases explained by the Mendelian factor (Vogel and Motulsky, 1986)

Two major problems associated with segregation analysis include sensitivity to biases in the ascertainment of families (for example, preferential inclusion of affected individuals may cause the penetrance to be overstated) and the difficulty of choosing between alternative models (Vogel and Motulsky, 1986). The close overlap of distributions may mean that it is often difficult to reject or accept a restricted model in preference to a more general one (Lander and Schork, 1994).

#### **7.2.1.3 Heritability**

The concept of heritability is widely used in quantitative genetics and is used to estimate the fraction of phenotypic variance contributed by genes. Phenotypic variance can be expressed as:

$$P = G + E$$

where P = phenotypic value, G = genotypic value and E = environmental value.

Heritability ( $h^2$ ) is a statistic parameter that expresses the (additive) genetic contribution to a trait under study. It is usually expressed as a percentage, with a value ranging from 0 to 100%. A low value implies few contributions of additive genes to the trait, whereas a high value suggests a larger contribution. The concept was developed for purposes of selection in plant and animal breeding of economically useful traits such as milk production in cows and egg laying in chickens. The additive component of genetic variability was most important for these purposes. In human genetics, the heritability defined as above is called the narrow sense heritability and can be expressed as:

$$h^2 = \frac{V_A}{V_P}$$

where  $V_A$  = additive component and  $V_P$  = phenotypic variance.

Heritability in the broad sense is defined as the total genetic variability, whether it is additive or not. This is expressed as:

$$h^2 = \frac{V_G}{V_P}$$

where  $V_G$  = genotypic variance and  $V_P$  = phenotypic variance. Broad sense heritability can be estimated from twin studies, while narrow sense heritability can be estimated from both twin and non-twin family studies. There are a number of methods available for estimating the heritability of a quantitative trait (Khoury *et al.*, 1993).

Calculation of heritability using conventional maximum likelihood variance components models assumes an underlying linear model, multivariate normal distribution of the phenotype and that genetic and environmental factors are independent and uncorrelated.

Variance component modelling attempts to partition the phenotype into genetic and non-genetic components. Depending on the available information, the environmental component can be broken down further into common family environment (environment shared by an entire family), shared sibling environment (environment unique to siblings) and the residual variance (arising from non-familial random effects). Similarly, the genetic component can be broken down to the additive genetic effects (the additive effects of genes) and variance due to dominant effects (deviation due to dominance and epistasis). The models can be modified depending on the type of information available and whether or not the narrow or broad sense heritability is estimated.

Heritability values have been estimated for a variety of complex diseases and conditions, including, asthma (Palmer *et al.*, 2001), cardiovascular disease (Knuiman *et al.*, 1996) (Mitchell *et al.*, 1996), left ventricular mass (Post *et al.*, 1997) and IDDM (Kyvik *et al.*, 1995).

### **7.2.2 Study design**

The demonstration that there is a significant genetic predisposition to such conditions depends on different types of samples: twin, family and population-based studies. In the following section, only twin and family based studies will be discussed.

#### **7.2.2.1 Twin studies**

Twin studies and in particular studies of adopted twins have provided opportunities for evaluating the role of genetic and environmental factors in the aetiology of diseases. Monozygotic (MZ) twins are, as a rule, genetically identical. Therefore, differences found between them must be nongenetic. Dizygotic (DZ) twins are no more similar than any normal sibs. Like MZ twins, however, they are usually reared together and therefore the influence of environmental factors may be assumed to be similar in approximation. Genetic factors should therefore result in a higher concordance in MZ than DZ twins. However, higher concordances in MZ compared with DZ twins might also be possible if the character is determined by environmental factors. It is more likely that MZ twins are very similar in appearance and in the way they are treated and hence share more of their environment than DZ twins.

A disadvantage of using twins is that they are an inherently selected population and hence do not represent the general population. To overcome these limitations, adoption studies are an even more powerful way to distinguish between genetic and environmental factors.

#### **7.2.2.2 Family studies**

Families form the basis of most studies aiming to investigate the genetics of human disease. Nuclear families, extended families or interrelated families from inbred populations may all be used. Nuclear and extended families allow the estimation of the mode of inheritance of phenotypes via segregation analysis. Narrow-sense heritability can be estimated from family-based studies, as can some components of the phenotypic variance. Family based studies may therefore allow the nature and degree of genetic influences on a trait to be estimated.

Some considerations need to be taken into account when conducting family studies. These include age and sex differences in the expression of disease and ascertainment bias. Ascertainment should always be clearly described as ascertainment bias may affect the results. What is needed is information on the proportion of affected relatives of specific degree, for example, the sibs of index cases or their children. The diagnosis of index patients must be reliable. Furthermore, it must be indicated whether two or more members are affected and which of them is the index case.

Environmental factors common to families will inflate intra-familial covariance and, if interpreted naively, can inflate epidemiological estimates of the relative importance of genetic factors in determining a trait (Hopper, 1983). It is important to adjust for known environmental exposures in family studies. Several modelling techniques allow for the adjustment of both known and unknown influences common to families.

#### **7.2.3 *Summary of the Literature Review***

The literature review has provided substantial evidence of phenotypic heterogeneity in ADPKD within families. While the role of PKD and RAS genes has been addressed, no study has quantitatively assessed the overall genetic contribution to this variance. Statistical techniques commonly used in the study of complex disease can in fact be

applied to Mendelian disorders expressing wide phenotypic variation. Therefore this study is designed to examine the phenotypic variance in ADPKD by applying quantitative techniques to estimate the resemblance between related individuals and the contribution of genetic factors to the phenotypic variance.

#### **7.2.4        *Aims***

- 1) To compare clinical findings (age at CRF, rate of deterioration of kidney function, age at development of hypertension) between individuals depending on genetic relatedness *i.e.* sib, cousin, parent-offspring and uncle, aunt, niece, and nephew relationships;
- 2) To examine the contribution of genetic effects on the ADPKD phenotype by calculating the correlation coefficient between individuals depending on genetic relatedness;
- 3) To evaluate the contribution of genetic effects to the observed phenotypic variance in ADPKD by estimating the narrow sense heritability, using family sets and multivariate component modelling by likelihood methods.

### **7.3                SUBJECTS AND METHODS**

#### **7.3.1        *Subjects***

The study included 70 ADPKD families from Australia, Bulgaria and Poland, comprising two or more affected individuals. The Australian sample included 64 individuals from 24 families, the Bulgarian sample included 119 individuals from 30 families and the Polish group included 69 individuals from 16 families. A total of 85 sib, 46 cousin and 71 parent-offspring pairs were included. Details on the diagnostic and clinical assessment are provided in Chapter Two.

#### **7.3.2        *Statistical Analysis***

The number of individuals, pairs of family members and number of families included in the intra-familial analysis conducted by Prof Nick De Klerk (Institute for Child Health Research, WA) is shown in Table 7.2



**Table 7.2: Number of Individuals and Pairs Included in the Phenotypic Variation Analysis**

Sets of relatives	Observed age at CRF	Extrapolated age at CRF	Slope of SCr	Age at development of hypertension
<b>Sibs:</b>				
Number of individuals	48	170	-	32
Number of pairs	24	85	-	16
<b>Cousins:</b>				
Number of individuals	16	92	-	8
Number of pairs	8	46	-	4
<b>Parent-offspring:</b>				
Number of individuals	18	142	-	14
Number of pairs	9	71	-	7
<b>Nuclear families*:</b>				
Number of individuals	-	203	200	-
Number of families	-	60	59	-

*\* Familial correlations were estimated using multivariate analysis. ANOVA was used for the analysis of pairs of relative*

**7.4 RESULTS:**

**7.4.1 General Characteristics of the ADPKD Families**

The general characteristics of the patients included in the intra-familial variation analysis are shown in Table 7.3.

**Table 7.3: Characteristics of the 59 ADPKD Families included in the Intra-familial Variation Analysis**

	Country of residence			
	Australia	Bulgaria	Poland	Overall
Number of Families	24	30	16	70
Number of Individuals	64	119	69	252
Male/female	29:35	64:55	36:33	130:122
Number with CRF	23 (36%)	54 (45%)	32 (46%)	109 (43%)
Number with ESRF	14 (22%)	30 (25%)	10 (14%)	54 (21%)

**7.4.2 Phenotypic Variation within ADPKD Families**

Table 7.4 shows examples of 28 ADPKD families demonstrating phenotypic variation within families. The clinical parameters included age at CRF and ESRF, ADPKD diagnosis, presence and age at development of hypertension, age and type of first symptom and presence or absence of liver cysts.

**Table 7.4: 28 ADPKD Families Displaying Phenotypic Variation (Inter- and Intrafamilial)**

Family #	Family ID	Birth year	Sex	Relationship to proband	CRF	CRF age	ESRF	ESRF age	Age at 1st symptom	Type of 1st symptom	Age at PKD diagnosis	Reason for PKD testing	HBP	HBP age	Liver cysts
A3	1	1939	M	Proband	1	unknown	1	42			34	F/hx	1	34	absent
	11	1932	F	Sib	1	47	1	58	37		40	unknown	1	49	present
	13	1954	M	Nephew	0		0		40		18	unknown	1	39	present
	19	1960	F	Niece	0		0				13	unknown	unknown		absent
A4	1	1948	M	Proband	1	38	1	44	29		29	Sx	1	39	absent
	4	1953	F	Sib	0		0				25	F/hx	1	unknown	present
	16	1975	F	Daughter	0		0				14	F/hx	unknown		absent
A15	1	1936	M	Father	1	unknown	1	52	44		44	Sx	1	unknown	present
	3	1957	F	Proband	1	40	0		12		23	F/hx	1	23	present
	35	1929	F	Aunt	1	59	1	64	42		42	Sx	1	41	absent
A25	1	1942	F	Proband	0		0		26	pre-eclamptic toxemia	26	Sx	1	26	absent
	22	1943	F	Cousin	1	55	0		19	back pain	44		1	43	absent
	24	1948	F	Cousin	1	48	0		43	peritonitis	45		1	25	absent
A30	1	1938	M	Proband	1	57	0				53	F/hx	1	unknown	absent
	3	1969	F	Daughter	0		0		17	R loin pain	17		unknown		absent
A53	1	1940	M	Proband	1	unknown	1	43	35	haematuria/ abdominal pain	35	Sx	1	unknown	absent
	4	1949	M	Sib	1	35	1	43			0		0		absent
	7	1943	F	Sib	1	39	1	44	39	back pain	40	Sx	1	unknown	absent
	8	1946	F	Sib							50	PKD study	0		absent
A61	1	1946	M	Proband	1	45	1	Unknown		FFFT's	35	F/hx	1	35	absent
	5	1942	M	Sib	1	unknown	1	Unknown	37	haematuria/ back pain	37	Sx	1	38	absent

Cont'd

Family #	Family ID	Birth year	Sex	Relationship to proband	CRF	CRF age	ESRF	ESRF age	Age at 1 <sup>st</sup> symptom	Type of 1 <sup>st</sup> symptom	Age at PKD diagnosis	Reason for PKD testing	HBP	HBP age	Liver cysts
B7801	1	1951	F	Proband	1	37	1	48	32	HBP, flank pain, pyelonephritis	38	Sx	1	unknown	absent
	2	1969	F	Daughter	0						20	PKD study	0		absent
B7801	3	1972	M	Son	0						17	PKD study	0		absent
	5	1934	M		0				65	unknown	65		0		absent
	8	1953	F	Niece	0						41	PKD study	0		absent
B7802	1	1939	F	Proband	1	44	0		40	HBP	45	Sx	1	unknown	absent
	2	1941	M	Sib	1	46	1	54	20	flank pain/ pyelonephritis	45	Sx	1	45	present
	3	1966	F	Niece	0		0				24		0		absent
	4	1959	M	Son	0		0				28		0		absent
	6	1962	M	Nephew	0		0				26		0		absent
B7803	1	1936	M	Proband	1	46	1	50			0		1	unknown	unknown
	2	1934	M	Sib	1	50	1	61			0		1	unknown	unknown
	3	1956	F	Niece	1	39	0				0		0		unknown
B7804	1	1935	F	Proband	1	44	1	51			0		1	unknown	unknown
	2	1959	F	Daughter	1	38	0				0		1	unknown	unknown
	4	1941	F	Cousin (half)	0		0				0		0		unknown
B7805	1	1931	F	Proband	1	unknown	1	57	50	CRF	56	Sx	1	unknown	absent
	2	1924	M	Sib	1	56	1	66	41	pyelonephritis/ CRF	64	Sx	1	unknown	absent
	4	1961	M	Son	0		0		31	HBP	30	Sx/ PKD study	0		absent
	6	1942	M	Nephew (parent B7805-2)	1	55	0				50	PKD study	1	unknown	absent
	12	1967	M	Nephew (2 <sup>nd</sup> degree)	0		0				26	PKD study	0		absent
B7807	1	1931	M	Proband	1	52	1	55	31	HBP, pyelonephritis, flank pain	52	Sx	1	unknown	present

Cont'd

Family #	Family ID	Birth year	Sex	Relationship to proband	CRF	CRF age	ESRF	ESRF age	Age at 1 <sup>st</sup> symptom	Type of 1 <sup>st</sup> symptom	Age at PKD diagnosis	Reason for PKD testing	HBP	HBP age	Liver cysts
	2	1913	F	Mother	1	unknown	1	77	40	renal colic	75	PKD study	1	unknown	present
	3	1957	F	Daughter	0		0				37		0		absent
	5	1933	M	Cousin	0		0		54	HBP	58	Sx/ PKD study	1	54	absent
	6	1963	F	Cousin (2 <sup>nd</sup> degree)	0		0			asymptomatic	28	PKD study	0		absent
B7808	1	1956	M	Proband	1	33	1	39	20	flank pain	20	Sx	1	unknown	present
	2	1926	F	Aunt	1	57	1	62			0		0		absent
	3	1931	F	Aunt					35	HBP, flank pain	45	Sx	1	35	absent
	4	1937	F	Aunt	1	55	1	62	49	HBP, flank pain	39	Sx	1	unknown	absent
	5	1939	M	Uncle	1	unknown	1	50	44	HBP, flank pain	50	PKD study	1	44	absent
	6	1926	M	Mother	1	72 (died)	0		60	HBP	61	Sx/ PKD study	0		absent
	7	1950	M	Sib	0		0		35	HBP	37	Sx	1	35	absent
	8	1964	M	Cousin	0		0				31	PKD study	0		absent
	13	1977	M	Son	0		0				17	PKD study	0		absent
	22	1950	M	Cousin	0		0				43		0		absent
B7852	1	1936	F	Proband	1	54	0		52	HBP, flank pain, pyelonephritis	52	Sx	1	unknown	present
	2	1957	F	Daughter	0		0		35	pyelonephritis	32		0		absent
	13	1933	M	Sib	1	49	1	61	55	HBP, flank pain, pyelonephritis	55	Sx	1	unknown	absent
	15	1932	F	Sib	1	65	0		48	flank pain	48	Sx/ PKD study	1	unknown	present
	17	1967	F	Daughter	0		0				15	PKD study	0		absent
	23	1960	M	Nephew	0		0				20		0		absent
	26	1939	F	Sib	0		0				41	PKD study	1	unknown	absent
B7858	1	1953	M	Proband	1	33	1	39			0		1	unknown	Absent
	4	1972	M	Son	0		0				0		0		absent
	5	1974	M	Son	0		0				0		0		absent
	6	1973	M	Son	1	27	0				0		0		absent

Cont'd

Family #	Family ID	Birth year	Sex	Relationship to proband	CRF	CRF age	ESRF	ESRF age	Age at 1 <sup>st</sup> symptom	Type of 1 <sup>st</sup> symptom	Age at PKD diagnosis	Reason for PKD testing	HBP	HBP age	Liver cysts
	7	1980	M	Son	0		0				0		0		absent
B7861	1	1958	F	Proband	1	33	0		33	HBP, flank pain, CRF	33	Sx	1	unknown	absent
	2	1980	F	Daughter	0		0				12	PKD study	0		absent
	3	1979	M	Son	0		0				13	PKD study	0		absent
	4	1956	F	Sib	1	34	0		24	HBP	32	PKD study	1	unknown	absent
	5	1975	M	Nephew	0		0				-9		0		absent
B7862	1	1949	F	Proband	1	41	1	47			0		1	unknown	unknown
	2	1972	F	Daughter	0		0				0		1	unknown	unknown
	4	1968	M	Cousin (2 <sup>nd</sup> degree)	1	27	0				0		1	unknown	unknown
	5	1940	F	Cousin	1	45	1	52			0		1	unknown	unknown
	8	1970	M	Nephew	0		0				0		0		unknown
	3	1969	F	Daughter	0		0				-9		0		unknown
	4	1967	M	Son	0		0				-9		0		unknown
	5	1970	M	Other (son of 73.7)	1	26	0				-9		0		unknown
	7	1942	F	Niece	1	unknown	1	54			0		1	unknown	unknown
	8	1931	M	Sib	1	55	1				0		1	unknown	unknown
	10	1955	M	Nephew	0		0				0		0		unknown
P101	1	1949	F	Proband	1	35	1	45	21	back pain	21	Sx	1	30	present
	2	1970	M	Son	0		0		23	back pain/ haematuria	16	F/hx	1	19	absent
	3	1922	M	Father	1	65	1	72	60	HBP	70	Sx/ F/hx	1	60	present
	6	1959	F	Sib	0		0		32	HBP	32	Sx/ F/hx	1	32	absent
P102	1	1926	M	Proband	1	55	1	61	45	HBP, back/loin pain, haematuria	45	Sx	1	34	present
	2	1956	M	Son	1	38	0		35	HBP	0	F/hx	1	35	absent
	3	1960	F	Niece	1	40	0		26	back/loin pain	28	Sx/ F/hx	1	30	present

Cont'd

Family #	Family ID	Birth year	Sex	Relationship to proband	CRF	CRF age	ESRF	ESRF age	Age at 1 <sup>st</sup> symptom	Type of 1 <sup>st</sup> symptom	Age at PKD diagnosis	Reason for PKD testing	HBP	HBP age	Liver cysts
	6	1962	F	Niece	0		0		26	Back/loin pain	30	Sx/ F/hx	1	28	present
P103	1	1947	M	Proband	1	47	1	53	40		40		1	40	absent
	4	1943	F	Sib	0		0		26		28		1	26	absent
	55	1940	F	Sib	0		0		50		54		1	50	absent
	63	1960	F	Niece	0		0		20		32		1	32	present
	64	1987	F	great niece	0		0				6		0		absent
P106	1	1950	M	Sib	1	44	0		41	back/loin pain	37	F/hx	1	40	present
	2	1949	M	Proband	1	51	0		37	HBP	39	Sx/ F/hx	1	37	absent
	4	1977	M	Son	0		0			asymptomatic	21	F/hx	0		absent
P106	54	1984	M	Son	0		0			asymptomatic	14		0		absent
P107	1	1940	M	Proband	1	37	1	46	30	HBP	41	Sx	1	30	absent
	2	1942	M	Cousin	1	42	1	51	32	UTI, renal colic	36	Sx	1	36	Present
	4	1969	F	2nd cousin (parent P107-2)	1	26	0		21	UTI	21	Sx/ F/hx	1	27	absent
P108	1	1960	M	Proband	1	27	1	31	29	UTI	29	F/hx	0		absent
	2	1951	M	Cousin	1	38	0		37	back pain	38	Sx	1	37	absent
	3	1961	M	Sib	0		0		31	HBP	30	F/hx	1	31	present
	4	1974	M	Sib	0		0			asymptomatic	19	F/hx	0	0	absent
	5	1949	M	Cousin	1	45	0		40	HBP	40	F/hx	1	40	absent
P110	1	1937	F	Proband	1	59	0		42	HBP	42	Sx	1	42	present
	2	1926	F	Sib	1	73	0		unknown	UTI	68	Sx	1	60	present
	7	1952	M	Nephew	0		0		30	back/loin pain	43	F/hx	0		absent

Con'd

Family #	Family ID	Birth year	Sex	Relationship to proband	CRF	CRF age	ESRF	ESRF age	Age at 1 <sup>st</sup> symptom	Type of 1 <sup>st</sup> symptom	Age at PKD diagnosis	Reason for PKD testing	HBP	HBP age	Liver cysts
P111	1	1938	F	Proband	1	44	1	52					0		present
	2	1940	F	Sib	1	56	0						1	unknown	absent
	3	1962	M	Son	1	30	0						1	unknown	absent
P113	1	1945	F	Proband	1	49	0						1	unknown	present
	2	1970	F	Daughter	0		0				0		0		absent
	5	1969	F	Niece	0		0				0		1	unknown	present
	6	1975	M	Nephew	0	0	0				0		1	unknown	absent
	7	1952	M	Sib	1	48	0						1	unknown	present
	8	1955	M	Sib	1	37	0						1	unknown	absent
	9	1957	F	Sib	0		0						0		absent
P114	1	1913	F	Proband	1	80	0				0		0		absent
	3	1951	F	Niece	0		0				0		0		present
	4	1956	F	Niece	1	39	0				0		0		present
	5	1957	M	Nephew	1	42	0				0		0		absent
	6	1968	M	Nephew	1	27	0				0		0		absent
	7	1954	M	Nephew	1	42	0				0		0		absent

HBP – high blood pressure; CRF – chronic renal failure; ESRF – end-stage renal failure; Sx – symptoms; F/hx – family history; UTI – urinary tract infection; FFFT'S – faints, fits and funny turns; 0 = no, 1 = yes, blank lines indicate no available information.

\* Highlighted areas indicate areas where phenotype variation is observed within a family.



### 7.4.3 Anticipation

The presence of anticipation, defined as a 10-year earlier onset of CRF in the offspring of any parent-offspring pair was examined to further investigate the clinical course of ADPKD within families. The analysis included 71 parent-offspring pairs with extrapolated age at CRF. Anticipation was observed in 58 (82%) parent-offspring pairs with a difference in age ranging between 10 and 33 years. The difference in extrapolated age at CRF between parents and offspring are shown in Table 7.5.

**Table 7.5: Evidence of Anticipation in 71 Parent-offspring Pairs**

Family	Difference in age at CRF	Evidence for anticipation	Family	Difference in age at CRF	Evidence for anticipation
A3	5	0	B52	26	1
A3	11	1	B55	14	1
A4	24	1	B57	0	0
A5	16	1	B58	9	0
A5	27	1	B58	10	1
A6	22	1	B58	10	1
A9	22	1	B58	16	1
A10	14	1	B61	21	1
A15	3	0	B61	21	1
A55	27	1	B61	16	1
A55	28	1	B73	22	1
B1	7	0	B73	20	1
B1	10	1	B73	15	1
B1	13	1	B74	28	1
B2	9	0	B74	30	1
B2	16	1	B74	17	1
B3	11	1	B75	22	1
B4	6	0	B75	28	1
B5	4	0	B77	21	1
B5	25	1	B77	24	1
B7	18	1	B77	19	1
B7	12	1	B77	21	1
B7	29	1	B78	5	0
B8	11	1	B78	3	0
B8	1	0	P101	33	1
B10	8	0	P101	6	0
B15	18	1	P102	19	1
B19	10	1	P107	16	1
B19	32	1	P109	22	1
B19	22	1	P110	26	1
B22	12	1	P111	13	1
B22	25	1	P113	24	1
B23	33	1	P117	23	1
B26	31	1	P117	28	1
B29	31	1	P118	26	1
B52	16	1			

*1 - evidence for anticipation; 0 - no evidence for anticipation*

#### **7.4.4            *Familial Relationships***

##### **7.4.4.1            Sib Versus Cousin Pairs**

One-way ANOVA was performed to compare the variance in age at observed and extrapolated age at CRF between sib and cousin pairs. For observed age at CRF the analysis included 24 sib and 8 cousin pairs and for extrapolated age at CRF the analysis included 85 sib and 46 cousin pairs. No significant difference in observed (8.6 and 7.8 years,  $p = 0.69$ ) or extrapolated (6.7 and 8.2 years,  $p = 0.23$ ) age to CRF was found between sib and cousin pairs. Results are shown in Table 7.6.

Subsequent analysis compared the variance in age at development of hypertension. A total of 16 sib pairs and 4 cousin pairs were included in the analysis. No significant difference in the variance in age at development of hypertension was found between sib and cousin pairs (8.8 and 5.3 years,  $p = 0.09$ ). Results shown in Table 7.6

##### **7.4.4.2            Parent-Offspring Pairs Versus Sib Pairs**

One-way ANOVA was performed to compare the variance in age at observed and extrapolated age at CRF between sib and parent-offspring pairs. For the difference in observed age at CRF the analysis included 24 sib pairs and 9 parent-offspring pairs. For extrapolated age at CRF the analysis included 85 sib and 71 parent-offspring pairs. A significant difference in observed (8.2 and 15.8 years,  $p = 0.02$ ) as well as extrapolated (6.8 and 14.7 years,  $p < 0.01$ ) age was found between sib and parent-offspring pairs (Table 7.6).

The analysis was repeated on 16 sib and 7 parent-offspring pairs to compare the difference in age at development of hypertension. The results are shown in Table 7.6. Although not significant, greater variation in the age at development of hypertension was also observed in parent-offspring pairs as compared to sib pairs.

**Table 7.6: Differences in Age at CRF (Observed and Extrapolated) and Development of Hypertension between Relatives**

Sets of relatives	Observed age at CRF			Extrapolated age at CRF			Age at development of hypertension		
	Mean difference	95% CI	Min-Max	Mean difference	95% CI	Min-Max	Mean difference	95% CI	Min-Max
<b>Sibs Vs Cousins</b>	8.6±1.6 (n=24)	[5.3,11.9]	0-15	6.7±1.5 (n=85)	[3.7,9.7]	0-28	8.8±0.9 (n=32)	[6.7,10.6]	0-18
	7.8±1.6 (n=8)	[4.5,11.1]	2-18	8.2±1.7 (n=46)	[4.9,11.6]	0-27	5.3±1.1 (n=8)	[2.8,7.8]	0-9
<b>P value</b>	<b>0.69</b>			<b>0.23</b>			<b>0.09</b>		
<b>Sibs vs Par-off</b>	8.2±2.6 (n=24)	[2.7,13.6]	0-15	6.8±1.7 (n=85)	[3.4,10.2]	0-28	8.4±0.9 (n=16)	[6.4,10.3]	0-19
	15.8±2.6 (n=9)	[10.6,21.2]	1-30	14.7±1.9 (n=71)	[11.0,18.4]	0-33	11.6±2.4 (n=7)	[6.4,16.8]	1-30
<b>P value</b>	<b>0.02</b>			<b>&lt;0.01</b>			<b>0.14</b>		

## 7.4.5 *Familial Correlations Using Multivariate Component Modelling*

### 7.4.5.1 Familial correlations

Familial correlations in regard to the age at CRF and slope of decline of kidney function were calculated for sibs, parent-offspring, cousins and uncle/aunt-niece/nephew relationships. The analysis included 60 families (203 individuals) for age at CRF and 59 (200 individuals) for the rate of progression to CRF.

The estimated familial correlations in the analysis for age at CRF and the rate of decline in renal function are shown in Table 7.7. The correlation coefficient was significantly higher between sibs than parent-offspring, cousins and uncle/aunt-niece/nephew relationships for age at CRF and the slope of decline in renal function. Cousins also showed a higher correlation for age at CRF than parent-offspring and uncle/aunt-niece/nephew however, the correlation for the slope of decline in renal function was only slightly higher than parent-offspring and uncle/aunt-niece/nephew relationships.

**Table 7.7: Familial Correlations (95% CI) for development of renal failure**

Familial relationship	Degree of relatedness	Correlation coefficients, 95% CI and p-values	
		Age at CRF	Slope of decline in renal function
Siblings	1/2	0.63 [0.48,0.79] <0.001	0.20 [0.10,0.31] <0.001
Parent-offspring pairs	1/2	0.30 [0.05,0.56] 0.02	0.07 [-0.04,0.18] 0.22
Cousin pairs	1/8	0.39 [-0.09,0.85] 0.12	0.08 [-0.10,0.26] 0.41
Uncle/aunt-niece/nephew	1/4	0.03 [-0.29,0.34] 0.87	0.06 [-0.08,0.20] 0.39

### 7.4.5.2 Heritability estimates

The narrow sense heritability, expressed as the phenotypic variance due to the additive effect of genes *i.e.* in addition to the PKD genes, was estimated as 5% for age at CRF and 0% for the slope of decline in renal function. This was based on the correlations on all relationships (Table 7.7).

## 7.5

## DISCUSSION

ADPKD exhibits a complex phenotype, which suggests the involvement of genetic and environmental factors. The factors that affect ADPKD disease progression, however, remain unclear. In the previous chapters, a small proportion of plausible factors that may modify the ADPKD phenotype were examined. The effect of inter-population differences, locus heterogeneity and modifying genes within the RAS were excluded as major variables influencing renal disease progression. Results on the role of smoking and lipidaemia as possible modifiers of ADPKD severity were suggestive but inconclusive. Previous studies examining phenotypic variation in ADPKD also remain inconclusive. To determine where future research should be directed to the study of modifying factors in ADPKD, this part of the study has applied concepts and methods traditionally used in complex diseases to an autosomal dominant condition, which can in fact be regarded as a complex trait at the organism level.

The design of this study was twofold. The first part aimed to extend on previous research and to examine the extent of phenotypic variation by comparing clinical findings between affected relatives and investigate the presence of anticipation. The second part aimed to analyse the phenotypic variance using multivariate component modelling, to estimate the correlation coefficient between relatives, and the 'narrow' sense heritability for age at CRF and the slope of decline of kidney function. As discussed in section 7.2.1.3 and in Chapter Two, section 2.7.6, multivariate component modelling partitions the phenotype into genetic and non-genetic components, which allows the genetic contribution to a trait to be estimated. The study included 60 nuclear and extended families residing in three different countries: Australia, Bulgaria and Poland. An ideal way to examine complex traits is to compare the resemblance of a trait between affected relatives. Family investigations are the most straightforward and most unsophisticated approach for assessing a genetic contribution to the trait. Sibs and parent-offspring share 50% of their genes, while more remote relatives share only a small fraction of their genes. A comparison of relatives using appropriate quantitative techniques is therefore likely to show genetic factors underlying that trait (Vogel and Motulsky, 1986).

In a growing effort to make sense and understand more about the intra-familial variation in ADPKD, another approach used by researchers has been to examine for the presence of anticipation in ADPKD (defined as a 10-year earlier development of age at onset of ESRF in offspring compared to parents). The reason for this is most likely the availability of affected parents and offspring. Other studies also investigating evidence of anticipation have focussed on very early onset ADPKD in offspring and have also examined this concept at the molecular level (Torra *et al.*, 1997; Perichot *et al.*, 2001). Very few studies have extended the analysis by including sibs (Levy *et al.*, 1995) and other relatives (Milutinovic *et al.*, 1992). Given the small number of patients with ESRF and again the flaws in the study design, the results of these studies remain inconclusive.

The development of renal failure is the most important renal outcome in ADPKD. Moreover, it is the most reliable indicator and measure of renal function. Whereas other studies have used ESRF as the primary renal outcome, we used age at development of CRF (observed and extrapolated values) to increase the sample size. As demonstrated throughout this thesis, the use of observed and extrapolated values has produced similar results, although extrapolated values have resulted in an overall shift of approximately 7 years earlier for age at CRF. Since the shift appears uniform across the sample, extrapolated values are a useful way of increasing sample size in different comparisons. In addition, we also compared the age at development of hypertension between related individuals because of the recognised relationship between hypertension and renal disease progression (Bell *et al.*, 1988).

Evidence for intra-familial variation and anticipation was obtained in this study (see Table 7.4 and 7.5). With respect to the observed variation between parent and offspring, this study also showed strong evidence of anticipation (82% of parent-offspring pairs) for age at onset of CRF using extrapolated values in a relatively large sample comprising 71 parent-offspring pairs. The frequency of anticipation is higher than shown by Fick *et al.* (1994) and Torra *et al.* (1995) who observed anticipation for age at ESRF in 24% and 44% of parent-offspring pairs. The age at development of CRF, using observed and extrapolated values was found to be more similar between sibs and cousins than between parent and offspring. The mean difference in age at development of CRF was essentially the same for cousins as for sibs, whereas decreasing similarity

would be expected if genetic factors played a major role in progression to renal failure. The findings imply the contribution of environmental factors since sibs and parent-offspring pairs are genetically more similar than cousins and share a higher proportion of possible modifying genes. An earlier study (Milutinovic *et al.*, 1992) also showed evidence of intra-familial variation in 38% of affected relatives by serum creatinine concentrations and in 53% by kidney sizes. The results from that study are difficult to interpret because it failed to indicate the type of pairs *i.e.* sibs, parent-offspring etc., and the number of each of these pairs. Reports in the literature suggest that unless larger samples and more methodological information are provided, namely the types of samples used *i.e.* sibs, cousins, parent-offspring, uncle/aunt-niece/nephew relationships, the results on such studies remain inconclusive. To investigate further the genetic contribution to the observed phenotypic variance, multivariate component modelling was performed in this study.

Multivariate analysis was performed in 60 ADPKD families comprising sibs, cousins, parent-offspring and uncle/aunt-niece/nephew relationships and the correlation coefficient and narrow sense heritability were estimated. Two parameters were examined. The first parameter was age at CRF, which was based on extrapolated SCr values to increase the sample size. The second parameter was the slope of decline of kidney function, which was calculated in individuals with >2 SCr values and included patients with normal renal function and those with renal impairment.

The analysis, based on sibs, parent-offspring, cousins and uncle/aunt-niece/nephew relationships, showed a significantly higher correlation coefficient between sibs than parent-offspring pairs for age at CRF (0.63[0.48,0.79]; 0.30[0.05,0.56]), and the slope of decline in renal function (0.20[0.10,0.31]; 0.07[-0.04,0.18]). Cousins were also found to have a higher correlation than parent-offspring and uncle/aunt-niece/nephew relationships in regard to the age at CRF (0.39[-0.09,0.85]), however the correlation for the slope of decline of kidney function was only slightly higher than parent-offspring and uncle/aunt-niece/nephew. Nonetheless, the results from ANOVA, the high proportion of families demonstrating anticipation and the correlation coefficient estimates, provide valuable support for the calculated heritability, which was estimated to be <5% for age at CRF and 0% for the slope of SCr.

The results from our study indicate that environmental factors including shared and non-shared factors contribute to the observed phenotypic variation in ADPKD. The higher correlation between sibs and cousins than parent-offspring, particularly for age at CRF, provides strong support for shared environmental factors. It is likely that sibs and cousins are of similar age and hence may, at some point in time, have shared a common environment. Evidence for non-shared environmental factors is provided by the variation and the low correlation coefficient estimates between sibs, cousins and parent-offspring. The non-shared factors may include factors in the work place.

Again, our results cannot be directly compared to earlier studies because of differences in samples and study design. However, Levy *et al.* (1995) also aimed to determine the genetic contribution to the phenotypic variance in ADPKD by estimating the correlation coefficient for age at ESRF between MZ and DZ twins. The study showed a higher correlation coefficient (0.94) for ESRF in MZ but this was insignificant for DZ twins and therefore comparisons between the two twin groups were not achievable.

This study is the first to quantitatively estimate the genetic contribution to the phenotypic variance in ADPKD. The heritability estimates support the earlier findings of this study, which excluded the effect of modifying genes, while the results on smoking and lipidaemia imply that these factors may contribute to the phenotypic variance.

The inclusion of close and more distantly related relatives has increased the power of the study to determine the contribution of genetic and environmental factors. Moreover, multivariate component analysis is a powerful statistical technique, which is widely used in the study of complex diseases to determine the genetic contribution of the disease by dissecting the phenotype into genetic and environmental components. The analysis reduces the variance by adjusting for within and between group variance, resulting in more accurate estimates of the correlation coefficient. However, this analysis does have limitations. Heritability is population specific and the differences in genetic and environmental factors common to a specific population may influence the heritability estimates. Such an effect is unlikely in this study because we have previously shown that renal disease progression is not significantly influenced by inter-



population differences. In addition, the analysis was adjusted for country. Ascertainment bias is another variable, which can confound the results of many studies, however, the analysis was also adjusted for this by conditioning the proband from each family. Furthermore, the analysis adjusted for the effects of sex, age, ACE I/D polymorphism and smoking. A limitation, similar to other studies, is obviously the small samples, especially when studying adult onset disorders. Because not all patients can be expected to reach ESRF, we used age at CRF as our primary renal outcome and the slope of decline in renal function, which enabled us to enlarge our sample size. It is expected that similar results would be estimated for age at onset of ESRF since ESRF is the final outcome of CRF.

Due to the many limitations involved in studying humans, mouse models have become an increasingly powerful tool to further assess the genetic contribution of a disease phenotype, as the genetic interactions can be probed more easily than it is possible in a randomly mating human population (Guay-Woodford *et al.*, 2000). In the case of ADPKD, numerous mouse models have been described in which the mutant phenotypes closely resemble human PKD with regard to morphology, cyst localisation, and disease progression (Schieren *et al.*, 1996). Using experimental crosses, the effects of genetic background on disease phenotype is suggested by the reports of increased disease severity when either *cpk* or *pcy* mutations were introduced into the DBA/2J genetic background (Fry *et al.*, 1985; Takahashi *et al.*, 1991). Such modifying effects can be dissected into discrete genetic factors referred to as quantitative trait loci (QTL) (Lander and Botstein, 1989), which are used to provide an estimate of the total trait variance explained by the QTL. These tools can be used to analyse genotypic and phenotypic information for specific families, populations, or crosses in order to localise the regions of chromosomes whose inheritance appears to correlate with quantitative variation. This approach has been especially well suited for genetic studies in the mouse, since there exist well diverged, genetically inbred laboratory strains that exhibit differences in quantitative phenotypes that are heritable and reproducible. Recently, QTL have been mapped to chromosome (Chr) 1 and Chr 10 for juvenile cystic kidney (*jck*) (Iakoubova, Dushkin, Beier, 1995), Chr 1 and Chr 19 for *Kat<sup>2J</sup>* (Upadhyia *et al.*, 1999), Chr 4 and Chr 16 for *pcy* (Woo *et al.*, 1997), and Chr 4 for *cpk* (Woo *et al.*, 1995 [Abstract]).

However, while the identification of QTL's in mouse crosses is technically feasible, there are some problems. Firstly, the non-genetic variation in these traits, as well as the logistic limits on the number of animals studied, usually results in the localisation of a QTL with only modest resolution. Secondly, the positional cloning strategies for mutations that have unambiguously scored phenotypes may not be readily applicable for the cloning of a modifying locus. Because the QTL is a continuum, one cannot assume with any degree of certainty that a putative recombinant is a member of a severely or mildly affected class, and not an outlier from the other class. Hence, the ability to localise the modifying trait to a small interval for positional cloning is greatly limited. In addition, quantitative traits are often polygenic in origin and may exhibit both complex interactions with environmental factors, as well as unexpected inter-genic effects as a consequence of epistasis. However, the importance of characterising the modifying loci should not be underestimated since these genes may provide useful targets for therapeutic intervention that could significantly ameliorate disease related morbidity in ADPKD.

Nonetheless, as a continuum of this part of the study, hypertension was also investigated because of its known association with renal disease progression (Bell *et al.*, 1988). Therefore the study aimed to compare the mean difference in age at development of hypertension between sib and cousin pairs and between sib and parent-offspring pairs. The results from the analysis showed intra-familial variation in relation to age at development of hypertension between sib and cousin pairs and between sib and parent-offspring pairs, although greater variation in age at development of hypertension was found between parent-offspring pairs. This parameter was not investigated further using multivariate analysis because of the small numbers and secondly because the age at diagnosis of hypertension is confounded by several variables including diagnostic procedures and definition of hypertension among many others.

Levy *et al.* (1995) also compared the age at development of hypertension and age at start of antihypertensive therapy between MZ and DZ twins by estimating the correlation coefficients. With respect to these two parameters, a higher correlation coefficient (0.74) was found for dizygotic twins than monozygotic twins (0.73 and 0.57 respectively). The results provide evidence for the influence of environmental factors,

since dizygotic twins are genetically less similar than monozygotic twins. However, in interpreting the results from this study, one has to again keep in mind that the diagnosis of hypertension and the age at start of antihypertensive therapy are influenced by many variables. Hence the results on hypertension remain inconclusive.

The strategies used in determining the contribution of modifying genes in humans and in mouse models offer important tools and provide important information to ADPKD research. As a result, using multivariate component analysis, this study has provided substantial evidence that it is environmental factors rather than modifying genes that contribute to the observed phenotypic variance in ADPKD. Efforts to identify genetic factors, such as variants in the RAS genes that modify the ADPKD phenotype thus appear to hold little promise. Hence, the results from this study suggest that the next stage in ADPKD research should be re-directed into identifying the specific environmental factors and investigate how these factors interact with the *PKD* genes or other pathways, which ultimately modify the phenotype.

## CHAPTER EIGHT: FINAL DISCUSSION

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### 8.1 DISCUSSION

ADPKD is one of the most common genetic disorders world-wide, with a frequency of approximately 1 in 1000 individuals (Dalgaard, 1957). ADPKD results from mutations in at least two causal genes, *PKD1* and *PKD2* (European PKD Consortium, 1994; Mochizuki, *et al.*, 1996). The identical phenotype in human patients and targeted *Pkd1* and *Pkd2* mutant mouse models provides evidence that both gene products act in the same pathway. The discovery that *PKD1* and *PKD2* do indeed interact in a common pathway suggests that the gene products of *PKD1* and *PKD2* play a functional role in the same molecular complex (Mochizuki *et al.*, 1996; Qian *et al.*, 1997; Tsiokas *et al.*, 1997). Despite its autosomal dominant mode of inheritance, ADPKD is recognised as a systemic disorder (Gabow *et al.*, 1993) and is characterised by extensive phenotypic variation between families and more importantly between individuals within the same family who carry the same germline mutation. While locus and allelic heterogeneity can explain (at least in part) differences between families, additional factors are expected to contribute to the observed variation within families.

Many studies have aimed to investigate factors that may modify ADPKD disease severity. These factors include locus and allelic heterogeneity, trans-heterozygous and somatic mutations, modifying genes, genomic imprinting, environmental factors and other factors including sex, hypertension, UTI and pregnancy (Gabow *et al.*, 1992; Peters and Breuning, 2001). However, the variables involved in modifying the ADPKD phenotype remain unclear because of the difficulties in performing genotype-phenotype correlations due to the high number of private mutations (*i.e.* confined to individual families), small sample numbers, differences in study design and ascertainment.

To address some of these inconsistencies, the aim of this PhD thesis was to investigate some of the major plausible variables on a large sample and examine a number of clinical parameters. These included 1) the rate of progression of renal impairment assessed by age at development of renal failure (CRF and ESRF) and cumulative renal survival analysis; 2) extent of development of renal cysts including the length of the

kidneys and number and size of cysts; 3) development of hypertension and 4) left ventricular mass as measured by echocardiography. The effect of inter-population differences, locus heterogeneity, sex, genetic variants within the renin-angiotensin system (RAS) and recognised environmental risk factors (including smoking, alcohol consumption, diet and physical activity patterns) were investigated.

The design of this study included 322 patients residing in three countries, namely Australia, Bulgaria and Poland, which are known to differ in terms of biological (locus heterogeneity and hypertension) and environmental (diet and lifestyle, socioeconomic status) variables. It was expected that these differences could in part explain some of the observed phenotypic variation between families. Therefore, the first component of this study aimed to examine the effect of inter-population differences on the ADPKD phenotype. The following clinical parameters were examined: age at onset and rate of progression to renal failure (CRF and ESRF), age at development and prevalence of hypertension, length of the kidneys and cyst size and number. Differences in terms of the prevalence of hypertension and ultrasound findings were found between the three countries. As expected, a higher frequency of hypertension (88%) was observed in the Bulgarians but not in the Poles (61%). The lower frequency in the Poles may be attributed to the younger age and the lower number of patients with ESRF, given that hypertension and cardiovascular disease is epidemic in Eastern Europe (Global Cardiovascular Infobase: <http://cvdinfobase.ic.gc.ca/>). On the other hand a reported frequency of 73% was found in the Australian sample and may be indicative of the age and hence the high number of patients with ESRF. In terms of ultrasound findings, the largest kidneys were found in the Poles, a higher number of cysts were observed in the Poles and Bulgarians, whereas the diameter of the largest cyst was greater in the Poles and the Australians. Because of the recognized relationship between hypertension, renal volume, cyst number and size and renal disease progression, differences observed between the three countries were expected to influence renal disease progression. However, such differences may exist, as suggested by the high proportion of CRF in the Polish group (despite its younger age), and the severe course of the disease in the Bulgarian patients (despite the substantial number of PKD2). The results suggest that environmental, health care and other differences may exert effects predominantly in the preclinical stage and that once a specific point of kidney impairment is reached, then

progression is irreversible. Further studies of inter-population differences appear justified, especially if designed to compare patients from wealthy industrialised countries to those in poorer societies. Our study failed to detect statistically significant differences, implying that, in our patient sample the observed phenotypic variance in ADPKD is less between, than within populations. Consequently, the three countries were pooled together and treated as a homogenous group, in the study of additional modifying factors of ADPKD severity.

The effect of genetic factors was addressed by examining locus heterogeneity, *i.e.* *PKD1* versus *PKD2* and modifying genes within the renin-angiotensin system (RAS). The majority of studies have reported that patients linked to *PKD2* express a milder phenotype in terms of later age at onset and rate of progression to renal failure, later age at onset and frequency of hypertension and later and slower development of cysts compared to *PKD1* linked individuals (Parfrey *et al.*, 1990; Ravine *et al.*, 1992; Wright *et al.*, 1993; Coto *et al.*, 1995; Torra *et al.*, 1996). The age at onset and rate of progression to renal failure varies widely between studies preventing the direct use of the published information in subsequent research. The results from this study are in agreement with an earlier study (Bogdanova *et al.*, 1995) that showed no significant differences between *PKD1* and *PKD2* individuals with regard to age at onset and rate of progression to renal failure and in the frequency of hypertension. The results suggest that the differences in clinical severity between the two genetic forms may not be as apparent as previously reported and that the prognosis in *PKD2* families should therefore be individualised and discussed carefully with the patient and family.

The effect of modifying genes was examined in view of the observed intra-familial variation. Genetic variants within the RAS were studied for a number of reasons. Firstly, early activation of the RAS has been demonstrated in both experimental and clinical studies of ADPKD (Chapman *et al.*, 1990). Secondly, because of the recognised relationship between hypertension and renal disease progression, and the known involvement of the RAS in blood pressure regulation (Bell *et al.*, 1988). Thirdly, angiotensin II, the end-product of the RAS, has growth-promoting effects that could contribute to cystogenesis in ADPKD (Norman *et al.*, 1987). This study has provided substantial evidence that genetic variants in the RAS do not influence renal disease

progression. In terms of the *ACE* gene, which has been studied extensively, we examined both the I/D polymorphism and the biological variable namely enzyme activity in the largest sample to date and showed no influence of the ACE on renal disease progression. Conversely, the association between the ACE I/D polymorphism and an increase in left ventricular mass merits further investigation. This study also examined the association between alleles of microsatellite polymorphisms in the renin and angiotensinogen (AGT) genes and renal disease progression. The study excluded a role for renin, although an association between allele 9 of the AGT microsatellite and renal disease progression was found. The results on AGT deserve further investigation.

We also examined the effect of environmental risk factors on renal disease progression. An association between smoking, alcohol consumption, lipidaemia and physical activity and the progression of cardiovascular and renal disease progression has been reported by several studies (D'Amico and Gentile 1993; Polat *et al.*, 1998; Perneger *et al.*, 1999; Hu *et al.*, 2000; Muntner *et al.*, 2000). In ADPKD, the effect of these factors has been studied less, however several reasons make these factors potential modifiers of ADPKD severity. For example, smoking may exert its effect directly on the kidney by affecting renal hemodynamics, water diuresis and electrolyte excretion causing changes in proximal tubular function. The indirect effects of smoking on the kidney may be caused by the development of hypertension, which adversely affects the kidneys (Orth *et al.*, 1997). Alternatively, the mutagenic effects of smoking may affect ADPKD by providing the second hit in the normal PKD gene, hence contributing to cystogenesis and phenotypic variation. The results from this study revealed subtle relationships between smoking and renal disease progression and smoking, lipidaemia and renal cystogenesis. The results are inconclusive because of the small samples due to subgroup analysis but deserve further investigation.

The results presented in this thesis suggest that apart from mutations in the PKD genes, environmental factors and sex contribute to the observed phenotypic variation in ADPKD. Again, it must be pointed out that we studied a small proportion of all possible factors likely to modify disease severity. There are many other possible modifiers of ADPKD severity that may directly or indirectly interact with the *PKD* genes or



associated pathways. For example, the RAS is only one pathway among many that is involved in the regulation of blood pressure.

On the basis of these findings and to determine where future research in the study of modifying factors should be directed, concepts and methods traditionally used in the study of complex diseases were employed to investigate an autosomal dominant condition. The rationale behind this was based on the fact that even the simplest genetic disease is complex, when examined at the level of the organism (this is discussed in Chapter Seven). The term 'complex trait' refers to any phenotype that does not exhibit classic Mendelian recessive or dominant inheritance attributable to a single gene locus. Complexities arise when the simple genotype and phenotype correlation breaks down, either because the same genotype can result in different phenotypes or different genotypes can result in the same phenotype. The category of complex traits is all-inclusive and comprises all diseases that express a complex or variable phenotype. Therefore, to infer as much as possible about the observed variation in ADPKD, such methods were undertaken in this study (Vogel and Motulsky, 1986).

Based on our sample of nuclear and extended families and the influence of genetic and environmental factors (shared and non-shared), multivariate component modelling was employed to estimate the resemblance between related individuals and estimate the heritability by partitioning the phenotype into genetic and environmental components. .

The results showed that the correlation coefficient is significantly higher between sibs and higher between cousins compared to parent-offspring and uncle/aunt-nephew/niece for both age at CRF and the slope of decline of renal function. The closer resemblance between cousins compared to parent-offspring pairs (who are genetically more similar than cousins) implies the effect of shared environmental factors. It is likely that sibs and cousins are similarly aged and may in fact have been exposed to similar environmental effects common to that generation or a shared family environment. The lower correlation between parent and offspring and the remaining variance observed between sibs also suggest the influence of non-shared environmental factors. The findings of this study were further supported by the estimated low heritability of <5% for age at CRF and 0% for the slope of decline of kidney function in ADPKD. In terms of the possible

role of environmental factors, one attractive hypothesis is that smoking may be one factor that initiates the second hit in the normal somatic PKD cell and thus modifies disease severity in ADPKD patients. In addition, smoking may contribute to cystogenesis by increasing plasma growth hormone, which is known to augment basement membrane thickening (Østerby *et al.*, 1978).

The results of this thesis have significant implications for the direction of future ADPKD research and the clinical management of the disease. On a scientific level, the next stage is to identify specific environmental determinants that are influencing the ADPKD phenotype and to investigate how these factors interact with the *PKD* genes and other associated pathways. From a clinical perspective, these findings further support the need to inform ADPKD patients to modify their behaviour and lifestyle in terms of smoking and diet.

#### **8.1.1      *Limitations and Possible improvements to the study***

Due to time and space, not all work conducted as part of this doctoral project can be presented, nor were all aspects of data analysis as fully explored as I could wish. This was partly due to the breadth of the initial research topic and partly to the doctoral project itself evolving and growing during the time frame of the thesis.

#### **8.1.2      *Limitations and improvements on the collection of data***

A limitation of this and many other studies relate to the use of questionnaire data. Data regarding disease history collected from questionnaires are subject to recall bias and recollection loss. For example, the self reported information on smoking, alcohol, physical activity and lipidaemia may have skewed the results. In addition, the questionnaire design did not allow the collection of more comprehensive information such as pack-years of smoking and past physical activity.

The collaboration with other researchers (as evidenced in this thesis) has advantages and limitations. The obvious and most important advantage is the possibility of increasing the sample size of the study and subsequently the power to detect associations. This was demonstrated in this thesis, although the problem of small samples was still encountered when performing subgroup analysis.

In terms of limitations, the delay in receiving clinical information and missing data is a problem of studies of this nature. Information on lifestyle and dietary patterns was not available for the Bulgarian sample, therefore comparisons between the three study populations was not possible in this part of the study (see Chapter Six). However, given that inter-population differences were not found to impact on the disease outcome in this study, it is unlikely that this would have influenced the results in any major way. Information on height and weight was also not available on every individual and hence the analyses on ultrasound findings could not be adjusted for these variables.

### **8.1.3      *Limitations of molecular analyses***

Appropriate care and quality control was undertaken during the genotyping described in Chapter Two, section 2.5. It is likely that some mistyping of genetic markers occurred. This is a known problem, particularly when genotyping is performed using microsatellite markers. However, all experiments were repeated where any doubt about the validity of the results existed.

### **8.1.4      *Statistical limitations***

A primary limitation of this thesis was the small number of patients particularly when performing statistical analyses according to various subgroups. The fact that not everyone in this study had reached renal failure meant that the small numbers were inevitable. Consequently, an issue of concern is that no formal measure of consistency or accuracy of predicted values based on only two data points was applied. However, to overcome these limitations, two sets of models were used to give some insight into the robustness of the analyses. For CRF, the analyses were performed twice, initially using actual values, whereas the repeated analyses consisted of estimates dependent on predicted values from regression models. The latter method was used to increase the sample size. The different analyses were run to see if the results were consistent. Similarly, for ESRF, the analyses were performed twice, given the multicentre sources of the data and possible misclassification among those not identified as having ESRF, again to provide confirmation. The methods are described extensively in Chapter Two. Empirically, the results of the analyses obtained using observed versus extrapolated values appeared consistent although, in terms of accuracy, the use of regression models to obtain the extrapolated values resulted in a displacement to an earlier age (see results

in Chapters Three, Four, Five and Six). The difference in age at CRF using both approaches may have depended on whether those patients who lacked data to calculate observed age at CRF were significantly older or younger, or had more or less advanced renal failure than the main group. In addition, a more accurate age at CRF was most likely calculated in patients with many SCr values compared to those individuals with only two values. Like any study, longitudinal data are necessary to obtain a more accurate picture of the natural progression of the disease.

Additionally, owing to the small numbers and the subsequent analyses multiple comparisons were performed, which is an issue of debate among epidemiologists and statisticians. Whereas, some authors question the validity of data because of the probability of generating some false positive results due to random error (Thompson, 1997) and that incorporation of a correction variable is crucial, other authors provide substantial evidence that multiple comparisons are acceptable, generate valid data and require no adjustment (Rothman, 1990; Savitz and Olshan, 1995, 1998). In this thesis, the reader may question the validity of the findings because adjustments for multiple comparisons were not undertaken. Nonetheless, the underlying aims and goals of the study determine the need for adjusting for multiple tests.

It is argued that studies that generate a large number of measures of association have a greater probability of generating some false positive results due to random error than does the stated alpha level for individual comparisons. A variety of procedures, for example, the Bonferroni correction require the establishment of a smaller critical  $p$  value for rejecting the null hypothesis on each individual test in light of multiple tests to 'preserve' the stated alpha level for the entire study. However, formal correction methods also diminish the statistical power (Cole, 1979; Jones and Rushson, 1982; Thomas *et al.*, 1985) of studies.

The statistical formulation of the multiple comparisons problem is predicted on the universal null hypothesis that 'only purely random processes govern the variability of all the observations in hand' (Rothman, 1990). The omnibus alternative is that some associations are present in the data. Under this formulation, the purpose of conducting a study is to gather data that can be evaluated for its compatibility with the null

hypothesis. If the null hypothesis were the focal point, then the statistical technology applied to the multiple comparisons would be entirely appropriate, in particular to protect against type I errors. However, in this thesis, the null hypothesis and the omnibus alternative hypothesis were not the primary aims. This thesis was designed to address questions (hypothesis generating) about possible associations between an end-point (CRF and ESRF) based on observational data and the disease (ADPKD), with the fortune of generating information that could contribute information to evaluate a number of hypotheses (Savitz and Olshan, 1995). Hence, it was not necessary to adjust for multiple comparisons.

According to Savitz and Olshan (1995, 1998), the concern with multiple comparisons is unwarranted. They suggest that the investigator's perspective – how and when the idea for collecting and analysing data occurred is irrelevant to assessing the validity of the product. Focus on such issues may lead to unjustified dismissal of meaningful results or exaggerated confidence in weak results and distract the readers from focusing on the real threats to validity (Savitz and Olshan, 1995, 1998). Rothman (1990) pointed out that by adjusting for multiple comparisons and reducing type I errors, consequently increases the type II error. The theoretical basis for advocating a routine adjustment for multiple comparisons is the 'universal null hypothesis' that chance serves as the first order explanation for observed phenomena. However, this hypothesis undermines the basic premises of empirical research, which holds that nature follows regular laws that may be studied through observations. A policy of not making adjustments for multiple comparisons is preferable because it will lead to fewer errors of interpretation when the data under evaluation are not random numbers but actual observations of nature (Rothman, 1990). In agreement with the above statement, Greenland (1993) also pointed out that the statistical analysis of observational data should be limited to pattern detection and recognition. This means that the data is examined and organised to facilitate a clear understanding of what it can tell the reader about the substantive phenomenon of interest. In this case, the questions addressed in this thesis provide substantial data to deduce a better understanding of the factors associated with the development and progression of ADPKD. From this perspective, statistics are not 'descriptive' or 'inferential', they can only be descriptive, since it is the user who draws any inferences.

Furthermore, another statistical limitation in this thesis due to the small samples was the inability to adjust for lipid levels in the survival analyses with hyperlipidaemia increasing the risk of coronary disease. Owing to the small samples, adjustment for multiple variables would substantially reduce the number of degrees of freedom, which would consequently present significant problems in the interpretation of results.

Similarly, in the analyses for ACE, which included small numbers of the I/I group and AGT, which included small numbers of individuals with allele 4, no special test was available to adjust for this limitation.

The issues discussed in the above paragraphs emphasize the need for larger samples to allow flexibility in the statistical approaches and hence avoid much criticism from the reader. However, in reality, the small samples and limited data available in such studies are unavoidable. Based on these limitations, the methods and strategies used in this thesis are valid and do provide reliable detection of patterns based on observational data culminating in the generation of various hypotheses, which can be addressed in future longitudinal studies.

#### **8.1.5      *Conclusions***

Over the past decade there has been an explosion of new knowledge into the pathogenesis of ADPKD, which has resulted in a better understanding of the disease. This goal is important to target preventative strategies, better diagnostic tools, the development of new and specific pharmacological therapies, the improvement of risk models and an improved understanding of the epidemiology of the disease. The high frequency of ADPKD and the financial burden on society further emphasises the need to investigate factors that may be preventable.

This thesis also highlights the necessity for interdisciplinary research involving geneticists, biostatisticians and clinicians. Further progress into dissecting and identifying factors involved in the pathogenesis of ADPKD will require the cooperation of all these disciplines.

Only recently have we begun to understand the factors associated with the development of ADPKD and it is now clear that research that attempts to elucidate the complex pathogenesis of ADPKD offers the only real hope for an improved understanding of the disease.

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# APPENDICES

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These appendices contain documents relating to data collection described in this thesis and publications arising directly and indirectly from this thesis.

**A.1            COVERING LETTER SENT TO INDEX CASES  
ASCERTAINED FROM THE RPH RENAL REGISTRY**

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**Dept. of Nephrology**  
Royal Perth Hospital  
PO Box X2213  
Perth 6001  
Tel: 9224 2550  
Fax: 9224 2160

Dear

**POLYCYSTIC KIDNEY DISEASE GENETICS RESEARCH PROJECT**

We are writing to invite you and other willing members of your family to participate in a new research project for patients and families with polycystic kidney disease. The information derived from this project will help advance new medical knowledge on the genetic abnormalities that may cause this condition.

If you are known to have a cystic kidney condition, all that would be required is for you to have a standardised medical examination when convenient, your ultrasound examination to be reviewed (or repeated if not available), and then to attend the Genetics Unit at either Princess Margaret Hospital or King Edward Memorial Hospital for a detailed family history and to give approximately 30 mls (2 tablespoons) of blood. Your routine care would not be affected whether or not you participate.

If your family member has not been diagnosed or examined for polycystic kidney disease before, the potential implications should be considered. These are discussed on the accompanying Information Sheet. No pressure to participate should be applied.

If you would like to participate or wish further information, please contact either your usual physician or one of us directly on page at Royal Perth Hospital (Tel. 9224 2244).

Yours sincerely

Dr Mark Thomas    Dr Barry Saker    Dr Geoff Thatcher    Dr Andrew Woodroffe

**A.2            INFORMATION SHEET/CONSENT FORM SENT TO  
PARTICIPATING INDIVIDUALS**

---

# POLYCYSTIC KIDNEY DISEASE RESEARCH PROJECT:

## Patient Information and Consent Form

You or one of your family have been shown to have polycystic kidney disease. This condition affects one in a thousand people, with a 50% chance of being passed on from an affected parent to child. If you are affected, cysts (fluid-filled spaces) will gradually develop in both kidneys and some organs. High blood pressure, kidney infection and episodes of bleeding in the urine may occur. Up to 50% of affected people develop slowly-progressive kidney failure, some of whom reach the point of requiring artificial kidney treatment (dialysis or transplantation), while others never need such therapy. The diagnosis is usually made on an abdominal ultrasound examination combined with a history of polycystic kidney disease in a family.

Recent genetic (DNA) research has identified the gene within our body cells that causes the majority of cases of polycystic kidney disease, although its prevalence seems to vary in different parts of the world and in the different racial types. Research workers at Edith Cowan University, in collaboration with Royal Perth Hospital Dept of Nephrology and overseas researchers, can now offer this technique in WA. The aim of this project is to determine the prevalence of this gene in the multi-ethnic mixture of races in Western Australia.

If you wish to participate, you will be asked to attend Royal Perth Hospital for about one hour for a standard medical consultation to check your personal and family history, looking for symptoms or signs of polycystic kidney disease, or any associated conditions (such as heart murmurs or herniae). Samples of urine and about 30 mls (2 tablespoons) of blood will be collected; occasionally blood sampling can cause mild bruising or discomfort at the elbow. If not already performed, an ultrasound examination of your abdomen will be arranged in the X-Ray Dept – this involves a probe from being rubbed over your kidneys and flanks for about 10 minutes, but does not expose you to radiation or any other risk. A data bank of DNA material for analysis will be established from the blood sample.

If you do not know whether you have polycystic kidney disease yourself, **it is very important** that you should consider the potential advantages and disadvantages of possibly making this diagnosis. Early identification of treatable problems such as high blood pressure and kidney failure can slow their rate of progress, and may also help those planning a family. However, some employers or medical insurance and life assurance companies may adversely view this condition. Psychological reactions to the diagnosis vary from happiness at the relief from uncertainty, to sadness at the discovery of an inherited and possibly progressive condition. Neither the genetic test nor abdominal ultrasound can diagnose the disease with 100% certainty, especially in people under the age of 30 years, so undergoing the screening tests may still not provide an absolute answer. Further information is available through RPH Dept of Nephrology if required, and genetic counselling can be provided by Princess Margaret Hospital.

There will be no charge or costs to you for participation, your identity will be kept confidential in any publications arising from these investigations, and you are free to withdraw at any stage without prejudice to your usual medical care. The Ethics Committees of Edith Cowan University and Royal Perth Hospital have approved this study. If you require any further information at any stage, you may contact either:

Dr Mark Thomas  
Department of Nephrology  
Royal Perth Hospital. Tel: 224 2550

Dr W Beresford  
Chairman, RPH Ethics Committee  
Royal Perth Hospital. Tel: 224 2280

I have read the above and had all the questions answered to my satisfaction. I consent to participate in the study, realising that I can withdraw at any stage. I agree that the research may be published provided I am not identified.

SUBJECT: Signed.....Name (print).....Date.....  
WITNESS: Signed.....Name (print).....Date.....



**A.3            GENETICS    INFORMATION    SHEET    SENT    TO**  
**PARTICIPATING INDIVIDUALS**

---

## WHAT YOU NEED TO KNOW ABOUT THE GENETICS OF POLYCYSTIC KIDNEY DISEASE

The way we look and the way our bodies function is very much determined by our genes. Genes come in pairs, with one gene in the pair inherited from the mother and the other one from the father. Genetic diseases develop when one or both genes in a pair are changed and can no longer function normally.

Polycystic kidney disease (PKD) is a genetic disorder, i.e. it is inherited from a parent and it can be passed on to the offspring. A defect in one gene of the pair is sufficient to cause the disease. Thus, individuals with polycystic kidney disease have one gene for the disease and one normal gene. They can pass either the disease gene or the normal gene on to their children (Refer to diagram of PKD Inheritance). The chance of any child inheriting the disease gene is 1 in 2, that is 50%. This chance remains the same for every pregnancy and is not affected by the outcome of previous pregnancies.

Individuals who carry the disease gene will all develop cysts in their kidneys. Cysts are detected by ultrasound scans, which is a painless procedure and has no risk. A younger individual from a polycystic kidney family may show no cysts at ultrasound, however this does not mean that such an individual will not develop the disease later in life. At age 30, the cysts are already detectable in about 90% of individuals who have inherited the polycystic kidney gene. This means that if you are 30 years of age or older and have no cysts in your kidneys, your chance of having inherited the disease gene from an affected parent is very low because if you had the gene the kidney cysts should already be present. On the other hand, if you are 20 or younger and have no cysts in your kidneys, you may still have inherited the gene from an affected parent but the cysts may be too small to see.

Kidney cysts alone cause no health problems. Although cysts will develop in all people who carry the polycystic kidney gene, during life they will cause problems in kidney function in only half of the individuals who have the gene and our present knowledge does not allow us to predict the course of polycystic kidney disease. This means that if you have a first-degree relative with the disorder and you do not have any complaints, you may still have inherited the gene and pass it on to your children. On the other hand, if the examination of your kidneys has failed to detect any cysts, your chance of carrying the gene and passing it on becomes very low (see previous paragraph).

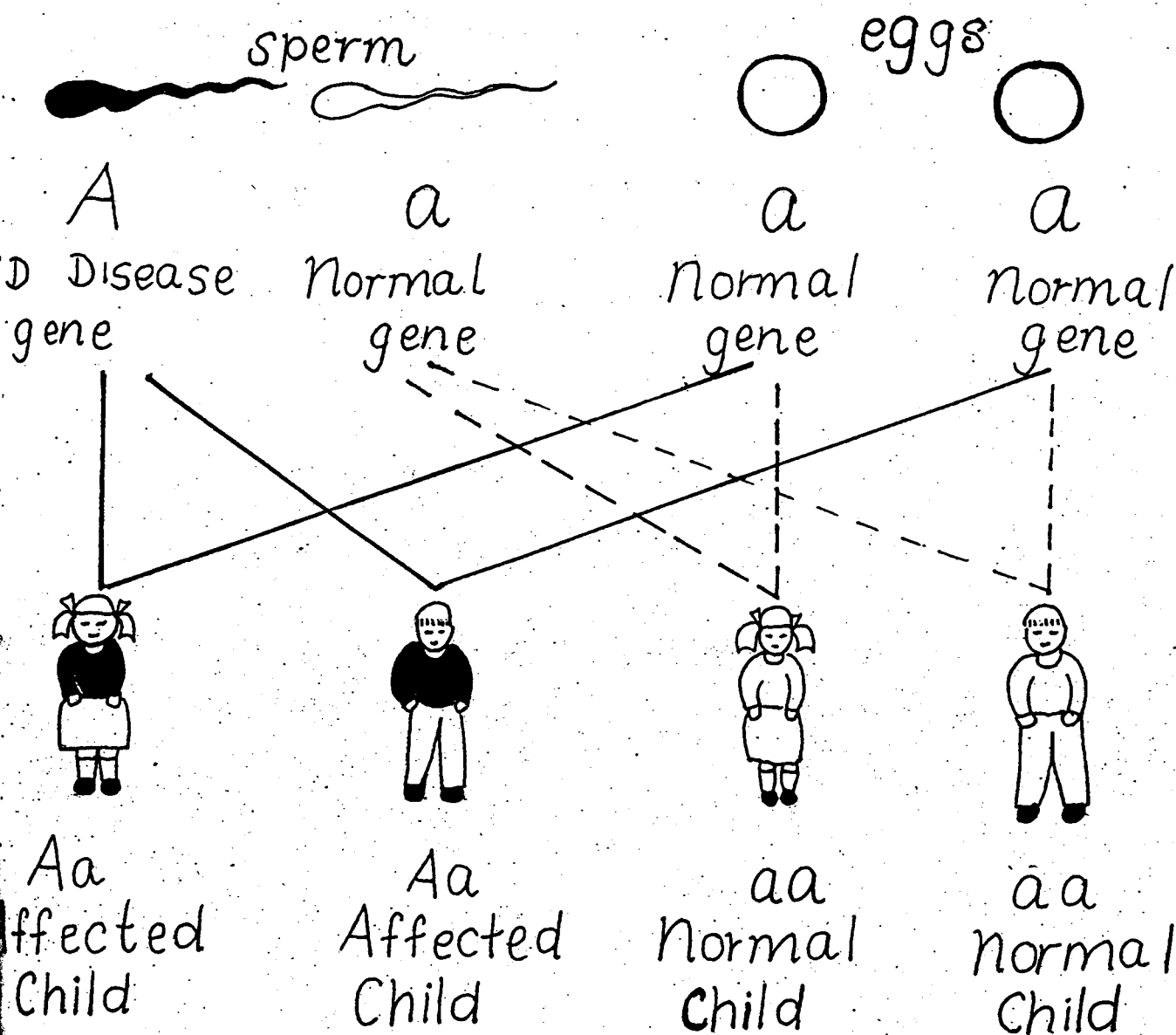
We hope that the present study will help us to understand better polycystic kidney disease and ultimately to be able to treat and prevent it. We thank you for your participation.

# Inheritance Of Polycystic Kidney Disease

Affected Male



Normal Female



**A.4            CLINICAL   PROTOCOLS   USED   TO   COLLECT  
INFORMATION ON PARTICIPATING INDIVIDUALS**

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# POLYCYSTIC KIDNEY DISEASE STUDY

## GENERAL INFORMATION

1.	RPH/SCGH ID NO.	_____	2.	STUDY NO.	_____
4.	PATIENT SURNAME	_____	3.	DATE	_____
5.	MAIDEN NAME	_____	5.	FIRST NAME	_____
6.	ADDRESS:	STREET _____			
7.		SUBURB _____		POSTCODE	_____
8.	PHONE:	HOME _____		WORK	_____
9.	SEX	M                      F			
10.	DATE OF BIRTH	_____	11.	COUNRTY OF BIRTH	_____
11.	THE PATIENT IS:	PROBAND/ SPOUSE/ AT RISK RELATIVE			
12.	IF SPOUSE OR RELATIVE:				
12a.	NAME OF PROBAND		_____		
12b.	RELATIONSHIP TO PROBAND		_____		
13.	IF AT RISK RELATIVE:				
13a.	ULTRASOUND TEST FOR PKD		Y	N	
	<u>IF NO:</u>				
14.	APPOINTMENT FOR ULTRASOUND:		Y	N	
14a.	DATE OF ULTRASOUND		_____	dy/mm/yy	
14b.	DAY OF ULTRASOUND		_____		
14c.	<u>RESULT OF ULTRASOUND</u>		POS	NEG	

**IF YES:**

14d.	RESULT OF ULTRASOUND	POS	NEG
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**IF YES: ASSIGN INDIVIDUAL TO CATEGORY:**

15. UNKNOWN/ DEFINITELY AFFECTED/  
DEFINITELY UNAFFECTED/ UNDEFINED

**IF NO:**

DATE OF LAST ULTRASOUND

AGE AT LAST ULTRASOUND  
BEFORE 30 YEARS

### BETWEEN 30 - 40 YEARS

OVER 40 YEARS

16. APPOINTMENT FOR ULTRASOUND: Y N

17. DATE OF ULTRASOUND \_\_\_\_\_ dy/mm/yy

18. DAY OF ULTRASOUND

19.	RESULT OF ULTRAOUND	POS	NEG
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20. **IF AFFECTED:** PREVIOUSLY DIAGNOSED/ NEWLY DIAGNOSED

**21. ETHNIC ORIGIN:**

21a. MATERNAL ORIGIN

22b. PATERNAL ORIGIN

**23. IF DECEASED:**

23a. YEAR OF DEATH yy

YEAR OF BIRTH \_\_\_\_\_ yy

23b. CAUSE OF DEATH \_\_\_\_\_

HOSPITAL \_\_\_\_\_

PHYSICIAN \_\_\_\_\_

24.	PARTICIPATION OF FAMILY MEMBERS IN PKD RESEARCH:	Y	N
25.	PARTICIPATION IN ENVIRONMENTAL/ LIFESTYLE FACTORS STUDY:	Y	N
26.	PATIENT REQUIRES RESULTS:	Y	N
	PARTICIPATION IN ENVIRONMENTAL/ LIFESTYLE FACTORS STUDY:	Y	N
	PATIENT REQUIRES RESULTS:		
	APPOINTMENT TO MADE TO COLLECT CLINICAL RESULTS	Y	N

PHYSICAL EXAMINATION

27.	DOCTOR	_____	27a.	RECORD NO.	_____
			27b.	DATE	_____
28a.	PATIENT SURNAME	_____	28b.	FIRST NAME	_____

MEDICAL HISTORY

29	DATE OF FIRST SYMPTOM	_____
30.	NATURE OF FIRST SYMPTOM	_____
_____		
31.	HOSPITAL PROVIDING CARE	_____
32.	DOCTOR PROVIDING CARE	_____
33.	AGE AT DIAGNOSIS	_____
34.	HOW WAS DIAGNOSIS MADE	_____
_____		
35.	IF DECEASED:	
35a.	POST MORTEM PERFORMED	Y N
35b.	POST MORTEM REPORT REQUESTED	Y N
35c.	AVAILABLE	Y N
36.	COMMENTS:	_____
_____		



**SYMPTOMS**

37. BACK/ LOIN PAIN                    Y            N

39. NOCTURIA                            Y            N

41. PROTEINURIA                    Y            N

43. UTI                                    Y            N

45. HEADACHES                        Y            N

47. RUPTURED  
ANEURYSMS                    Y            N

48. OTHER CONDITIONS

38. DYSURIA                            Y            N

40. HAEMATURIA                    Y            N

42. F.F F/T.S                        Y            N

44. STONES                              Y            N

46. VISUAL  
DISTURBANCE                    Y            N

49. WEIGHT                            \_\_\_\_\_ KG

50. HEIGHT                            \_\_\_\_\_ CM

51a. ABDOMEN:                        LIVER

51b.                                        SPLEEN

51c.                                        R. KIDNEY

51d.                                        L. KIDNEY

52. COLONIC DIVERTICULI

53. HERNIAE

54. CARDIAC VALVE DEFECTS

55. BONE DEFORMITIES

56. OTHER

ABDOMINAL ULTRASOUND EXAMINATION

57.	ULTRASONOGRAPHER	_____	58.	DATE	_____
59.	X-RAY RECORD NO.	_____	60.	STUDY NO.	_____
61.	PATIENT SURNAME	_____	62.	FIRST NAME	_____

63.	LENGTH	_____ mm	64.	LENGTH	_____ mm	<div></div>
65.	NO. OF CYSTS	_____	66.	NO. OF CYSTS	_____	<div></div>
67.	DIAMETER OF LARGEST CYST	_____ mm	68.	DIAMETER OF LARGEST CYST	_____ mm	<div></div>
69.	DIAMETER OF 2nd LARGEST CYST	_____ mm	70.	DIAMETER OF 2nd LARGEST CYST	_____ mm	<div></div>
71.	DIAMETER OF SMALLEST CYST	_____ mm	72.	DIAMETER OF SMALLEST CYST	_____ mm	<div></div>

73.	<u>LOCATION</u>				
73a.	CENTRAL	_____	73b.	CENTRAL	_____
74a.	PERIPHERAL	_____	74b.	PERIPHERAL	_____
75a.	BOTH	_____	75b.	BOTH	_____

CYSTS IN OTHER ORGANS

76a.	LIVER	_____	Y	N
76b.	NO. OF CYSTS	_____		
76c.	SIZE OF CYSTS	_____ mm		
77a.	OVARY	_____	Y	N
77b.	NO. OF CYSTS	_____		
77c.	SIZE OF CYSTS	_____ mm		
78a.	PANCREAS	_____	Y	N
78b.	NO. OF CYSTS	_____		
78c.	SIZE OF CYSTS	_____ mm		

79.	OTHER RENAL OR HX FINDINGS	_____
-----	----------------------------	-------

KIDNEY FUNCTION

80.

DATE									
WEIGHT									
SERUM CREAT.									
GFR									

RENAL FAILURE

81. DATE OF FIRST DIALYSIS
82. DATE OF FIRST TRANSPLANT
83. NUMBER OF TRANSPLANTS
- \_\_\_\_\_ yr/mm/dy
- \_\_\_\_\_
- \_\_\_\_\_



**AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE CLINICAL/GENETICS  
RESEARCH PROJECT**

**DIETARY QUESTIONS (CODING SHEET)**

UMRN \_\_\_\_\_

**1) WHEN YOU EAT MEAT WITH FAT ON IT, DO YOU EAT**

- |    |                            |     |
|----|----------------------------|-----|
| 1) | ALL OF THE FAT?            | (2) |
| 2) | MOST OF THE FAT?           | (2) |
| 3) | ABOUT HALF OF THE FAT?     | (1) |
| 4) | LITTLE OR NONE OF THE FAT? | (0) |
| 5) | I DO NOT EAT MEAT.         | (0) |


**1)  
SCORE**

--

**2) WHEN YOUR MEAT IS FRIED OR ROASTED, WHICH DO YOU (OR THE PERSON WHO  
USUALLY COOKS YOUR FOOD) USE MOST OFTEN?**

- |    |   |     |
|----|---|-----|
| 1. | SATURATED (BUTTER, DRIPPING, LARD, COOKING<br>OR TABLE MARGARINE)   | (1) |
| 2. | POLYUNSATURATED MARGARINE/ VEGETABLE<br>OILS (OLIVE/ SUNFLOWER OIL) | (0) |
| 3. | COOKED IN OWN JUICES / MICROWAVE / ON A<br>RACK                     | (0) |
| 4. | I NEVER ROAST OR FRY MEAT   | (0) |
| 5. | NOT APPLICABLE  | (0) |


**2)  
SCORE**

**3) HOW MANY EGGS DO YOU EAT A WEEK (e.g., raw, boiled, scrambled, poached, quiche)?**

- |    |   |       |
|----|---|-------|
| 1) | NUMBER OF EGGS PER WEEK (> 2 EGGS SCORE =<br>1; > 2 EGGS SCORE 0) | _____ |
| 2) | I RARELY OR NEVER EAT EGGS ( 0 )                                  |       |


**3)  
SCORE**

--

4) WHICH OF THE FOLLOWING FATS DO YOU USUALLY SPREAD ON BREAD OR CRACKERS?

- 1) BUTTER (1)
- 2) COOKING OR TABLE MARGARINE (1)
- 3) POLYUNSATURATED MARGARINE (0)
- 4) NOTHING (0)
- 5) OTHER FATS (0)


4)  
SCORE

--

5) WHICH MILK DO YOU MOSTLY / ALWAYS USE?

- 1) FULL-CREAM (WHOLE) MILK (2)
- 2) HI-LO MILK (APPROX. HALF-FAT MILK) (1)
- 3) SHAPE MILK (FAT CONTENT OF SKIM MILK) (0)
- 4) SKIM MILK (0)
- 5) I DO NOT USE MILK AT ALL (0)
- 6) SOYA MILK, RICE MILK, OTHER (0)


5)  
SCORE

6) HOW MUCH FULL-CREAM MILK DO YOU (YOURSELF) USE IN A WEEK (INCLUDING ON CEREALS?)

- 1) I DON'T DRINK FULL-CREAM MILK (0)
- 2) UP TO HALF A LITRE A WEEK (0)
- 3) MORE THAN HALF A LITRE A WEEK, UP TO 1 LITRE (0)
- 4) MORE THAN 1 LITRE UP TO 2 LITRES (1)
- 5) MORE THAN 2 LITRES UP TO 3 LITRES (1)
- 6) MORE THAN 3 LITRES UP TO 4 LITRES (1)
- 7) MORE THAN 4 LIRES A WEEK (1)


6)  
SCORE

--

7) **HOW MUCH SKIM MILK OR LOW-FAT MILK (e.g., Hi-Lo / Shape) DO YOU (YOURSELF) USUALLY DRINK IN A WEEK (INCLUDING ON CEREALS)?**

- 1) I DON'T DRINK SKIM / LOW-FAT MILK (0)
- 2) UP TO A LITRE A WEEK (0)
- 3) MORE THAN HALF A LITRE UP TO A LITRE (0)
- 4) MORE THAN 1 LITRE UP TO 2 LITRES (0)
- 5) MORE THAN 2 LITRES UP TO 3 LITRES (0)
- 6) MORE THAN 3 LITRES UP TO 4 LITRES (0)
- 7) MORE THAN 4 LIRES A WEEK (0)


7)  
SCORE

--

8) **HOW MUCH SOYA OR RICE MILK DO YOU (YOURSELF) USUALLY DRINK IN A WEEK (INCLUDING ON CEREALS)?**

- 1) I DON'T DRINK SKIM / LOW-FAT MILK (0)
- 2) UP TO A LITRE A WEEK (0)
- 3) MORE THAN HALF A LITRE UP TO A LITRE (0)
- 4) MORE THAN 1 LITRE UP TO 2 LITRES (0)
- 5) MORE THAN 2 LITRES UP TO 3 LITRES (0)
- 6) MORE THAN 3 LITRES UP TO 4 LITRES (0)
- 7) MORE THAN 4 LIRES A WEEK (0)


8)  
SCORE

--

**AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE CLINICAL/GENETICS RESEARCH  
PROJECT**

**DIETARY AND LIFESTYLE QUESTIONS**

UMRN \_\_\_\_\_

1) WHICH OF THE FOLLOWING BEST DESCRIBES YOUR CURRENT EXERCISE PATTERN

- 1. SUBSTANTIAL AND REGULAR
- 2. MODERATE AND REGULAR
- 3. MODERATE AND IRREGULAR
- 4. PHYSICALLY INACTIVE

1.
2.
3.
4.

2) WHICH IS YOUR MODE OF COMMUTING TO AND FROM WORK?

- 1. WALK
- 2. BICYCLE
- 3. DRIVE CAR OR VEHICLE/PASSENGER CAR/  
VEHICLE
- 4. NOT APPLICABLE

1.
2.
3.
0.

3) HOW MANY HOURS PER WEEK WOULD YOU SPEND IN VIGOROUS PHYSICAL EXERCISE?  
hours \_\_\_\_\_

4) NAME THE VIGOROUS PHYSICAL EXERCISE?

CODE NO. 1. \_\_\_\_\_  
CODE NO. 2. \_\_\_\_\_  
CODE NO. 3. \_\_\_\_\_

5) HAS YOUR LEVEL OF PHYSICAL ACTIVITY CHANGED NOTICEABLY IN THE PAST 6 MONTHS?

Y N  
(YES = 1) (NO = 0)


**IF YES:**

6) HAS YOUR LEVEL OF ACTIVITY INCREASED OR DECREASED?

INCREASED Y N  
(YES = 1) (NO = 0)  
DECREASED Y N  
(YES = 1) (NO = 0)  
MISSING INFORMATION 99




7) IN YOUR OPINION ARE YOU MORE PHYSICALLY ACTIVE OR LESS PHYSICALLY ACTIVE THAN MOST PEOPLE YOUR AGE?

1. MUCH MORE ACTIVE
2. A LITTLE MORE ACTIVE
3. ABOUT AS ACTIVE
4. A LITTLE LESS ACTIVE
5. MUCH LESS ACTIVE

1.
2.
3.
4.
5.

8) WHAT ARE YOUR SMOKING HABITS?

1. SMOKE
2. USED TO SMOKE
3. NEVER SMOKED BEFORE

1.
2.
0.

IF C GO TO QUESTION 15

9) HOW OLD WERE YOU WHEN YOU FIRST BEGAN TO SMOKE ?

1. UNDER 16 YEARS OLD
2. 16 – 30 YEARS OLD
3. 31 – 50 YEARS OLD
4. 51 YEARS OLD OR OLDER
5. NOT APPLICABLE

1.
2.
3.
4.
0.

10) HOW LONG HAVE YOU OR DID YOU SMOKE FOR?

1. LESS THAN 1 YEAR
2. 1 – 5 YEARS
3. 5 – 10 YEARS
4. 10 – 20 YEARS
5. 21 YEARS OR MORE
6. NOT APPLICABLE

1.
2.
3.
4.
5.
0.

11) DO YOU SMOKE

- 1. CIGARETTES
- 2. A PIPE
- 3. CIGARS
- 4. CIGARETTES/PIPE (1, 2)
- 5. CIGARETTES/CIGARS (1, 3)
- 6. PIPE/CIGARS (2, 3)
- 7. CIGARETTES/PIPE/CIGARS (1, 2, 3)
- 8. NOT APPLICABLE

1.
2.
3.
4.
5.
6.
7.
0.

12) USED TO SMOKE ? (AMOUNT PER DAY) \_\_\_\_\_ no.

☐

13) HAVE NOT SMOKED FOR ? (AMOUNT IN YEARS) \_\_\_\_\_ yy

☐

14) HOW OLD WERE YOU WHEN YOU FIRST BEGAN TO SMOKE AT LEAST ONE CIGARETTE PER DAY?

\_\_\_\_\_ yy

☐

15)		EVERY DAY	5-7 TIMES PER WEEK	1-4 TIMES PER WEEK	1-4 TIMES PER MONTH	LESS THAN ONCE PER MONTH
	A) BEER	5	4	3	2	1
	B) WINE	5	4	3	2	1
	C) SPIRITS	5	4	3	2	1

WHAT IS THE HEAVIEST THAT YOU HAVE EVER DRUNK FOR A PERIOD OF THREE MONTHS OR MORE?

16)		AMOUNT PER WEEK (e.g. NUMBER OF BOTTLES, CANS, GLASSES, ETC)
	BEER	ALCOHOL CODING; MISSING INFORMATION 99
	WINE	
	SPIRITS	

17) FOR HOW LONG HAVE YOU CONSUMED THE AMOUNT OF ALCOHOL CONTAINING BEVERAGES  
YOU NOW CONSUME/

- 1. FOR LESS THAN ONE YEAR
- 2. 1-2 YEARS
- 3. 2-5 YEARS
- 4. MORE THAN 5 YEARS

1.
2.
3.
4.



# POLYCYSTIC KIDNEY DISEASE CLINICAL/GENETICS RESEARCH

## PROJECT

### *Genetic Questionnaire*

1)	If you have a parent with PKD, what is your chance of having inherited the gene?	
2)	If you have a brother or sister with PKD, what is the chance that you have inherited the gene as well?	
3)	If you have the PKD gene, can you pass it on to your children?	
4)	If one of your children has inherited the PKD gene, what is the chance of your next child having the gene as well?	
5)	Does having the gene mean that you will have all the symptoms of the disease?	
6)	If you have no symptoms of kidney disease, can you still have the gene?	
7)	If you have no symptoms of kidney disease can you still pass the gene on to your children?	
8)	Are you worried about PKD and the risks of children inheriting it?	
9)	Are you interested in discussing family planning options with a genetic counsellor?	

**A.7 LETTER WRITTEN TO CLINICIANS ADVISING  
THEM OF THE STUDY AND REQUESTING CLINICAL  
INFORMATION ON THEIR PATIENTS**

---

Date

[Name]

[Address]

[State Postcode]

Dept. of Nephrology, RPH  
GPO Box X2213, Perth, WA, 6001  
Ph: 9224 2550

Dear [Doctor]

RE: [Patient}

I am writing to inform you that your patient Mr/Mrs/Miss [Name], who has polycystic kidney disease, has willingly agreed to participate in the Polycystic Kidney Disease Genetics Research project conducted in WA. The study is a collaborative project involving many clinical departments at Royal Perth Hospital and the Molecular Genetics Laboratory at Edith Cowan University.

As you are aware, autosomal dominant polycystic kidney disease (ADPKD) is a very common genetic disorder, which affects 1 in 1000 individuals. Medical research in the last 10 years has provided us with an enormous amount of knowledge about ADPKD, although there are still things we know little about or which we still do not understand.

The study will provide a unique opportunity to investigate the prevalence of different genetic forms in various ethnic groups with Western Australia and will contribute to understanding of the wide variation in disease severity caused by PKD1 and PKD2.

The Project will aim to investigate; the main genetic forms of polycystic kidney disease and the effect on the severity on the phenotype. It will also investigate modifying genetic variants in the renin-angiotensin system, as well as diet and lifestyle factors which may contribute to the severity of the clinical expression of polycystic kidney disease.

Occasionally it is useful to confirm some of our findings with the patients' doctor. Should this be the case, your assistance would be greatly appreciated.

A brief outline of the project is attached for your interest.

If you have any queries, please contact me on 9224 2550.

Regards

Best wishes

Mark Thomas  
(Renal Physician (Head of Nephrology Department, RPH))

Date

[Name]

[Address]

[State Postcode]

Dept. of Nephrology, RPH  
GPO Box X2213, Perth, WA, 6001  
Ph: 9224 2550

Dear [Doctor]

RE: [Patient]

I am writing to request clinical information on your patient Mr/Mrs/Miss [Name], who has polycystic kidney disease and has willingly agreed to participate in the Polycystic Kidney Disease Genetics Research project conducted in WA. The study is a collaborative project involving many clinical departments at Royal Perth Hospital and the Molecular Genetics Laboratory at Edith Cowan University.

As you are aware, autosomal dominant polycystic kidney disease (ADPKD) is a very common genetic disorder, which affects 1 in 1000 individuals. Medical research in the last 10 years has provided us with an enormous amount of knowledge about ADPKD, although there are still things we know little about or which we still do not understand.

The study will provide a unique opportunity to investigate the prevalence of different genetic forms in various ethnic groups with Western Australia and will contribute to understanding of the wide variation in disease severity caused by PKD1 and PKD2.

The Project will aim to investigate; the main genetic forms of polycystic kidney disease and the effect on the severity on the phenotype. It will also investigate modifying genetic variants in the renin-angiotensin system, as well as diet and lifestyle factors, which may contribute to the severity of the clinical expression of polycystic kidney disease.

We would greatly appreciate your assistance in providing the clinical information on [Patient Name]. That includes ultrasound finding, if possible the initial as well as the latest, the patients blood pressure values over the years and anti-hypertensive medication and biochemistry results, including creatinine values and the patient's weight which will allow us to calculate GFR. In addition, information on symptoms at the time of diagnosis and during the years under the care of the hospital or clinical, these include: loin pain, haematuria, dysuria, kidney stones, urinary tract infections, evidence of a herniae, cardiac valve defects, or aneurysms. This information is needed to compare the severity of the disorder in different families as well as between members of the same family.

Enclosed please find data sheets to complete the above information.



Thank you very much in advance. If you have any queries, please contact me or Tina Schiavello our Research assistant on (08) 9224 2550.

Regards

Best wishes

Mark Thomas  
(Renal Physician (Head of Nephrology Department, RPH))



Dept. of Nephrology  
Royal Perth Hospital  
GPO Box X2213,  
Perth, Western Australia, 6001  
Ph: (08) 9224 2550

[Date]

Dear [Name]

As you are aware your family has kindly agreed to participate in the WA Polycystic Kidney Disease Project. Polycystic Kidney Disease (PKD) is a very common genetic disorder, which affects 1 in 1000 individuals. Although medical research in the last ten years has provided us with an enormous amount of medical knowledge about the disease, there are still many aspects, which we know little about. This study will provide a unique opportunity to investigate the prevalence of different genetic forms of PKD in various ethnic groups within WA and will contribute to understanding the wide variation in disease severity between affected families and between members within the same family. A better understanding of the genetic basis of PKD may ultimately result in better treatment and management of the disease.

To investigate the genes involved in the development of PKD, we need participation of whole families, not just patients with PKD. We realise it may be difficult for some family members to attend RPH as part of the study routine and have tried to simplify the procedure in order to make it possible for such individuals to still participate in the study. Participation "from a distance" involves the collection of several drops of blood by a simple procedure, which you can perform at home, using the filter paper and the lancet enclosed. We will use this tiny amount of blood to extract DNA and conduct analyses. Also attached are two forms with questions on family history and personal medical history. The answers to the questions are important to us for two main reasons: 1) in order to interpret the results of our genetic analyses, we need to know whether the individual investigated has PKD; b) in order to compare the severity of the disease between families, we need information on as many members of your family as possible. The information you provide will be kept confidential and will only be used for the purposes of the present study.

We thank you again for your support for this research project and take the occasion to inform you that a meeting of the families, medical staff and academic staff participating in the study is planned for early May this year. The meeting will address; a description on PKD including; history, prevalence, signs and symptoms, management and treatment, the aims of the study and how each aim will be achieved, the departments involved and the role of each department, discussion on the genetics of PKD and how the genetic analyses will be carried out. All relevant suggestions for further

advancement of this study will be considered. Your involvement in this matter will be greatly appreciated.

With our best wishes:

Dr Mark Thomas  
(Head of Nephrology Dept., RPH)

Tina Schiavello  
(Research Assistant)

## **A.9 PUBLICATIONS ARISING DIRECTLY AND INDIRECTLY FROM THIS THESIS**

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RESEARCH ARTICLE

# Mutation Detection in the Duplicated Region of the Polycystic Kidney Disease 1 (*PKD1*) Gene in PKD1-Linked Australian Families

Marie McCluskey,<sup>1</sup> Tina Schiavello,<sup>1</sup> Michael Hunter,<sup>1</sup> Janina Hantke,<sup>1,4</sup> Dora Angelicheva,<sup>1</sup> Nadja Bogdanova,<sup>2</sup> Arseni Markoff,<sup>2</sup> Mark Thomas,<sup>3</sup> Bernd Dworniczak,<sup>2</sup> Juergen Horst,<sup>2</sup> and Luba Kalaydjieva<sup>1,4\*</sup>

<sup>1</sup>Centre for Human Genetics, Edith Cowan University, Joondalup, Australia

<sup>2</sup>Institut für Humangenetik, Westfälische Wilhelms-Universität, Münster, Germany

<sup>3</sup>Department of Nephrology, Royal Perth Hospital, Perth, Australia

<sup>4</sup>WAIMR, West Australian Institute of Medical Research, Perth, Australia

Communicated by Peter Pearson

Screening for disease-causing mutations in the duplicated region of the *PKD1* gene was performed in 17 unrelated Australian individuals with PKD1-linked autosomal dominant polycystic kidney disease. Exons 2–21 and 23–34 were assayed using PKD1-specific PCR amplification and direct sequencing. We have identified 12 novel probably pathogenic DNA variants, including five truncating mutations (Q563X, c.5105delAT, c.5159delG, S2269X, c.9847delC), two in-frame deletions (c.7472del3, c.9292del39), and two splice-site mutations (IVS14+1G>C, IVS16+1G>T). Three of the mutations (G381C, Y2185D, G2785D) were predicted to lead to the replacement of conserved amino acid residues, with ensuing changes in protein conformation. Defects in the duplicated region of PKD1 thus account for 63% of our patients. Together with the previously detected mutations (Q4041X, R4227P) in the 3' region of the gene, the study has achieved an overall mutation detection rate of 74%. In addition, we have detected 31 variants (nine novel and 22 previously published) that did not segregate with the disease and were considered to be neutral polymorphisms. Three of the nine novel polymorphisms were missense mutations with a predicted effect on protein conformation, emphasizing the problems of interpretation in PKD1 mutation screening. Hum Mutat 19:240–250, 2002. © 2002 Wiley-Liss, Inc.

**KEY WORDS:** KD1; polycystic kidney disease, autosomal dominant; ADPKD; polycystin-1; genotype-phenotype; homologous genes; pseudogenes; SNP

**DATABASES:**

PKD1 – OMIM:601313, 173900 (PKD); GDB:120293; GenBank:L39891 (PKD1); AC002039, AF320593, AF320594, AC010488, AC040158 (PKD1-homologous genes)

## INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD; MIM# 173900) is a common monogenic disorder with an incidence of approximately one in 1,000 [Dalgaard, 1957]. The disease manifests with the development of bilateral kidney cysts followed by renal failure in most affected individuals. Eight to 10% of all patients requiring hemodialysis or renal transplantation have ADPKD [Gabow, 1993]. Additional symptoms, such as hypertension, urinary tract infections, hematuria, proteinuria, extra-renal cysts,

cerebral aneurysms, herniae, cardiac valve defects, renal calculi, and gastrointestinal diverticula, vary both between and within families [Roscoe et al., 1993; Schievink and Torres, 1997]. The rate of progression toward kidney failure is also variable: end-stage renal failure

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(ESRF) occurs most commonly in the fifth decade [Milutinovic et al., 1984]. However, it can present as early as in utero, while some individuals may never progress to renal failure [Choukroun et al., 1995; Parfrey et al., 1990; Perrichot et al., 2001]. The wide range of clinical manifestations and severity is probably a compound product of heterogeneous germline mutations, and of the random nature of the "second hit," namely somatic mutations in the two characterized genes for ADPKD, PKD1 (MIM# 601313), and PKD2 (MIM# 173910) [Qian et al., 1996; Watnick et al., 1998; Koptides et al., 1999].

Up to 85% of germline mutations in Caucasian ADPKD individuals affect the *PKD1* gene [Peters and Sandkuijl, 1992], located on chromosome 16p13.3 [The European Polycystic Kidney Disease Consortium, 1994]. The *PKD1* gene consists of 46 exons spanning ~52 kb of genomic DNA [The International Polycystic Kidney Disease Consortium, 1995; The American PKD1 Consortium, 1995; Hughes et al., 1995]. A large part of the gene is duplicated in at least six homologous genes (HGs), which show > 95% homology with the *PKD1* gene [Hughes et al., 1995; Bogdanova et al., 2001]. The homologous genes cause problems of co-amplification, thus creating major obstacles to the mutation analysis of patients and families with the most common form of ADPKD.

Here we present the results of a screening for mutations in the duplicated region of *PKD1* in 17 Western Australian ADPKD families. Using *PKD1*-specific oligonucleotide primers and direct automated DNA sequencing, we have identified 12 putative pathogenic mutations and achieved an overall *PKD1* mutation detection rate of 74%.

## SUBJECTS AND METHODS

### Subjects

Nineteen ADPKD families, 18 of British and one of Spanish descent, were recruited through index patients at the Department of Nephrology, Royal Perth Hospital, Western Australia, as part of a large collaborative study of ADPKD. The diagnosis was based on standard ultrasound criteria [Bear et al., 1992]. Preliminary analysis of these families had shown linkage to the *PKD1* gene. Two families had mutations in the unique part of the *PKD1* gene [Bogdanova et al., 2000]. The screening for mutations in the duplicated

region of *PKD1* included the remaining 17 affected individuals.

### DNA Isolation and PCR Amplification

DNA was extracted from whole blood using standard procedures [Miller et al., 1988]. Primers for long-range amplification were chosen to be specific to *PKD1* when compared with its homologous genes. All long-range polymerase chain reaction (XL-PCR) primers are specific to *PKD1* when compared to HG1 and HG2 [Loftus et al., 1999]. The *PKD1* sequence between exons 2 to 14 was compared also to HG3, HG4, and HG5 [Bogdanova et al., 2001]. All primers in this region are specific to *PKD1* when compared to HG3 and HG5. Our XL-PCR products are not specific to *PKD1* when compared with HG4 between exons 6 and 11, due to the identity between the two sequences. However, the nested primers used for amplification of exons 6, 9, 10, and 11 (fragment A) are specific to *PKD1* when compared to HG4.

XL-PCR was performed on 400 ng of genomic DNA in a final volume of 100  $\mu$ l using 4 units of Applied Biosystems XL-rTth Taq polymerase with 200  $\mu$ M of each dNTP, 120 ng each of forward and reverse primer, 1.5 mM MgOAc, and 1  $\times$  buffer (Applied Biosystems, Foster City, CA). The optimized conditions for XL-PCR are listed in Table 1.

XL-PCR products (5  $\mu$ l aliquots) were electrophoresed in 1% agarose gels and visualized with ethidium bromide staining in order to confirm an amplification product of the expected length and to rule out large deletions or insertions.

Nested PCR reactions utilized 1  $\mu$ l of XL-PCR template diluted by a factor of at least  $1 \times 10^{-4}$  to prevent co-amplification of the *PKD1* homologous genes [Watnick et al., 1997; Bogdanova et al., 2001]. Nested PCR was performed in a final volume of 25  $\mu$ l with 0.2 units of QIAGEN<sup>®</sup> Taq DNA polymerase with 200  $\mu$ M of each dNTP, 60 ng each of forward and reverse primer, 20% Q-solution (QIAGEN, Hilden, Germany), 1.5 mM MgCl<sub>2</sub>, and 1  $\times$  buffer (QIAGEN). The optimized conditions and specific oligonucleotide primers for nested PCR amplification of individual fragments are listed in Table 2.

### DNA Sequencing

All samples were subjected to cycle sequencing in both directions with the BigDye<sup>™</sup> Termini-

TABLE 1. Primers for XL-Template Amplification

Exons	Primer sequence	Cycling conditions	Genomic position	Product size (bp)
Ex 2-5	IVS1F 5' cccagcgtctcatctgtctgg 3' <sup>a</sup>	68°C (X 40)	17049-69	4,542
XL-1	IVS5R 5' ggtgggaacgaggggtgtcaac 3'	2-step (0.5/5')	21579-90	
Ex 6-11	2927 5' gccgcctacagcatcgtggccc 3'	68°C (X 40)	21447-470	4,198
XL-2	11R 5' cagttagggaggacacactact 3'	2-step (0.5/4')	25622-644	
Ex 12-14	12F 5' cagttaggtgctcccctcactg 3'	68°C (X 40)	25621-644	1,810
XL-3	3130 5' ccagcacggtcaggaggact 3'	2-step (0.5/2')	27410-430	
Ex 15	IVS13F 5' ctgtcccggtcactcactgc 3'	68°C (X 40)	26769-89	3,861
XL-4	Ex15XLR 5' caacgtgggctccaagtagttg 3'	2-step (0.5/4')	30607-29	
Ex 15-21	Ex15XLF 5' gcaactacttggaggcccacg 3'	68°C (X 40)	30606-26	3,375
XL-5	IVS21R 5' gcagggtgagcagggtggggcctc 3'	2-step (0.5/4')	33957-80	
Ex 23-24	Ex23F 5' ctgcactgacctacgcatgt 3' <sup>b</sup>	68°C (X 40)	37674-694	6,749
XL-6	Ex34R 5' atgtgggtgtctgggtagg 3'	2-step (0.5/6')	44403-422	

<sup>a</sup>IVS1F used by Thomas et al. [1999] designated 17EXT.

<sup>b</sup>Ex23F used by Watnick et al. [1997] designated TWF1.

nator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), using the nested PCR primers listed in Table 2. The products were electrophoresed on an automated 377 DNA Analyzer (Applied Biosystems).

Deletions and insertions were characterized by performing a fresh PCR reaction, cloning the products using the TOPO™ TA Cloning® Kits with the pCR2.1-TOPO™ vector (Invitrogen, San Diego, CA), and subsequent sequencing.

#### Data Analysis

Sequences were analyzed using Sequence Navigator 1.0.1 (Applied Biosystems) and Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI). Missense mutations were analyzed further for the conservation of the amino acid residue in the mouse and pufferfish protein sequences, using Pfam (www.sanger.ac.uk/Software/Pfam/). PHDsec [Rost and Sander, 1994] was used to predict the effect of the mutation on secondary protein structure.

Confirmation of putative pathogenic sequence variants was achieved through repeated analysis of the index patient and a segregation study including affected and unaffected family members. Segregation analysis was not possible in one out of 12 families, where linkage analysis had been performed on DNA extracted from blood spots collected on Guthrie cards.

Mutations are named following the recommendations of Cotton and Scriver [1998], whereas nucleotide positions are numbered in the traditional way, where the first coding nucleotide of the cDNA is at position 212 for genomic sequence Accession # L33243 and position 3648 for Accession # L39891.

#### RESULTS

Using the techniques outlined above, we have identified a total of 43 DNA sequence variations in the duplicated region of the *PKD1* gene in the 17 unrelated affected individuals. The detected variants include 12 probable pathogenic mutations, all of which are reported for the first time (Table 3 and Fig. 1), and 31 neutral polymorphisms, including nine novel and 22 previously reported (Table 4).

#### Probable Disease-Causing Mutations

**Protein truncating mutations.** Two nonsense and three frameshift mutations were detected in the study.

Q563X. The C>T transition at c.1898 (g.22716) in exon 8 is predicted to truncate polycystin-1 by 3,739 amino acids, corresponding to the loss of most of the protein.

S2269X. S2269X results from a C>G transversion at c.7017 (g.30915) in exon 15. It is predicted to truncate polycystin-1 by 2,033 amino acids, spanning the REJ domain and all of the transmembrane and cytoplasmic regions of the protein.

c.5105-5106delAT. A 2 bp deletion, c.5105-5106delAT (g.29004-29005delAT), was found in exon 15 of the *PKD1* gene. The mutation causes a frameshift at amino acid position 1632 and a premature termination signal at codon 1656, corresponding to the loss of most of the PKD repeats, the REJ domain, the transmembrane domains, and the entire cytoplasmic region of the protein.

c.5159delG. A single bp deletion, c.5159delG (g.29058delG), was detected in exon 15 of the gene, leading to a shift in the reading frame at



TABLE 2. Primers and Conditions for Nested PCR Amplification of PKD1

Exons	Primer sequence	Cycling conditions	Genomic position	Product size (bp)
Ex 2	Ex2F 5' ggaaggcctctggcctacta 3'	64°C (X 35)	19845-64	230
XL-1	Ex2R 5' ggggattcggcaagctgat 3'	(0.5/0.5/1')	20064-74	
Ex 3	Ex3F 5' ctcagctgtgggggattcca 3'	58°C (X 35)	20045-64	186
XL-1	Ex3R 5' ggcagaaggatattggggg 3'	(0.5/1/1')	20211-30	
Ex4	Ex4F 5' ggctggcatagacccttccc 3'	64°C (X 35)	20378-97	273
XL-1	Ex4R 5' cctggctgggaaggacagag 3'	(0.5/0.5/1')	20630-50	
Ex 5(a)	Ex5aF 5' gtggagccaggaggagca 3'	58°C (X 35)	20770-87	329
XL-1	Ex5aR 5' gggaagacgtgctggagg 3'	(0.5/1/1')	21081-98	
Ex 5(b)	Ex5bF 5' cctccagcagctctccc 3'	64°C (X 35)	21081-98	272
XL-1	Ex5bR 5' ccgctccaccctgcactg 3'	(0.5/0.5/1')	21335-52	
Ex 5(c)	Ex5cF 5' ctcagccctgctgggacaga 3'	64°C (X 35)	21315-35	275
XL-1	IVS5 R 5' ggtgggaacgagggtgtcaac 3'	(0.5/0.5/1')	21579-89	
Ex 6	4349 5' gtgagtgtctgtgccca 3'	58°C (X 30)	21491-508	396
XL-2	4350 5' cctccttctctctgagact 3'	(1/1/1')	21868-886	
Ex 7	4351 5' ggctctgagcctcagttt 3'	58°C (X 30)	22104-121	391
XL-2	4352 5' taaccacagccagcgtct 3'	(1/1/1')	22476-493	
Ex 8	4353 5' gtctgttctctctggtgt 3'	58°C (X 30)	22575-592	306
XL-2	4354 5' ccattctcactgggcaca 3'	(1/1/1')	22863-880	
Ex 9	4345 5' gttcgggtagggggagttct 3'	60°C (X 35)	23090-108	271
XL-2	4346 5' gtgaaagctcagagaggcca 3'	(0.8/0.8/0.8')	23341-360	
Ex 10	4347 5' ggtggcctgtgggcaaatca 3'	61°C (X 30)	23608-627	340
XL-2	4348 5' gccctgaggagatgcaggga 3'	(1/1/1')	23929-947	
Ex 11-11a	11AF 5' ggggtccacgggcatgaccgt 3'	66°C (X 25)	24300-320	331
XL-2	11AR 5' cgcagtcagggttgggcctc 3'	(0.5/1/1')	24610-630	
Ex 11-11b	11BF 5' acggaacagctcaccgtgtc 3'	66°C (X 25)	24581-600	320
XL-2	11BR 5' aaggtggccaccaggcagggg 3'	(0.5/1/1')	24880-900	
Ex 11-11c	11CF 5' gcagtgacagcggcctgttg 3'	66°C (X 25)	24849-869	321
XL-2	11CR 5' agcaccctgtctgcaggcacc 3'	(0.5/1/1')	25150-170	
Ex 12	4355 5' ccaggaggcgacaggcta 3'	61°C (X 30)	25923-940	352
XL-3	5135 5' cctggccctgattggcgtc 3'	(0.8/0.8/0.8')	26256-74	
Ex 13	5228 5' tcacctgccacctgggctcac 3'	68°C (X 35)	26279-299	320
XL-3	5136 5' ccgaggctcagaaaagca 3'	2-step (0.5/2')	26581-598	
Ex 14	5002 5' ctgtcccggttactcactgc 3'	68°C (X 35)	26769-789	275
XL-3	4140 5' gagggctgttggggagggaagg 3'	2-step (0.5/2')	27023-043	
Ex 15	Amplified as 16 fragments using primers and conditions described previously by Watnick et al. [1999]			
XL-4				
Ex 15-15(a)	Ex15XLF 5' gcaactacttggaggcccacg 3'	66°C (X 35)	30606-26	287
XL-5	Ex15aR 5' ctcaatgatgggaccaggcg 3'	(0.5/0.5/1')	30872-92	
Ex 15-15(b)	Ex15bF 5' catccaggccaatgtgacggt 3'	64°C (X 35)	30841-61	266
XL-5	Ex15bR 5' cctgggtggcaagctgggtgtt 3'	(0.5/0.5/1')	31086-106	
Ex 16	Ex16F 5' ctggatggggctctcagg 3'	56°C (X 30)	31197-214	289
XL-5	Ex16R 5' cctgggtggcaagctgggtgtt 3'	(0.5/0.5/1')	31468-85	
Ex 17	Ex17F 5' ggggtcccccagtccttccag 3'	66°C (X 35)	32262-81	247
XL-5	Ex17R 5' ccattcccccagcccgcaca 3'	(0.5/0.5/1')	32489-508	
Ex 18	Ex18F 5' gccctctcaccaccttct 3'	66°C (X 35)	32571-90	344
XL-5	Ex18R 5' gatcccgctgctccccca 3'	(0.5/0.5/1')	32895-914	
Ex 19	Ex19F 5' gtgatgccgtggggaccgtc 3'	66°C (X 35)	32946-65	287
XL-5	Ex19R 5' gtgagcaggtggcagtcctg 3'	(0.5/0.5/1')	33213-32	
Ex 20	Ex20F 5' ctgtcaccacccctctg 3'	64°C (X 35)	33225-43	239
XL-5	Ex20R 5' ggtcccaagcacgcatga 3'	(0.5/0.5/1')	33445-63	
Ex 21	Ex21F 5' cgctgtgacagctgtgtgccc 3'	66°C (X 30)	33763-86	218
XL-5	IVS21R 5' gcagggtgagcaggtggggccatc 3'	0.5/0.5/1'	33957-80	
Ex 23-23a	23aF 5' ctgcactgacctcacgcatg 3'	58°C (X 30)	37674-694	353
XL-6	23aR 5' caccagaaagatgggctgcac 3'	(0.5/0.5/1')	38006-026	
Ex 23-23b	23bF 5' ggggcccctggccaacctcag 3'	68°C (X 35)	37979-998	377
XL-6	23bR 5' cgtgtgccccaccgctgca 3'	2-step (0.5/2')	38336-355	
Ex 24	24F 5' tgtgacctgcgcgttctg 3'	68°C (X 35)	38600-617	204
XL-6	24R 5' ccaggctggcccgacag 3'	2-step (0.5/2')	38786-803	
Ex 25	25F 5' ctgggtcacgtccgtacc 3'	68°C (X 35)	38927-946	329
XL-6	25R 5' cctcgactctgcagagctc 3'	2-step (0.5/2')	39236-255	
Ex 26	26F 5' cggctctatctgagaaggc 3'	68°C (X 35)	39272-291	315
XL-6	26R 5' cagcacagccagtgagagca 3'	2-step (0.5/2')	39567-586	

(Continued)

TABLE 2. Continued.

Exons	Primer sequence	Cycling conditions	Genomic position	Product size (bp)
Ex 27	27F 5' cctccaccctccctcttg 3'	68°C (X 35)	41000-019	225
XL-6	27R 5' cagagcttggcagggtccgc 3'	2-step (0.5/2')	41205-224	
Ex 28	28F 5' cgagcctgacctccctcctg 3'	58°C (X 30)	41254-273	204
XL-6	28R 5' cggagtgaggaccatggaacg 3'	(0.5/0.5/1')	41438-457	
Ex 29	29F 5' cgtggcctcctgcagtgccg 3'	68°C (X 35)	41486-505	285
XL-6	29R 5' ggcaggaagaggctgccccg 3'	2-step (0.5/2')	41751-770	
Ex 30	30F 5' cacctgtgtggcctcctc 3'	68°C (X 35)	41794-813	199
XL-6	30R 5' ggctccattccagctactcc 3'	2-step (0.5/2')	41973-992	
Ex 31	31F 5' gctgaccactgccctcgtc 3'	68°C (X 35)	43585-603	207
XL-6	31R 5' agtccaagctgcgccaagg 3'	2-step (0.5/2')	43773-791	
Ex 32	32F 5' ttggcgcagcttgactc 3'	58°C (X 30)	43775-792	123
XL-6	32R 5' cagggtcagaggtttctc 3'	(0.5/0.5/1')	43879-896	
Ex 33	33F 5' tacctgtgacccgcgc 3'	68°C (X 35)	44064-081	250
XL-6	33R 5' ggtgagcttcagagcccc 3'	2-step (0.5/2')	44296-313	

codon 1650 and a termination signal at position 1721. The mutation is predicted to truncate polycystin-1 by 2,581 amino acids, including the domains described in the above paragraph.

**c.9847delC.** A single bp deletion in exon 28, c.9847delC (g.41352delC), causes a frameshift at position 3213 and generates a premature stop codon at residue 3315. Polycystin-1 is truncated by 987 amino acids, corresponding to most of its cytoplasmic region.

**Splicing mutations.** We have identified two nucleotide substitutions affecting donor splice sites and therefore predicted to result in aberrant RNA splicing.

**IVS14+1G>C.** A G>C transversion at g.26942, 1 bp downstream of exon 14, abolishes the consensus donor splice signal in IVS 14.

**IVS16+1G>T.** A G>T transversion at g.31394G>T, 1bp downstream of exon 16, affects the consensus sequence of the donor splice site in IVS 16.

**In-frame deletions and missense mutations.** These mutations were classified as probably pathogenic on the basis of family segregation analysis, the evolutionary conservation of the amino acid residues involved and/or the predicted changes in the secondary structure of the protein.

**c.7472-7474del3.** A three base pair deletion in exon 18, c.7472-7474delACC (g.32650-32652del), results in the deletion of threonine at codon 2421 in the REJ domain of polycystin-1. This is the second threonine in a group of three that are conserved in the mouse and *Fugu* proteins.

TABLE 3. Summary of Novel, Probable Disease-Causing Mutations in the 5' Duplicated Region of PKD1

Location	Sequence variation	Amino acid change (and protein region or domain)	Predicted effect on protein	Affected family	Family segregation
Ex 8	c.1898C>T g.22716C>T	Q563X (Extracellular)	Truncation (nonsense)	A4	Yes
Ex 15	c.7017C>G g.30915C>G	S2269X (REJ domain)	Truncation (nonsense)	A15	Yes
Ex 15	c.5105-5106del (AT)	I1632fsX1656 (PKD domain)	Truncation (frame-shift deletion)	A52	Not possible
Ex 15	g.29004delAT	V1650fsX1721 (PKD domain)	Truncation (frame-shift deletion)	A36	Yes
Ex 15	c.5159delG g.29058delG	F321fsX3315 (PLAT domain)	Truncation (frame-shift deletion)	A10	Yes
Ex 28	c.9847delC g.41352delC				
IVS 14	IVS14+1G>C g.26942G>C	Intronic	Aberrant splicing	A22	Yes
IVS 16	IVS16+1G>T g.31394G>T	Intronic	Aberrant splicing	A5	Yes
Ex 5	c.1352G>T g.21430G>T	G381C (Extracellular)	Missense (loop and helix shortened)	A9	Yes
Ex 15	c.6764T>G g.30662T>G	Y2185D (PKD domain)	Missense (loop lost)	A61	Yes
Ex 23	c.8565G>A g.37893G>A	G2785D (Extracellular)	Missense (loop slightly shortened)	A6	Yes
Ex 18	c.7472-74del (ACC) g.32650-52del	2421delT (REJ domain)	In-frame deletion (helix replaced by extended sheet)	A46	Yes
Ex 25	c.9292-9331del g.39090del39	3027del13 aa (Extracellular)	In-frame deletion (loop lost)	A42	Yes

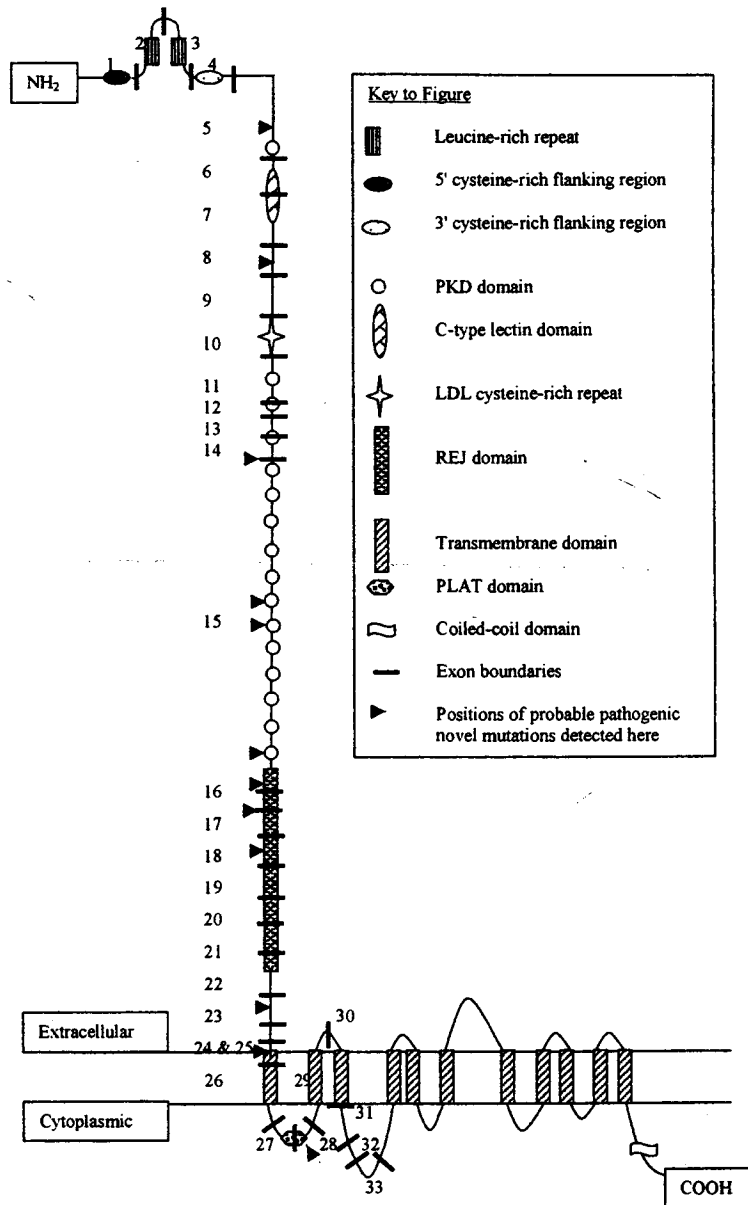


FIGURE 1. Schematic representation of polycystin-1 [adapted from Phakdeekitcharoen et al., 2000]. The locations of mutations detected in this study are marked with an arrow.

**c.9292-9331del.** This 39 base pair deletion in exon 25 is expected to result in the deletion of 13 highly conserved amino acid residues (positions 3027 to 3039) preceding the first transmembrane domain of polycystin-1. An example of the predicted topology change for this deletion is shown in Figure 2.

**G381C.** A G>T transversion at c.1352 (g.21430) in exon 5 leads to the replacement of a conserved glycine by a cysteine residue at position 381, between the first PKD domain and the c-type lectin domain.

**Y2185D.** A T>G transversion at c.6764 (g.30662) in exon 15 results in the substitution of histidine for tryptophan at the conserved position 2185 in the PKD repeat region of polycystin-1. An example of the evolutionary conservation and topology prediction for this missense change is shown in Figure 2.

**G2785D.** A G>A transition at c.8565 (g.37893) in exon 23 is predicted to result in a histidine for glycine substitution at conserved position 2785, immediately downstream of the REJ domain.

TABLE 4. Polymorphisms in the 5' Duplicated Region of PKD1

Location	Number of patients with change	Sequence variation	Amino acid position	Type of change	Previous reference
Exon 2	1	c.487G>A g.19963G>A	A92A	Silent exonic	Rossetti et al. [2001]
Exon 5	1	c.1234C>T g.21312C>T	A341A	Silent exonic	Thomas et al. [1999]
Exon 5	6 (A15.1) <sup>a</sup>	c.1330T>C g.21408T>C	L373L	Silent exonic	Rossetti et al. [2001]
IVS 6	1	g.22160G>A	—	Intronic	Novel
IVS 9	3	g.23307del7 (VNTR)	—	Intronic	Novel
Exon 11	1	c.2427A g.24474G>A	Q739R	Missense	Novel
Exon 11	1	c.2905A>C g.24952A>C	A898A	Silent exonic	Thomas et al. [1999]
Exon 11	3	c.2911G>A g.24958G>A	P900P	Silent exonic	Thomas et al. [1999]
Exon 11	3	c.2941C>T g.24988C>T	D910D	Silent exonic	Thomas et al. [1999]
Exon 13	3	c.3274T>C g.26395T>C	G1021G	Silent exonic	Thomas et al. [1999]
Exon 13	1	c.3319G>T g.26440G>T	T1036T	Silent exonic	Novel
Exon 13	4	c.3322A>G g.26443A>G	L1037L	Silent exonic	Thomas et al. [1999]
Exon 14	3	c.3486T>C g.26921T>C	M1092T	Missense	Novel
Exon 15	2	c.3583C>T g.27483C>T	A1124A	Silent exonic	Thomas et al. [1999]
Exon 15	2	c.3586C>T g.27486C>T	S1125S	Silent exonic	Thomas et al. [1999]
Exon 15	2	c.4406T>C g.28306T>C	W1399R	Missense	Watnick et al. [1999]
Exon 15	5	c.4876A>C g.28775A>C	A1555A	Silent exonic	Watnick et al. [1999]
Exon 15	2	c.4885G>A g.28784G>A	T1558T	Silent exonic	Watnick et al. [1999]
Exon 15	1	c.5157C>T g.29056C>T	T1649M	Missense	Novel
Exon 15	5	c.5383C>T g.29280C>T	T1724T	Silent exonic	Watnick et al. [1999]
Exon 15	3	c.5974G>A g.29872G>A	L1921L	Silent exonic	Thomas et al. [1999]
Exon 16	1	c.7138C>T g.31255C>T	G2309G	Silent exonic	Perrichot et al. [2000b]
IVS 16	1	g.31414G>A	—	Intronic	Novel
Exon 17	4	c.7376T>C g.32427T>C	L2389L	Silent exonic	Watnick et al. [1999]
Exon 18	4	c.7652C>T g.32830C>T	L2481L	Silent exonic	Perrichot et al. [2000b]
Exon 21	1	c.8098G>A g.33824G>A	A2629A	Silent exonic	Novel
Exon 21	2	c.8124A>G g.33849A>G	H2638R	Missense	Watnick et al. [1999]
Exon 23	2	c.8504C>T g.37854C>T	R2765C	Missense	Rossetti et al. [2001]
Exon 25	5	c.9406GT>CC g.39207GT>CC	F3066L	Missense	Peral et al. [1997]
Exon 26	5	c.9541T>C g.39466T>C	P3110P	Silent exonic	Peral et al. [1997]
IVS 31	1	g.43740T>C	—	Intronic	Novel

<sup>a</sup>A15.1 is homozygote for c.1330T>C. All other individuals are heterozygotes for polymorphisms listed.

### Probable Neutral Polymorphisms

The 31 polymorphic variants detected during our analysis of the duplicated region of PKD1 are shown in Table 4. Here we present in more detail the novel missense variants.

**Q739R.** A G to A transition (c.2427, g.24474) in exon 11 was predicted to result in an amino acid substitution at codon 739. Human sequence Accession # L33242 and mouse Pkd1 Accession # O08852 suggest a conserved glutamine at that position. However, another published human sequence, Accession # P98161, lists the same residue as arginine, suggesting a polymorphic variant. Computer prediction models show a marked difference in protein secondary structure (Fig. 2). In this study, the glutamine to arginine change was detected in a single affected subject (A42.1). The same substitution has been found subsequently in an ADPKD family from Bulgaria, where it did not segre-

gate with the disease (N. Bogdanova, unpublished results).

**M1092T.** A T to C substitution (c. 3486, g.26921) in exon 14 was found to occur in three unrelated affected subjects. Published human sequences have a methionine residue, while the mouse protein contains threonine. Again, a difference in secondary protein structure, albeit less pronounced, is predicted by computer analysis.

**T1649M.** A C to T substitution (c.5157, g.29056) in exon 15 results in the replacement of the conserved threonine at position 1649 by methionine. Computer models predict a slight change in secondary structure. This variant was detected in patient A52.1, where family segregation analysis was not possible. However, the cloning experiment performed to characterize the frameshift deletion (c.5105delAT) of the same subject demonstrated that the missense variant was carried on the other chromosome.



cluded long-range amplification, followed by nested PCR and direct sequencing.

We have tested 17 affected subjects for mutations in exons 2–21 and 23–34, which account for about 69% of the PKD1 coding sequence. A total of 12 possible disease-causing mutations were identified, spread from exon 5 to exon 28. The results of this study and others [Krawczak and Cooper, 1997; Afzal et al., 2000; Aguiari et al., 2000; Bogdanova et al., 2000; Kim et al., 2000; Koptides et al., 2000; Perrichot et al., 2000a,b; Phakdeekitcharoen et al., 2000, 2001; Rossetti et al., 2001] suggest that mutations are dispersed across the entire PKD1 gene, without any apparent clustering in mutation “hot spots.”

Similar to previous studies [Krawczak and Cooper, 1997; Afzal et al., 2000; Aguiari et al., 2000; Phakdeekitcharoen et al., 2000, 2001], all mutations that we have identified were private, i.e. confined to individual families.

Our analytical procedure is not problem-free: it is relatively costly and includes sequential amplification steps that can introduce sequence errors and therefore should be avoided in diagnostic protocols. However, in the case of ADPKD, the choice is limited by the characteristics of the PKD1 gene and of the pathogenic mutations. The risk of non-specific PCR amplification, due to the homologous genes, imposes the need of consecutive amplification steps. The spread of mutations across the PKD1 gene, their diversity (with truncating defects accounting for a little over 50% of our patients), and their private nature, all interfere with designing a hierarchical diagnostic procedure, prioritizing common defects and mutation hot spots.

The large number of private ADPKD mutations, together with the proposed random second-hit mechanism [Qian et al., 1996], make phenotype-genotype correlations difficult to assess. The analysis of such correlations will have to be limited to comparing the general effects of the nature of mutations (truncating vs. missense) and localization in specific protein domains. In our small group of patients, there was no significant difference between the age at onset of ESRF as related to the type of germline mutation (data not shown).

In view of the lack of functional assays and the prohibitively high costs of analyzing the PKD1 gene in a sufficient number of control chromosomes, the distinction between disease-

causing missense mutations, and “neutral variants” resulting in amino acid substitutions, has to rely on family segregation studies, assessment of the evolutionary conservation of the amino acid residue involved, the nature of the substitution, and the predicted effect on protein conformation. The predictive value of segregation analysis is limited in small families, as co-inheritance can be due to chance. As for evolutionary conservation and conformational changes, all three novel missense mutations, classified as non-pathogenic in our study due to lack of cosegregation, were predicted to result in conformational changes and one of the three (T/M1649) was conserved between mouse and human, emphasizing the problems of interpretation in PKD1 mutation analysis. Moreover, while independent segregation of missense mutations and the ADPKD phenotype indicates a lack of drastic effect on protein function, modifying effects on disease severity remain a possibility and merit further studies. In addition, the accumulation of “neutral” polymorphisms may affect protein function through numerous changes in topology even in the absence of an obvious pathogenic mutation. The existence of such “hyper-morphic” alleles has been proposed by Watnick et al. [1998].

Differences between the PKD1 nucleotide sequence and those of the homologous genes can provide an indication of the age of single nucleotide polymorphisms (SNPs) and the order of duplication events. In the case of M/T1092, the mouse sequence encodes threonine, while both published human PKD1 sequences, as well as all homologous genes, code for methionine at that amino acid position. The current data would suggest a very recent origin, subsequent to the duplication events, of the back mutation resulting in the human M/T1092 polymorphism.

At position 1649, threonine is conserved between the mouse and human PKD1 orthologs and in HG2, while the other HGs encode methionine. The findings may indicate that the origin of T/M1649 variant preceded some of the duplication events and that HGs 1, 3, 4, and 5 were derived from the M1649 allele.

The Q/R739 polymorphism is evident in the two published human PKD1 sequences, with Q739 encoded by sequence #L33242, and R739 encoded by #P98161. The homologous genes show differences. Based on the mouse PKD1

sequence (Accession # O08852), one could argue that Q739 is the ancestral allele, which gave rise to HG3 and HG4, whereas HGs 1,2, and 5 appear to be the products of duplication events involving the derived R739 allele.

Reliable conclusions on PKD1 gene genealogy and the origins and age of the HGs have to await the accumulation of SNP frequency data in humans, as well as information on other mammalian species. Such data will also allow conclusions on the role of the homologous genes as reservoirs of mutations in gene conversion events.

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*Original Article*

**Angiotensin-converting enzyme activity and the ACE Alu polymorphism in autosomal dominant polycystic kidney disease**

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**Abstract**

**Background.** Previous studies concerning Alu I/D polymorphism in the ACE gene and ADPKD severity have used the Alu genotypes as a representative of the true biological variable, namely ACE activity. However, wide individual and ethnic differences in the proportion of variance in ACE activity explained by the I/D genotype may have confounded these studies. This investigation examines the association between ADPKD severity and ACE in terms of plasma enzyme activity and I/D genotypes in individuals from three different countries.

**Methods.** Blood samples were collected from 307 ADPKD patients (116 Australian, 124 Bulgarian and 67 Polish) for determination of ACE activity levels and I/D genotypes. Chronic renal failure (CRF) was present in 117 patients and end-stage renal failure (ESRF) in 68 patients.

**Results.** ACE activity was related to the I/D genotype, showing a dosage effect of the D allele ( $P = 0.006$ ). The proportion of variance due to the Alu polymorphism was 14%. No difference in ACE activity and I/D genotype distribution was found between patients with CRF versus normal renal function ( $P = 0.494$ ;  $P = 0.576$ ) or between those with ESRF versus those without ESRF ( $P = 0.872$ ;  $P = 0.825$ ). No effect of the

I/D genotype on age at development and progression to renal failure (CRF; ESRF) was detected in the overall group, and in subgroups based on ethnic origin, linkage status and sex.

**Conclusion.** ACE is not likely to play a role as a determinant of ADPKD phenotype severity.

**Keywords:** autosomal dominant polycystic kidney disease; angiotensin converting enzyme; chronic renal failure; end-stage renal failure

**Introduction**

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder characterized by extensive inter- and intra-familial variation in clinical severity [1]. Since mutations in the PKD1 and PKD2 genes are the primary genetic cause of ADPKD, locus and mutation heterogeneity [2,3], as well as the random nature of the somatic mutations proposed by the “two-hit” model [4] are major contributors to phenotype diversity. Nonetheless, the complex pathogenesis of the disorder suggests that additional modifying factors may play a role. Activation of the renin-angiotensin system (RAS), present already in the early stages of ADPKD pathogenesis [5], could promote renal impairment and cyst growth through intrarenal vascular disease, as well as through the ability of angiotensin II to potentiate growth in tubular epithelial cells [6]. A number of studies have therefore

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focused on the role of the angiotensin converting enzyme (ACE) and tested the association between different measures of ADPKD severity and an intra-genic Alu insertion (I) or deletion (D) polymorphism in the *ACE* gene. The results have been controversial, with some studies suggesting a greatly increased risk of early kidney failure in individuals homozygous for the D allele [7,8], and others failing to find an effect [9,10]. The basic assumption of such studies is that the Alu genotypes are representative of the true biological variable, namely ACE activity. However, estimates of the proportion of the variance in enzyme activity explained by the Alu polymorphism vary substantially [11,12], and individual and population differences in the genetic control over *ACE* expression can be expected to have a confounding effect on studies where the I/D genotype is used as a substitute for ACE activity.

In an attempt to address this problem, we have studied subjects originating from three different populations, and assessed the association between ACE and ADPKD severity in terms of both plasma enzyme activity and I/D genotypes.

## Subjects and methods

### Subjects

The study included 307 ADPKD patients, 154 males and 153 females, aged from 9 to 85 years (mean  $45.2 \pm 0.9$  years). According to ethnic origin, they were grouped into 116 Australian (mostly of British descent), 124 Bulgarian, and 67 Polish. Informed consent was obtained from all participating individuals. The study complied with the ethical guidelines of the institutions involved.

The diagnosis of ADPKD was based on accepted ultrasonographic criteria [13]. Renal function and rate of deterioration were evaluated by serum creatinine (SCr) values, collected retrospectively over periods ranging from 1 to 7.2 years (mean  $6.8 \pm 0.31$ ). The number of measurements per patient varied between 1 and 16 (mean  $3.4 \pm 0.15$ ). Deterioration of kidney function was assessed using two end points: chronic renal failure (CRF), defined as SCr level of  $150 \mu\text{mol/l}$  ( $2.25 \text{ mg/dl}$ ), and end-stage renal failure (ESRF). In our patient population, 117 individuals had reached CRF and 68 had ESRF.

The definition of hypertension followed WHO criteria [14]. The overall number of hypertensive individuals was 192. Information on the use of ACE inhibitors at the time of the study was available for both the Australian and Polish patients, where 54 out of 85 Australian and all 41 Polish hypertensive subjects received such treatment.

Based on genetic linkage data, the patients were subdivided into 184 PKD1, 38 PKD2 and 85 subjects where linkage analysis was not feasible. Since the probability of linkage to the PKD1 gene is around 90% in affected individuals of European descent [15], the 85 undefined individuals were included in the PKD1 group, adding to a total of 269 subjects. Statistical analysis was performed separately on the definite and on the expanded PKD1 group.

The information on the affected individuals is summarized in Table 1.

### Methods

**Laboratory analyses.** Plasma ACE activity was measured in 92 Australian patients using the kinetic method [16], with a reference range of 23–100 U/l (covariance 3.2% at 125 U/l and 8.1% at 31 U/l).

The *ACE* I/D polymorphism was detected by PCR amplification as described [17]. To avoid mistyping, all D/D homozygous samples were re-typed using an insertion-specific PCR primer [18]. PCR products were separated on 3% agarose gels and visualized by ethidium bromide staining.

**Statistical analysis.** *ACE* genotype frequencies were compared between countries using the Chi-square test. Hardy-Weinberg equilibrium (HWE) was assessed with the probability test in the overall sample and with Fisher's exact test within each of the three populations. The relationship between *ACE* I/D genotypes and plasma ACE activity was assessed by general linear models (GLM). Analysis of covariance (ANCOVA) was used to correct for treatment with ACE inhibitors and analysis of variance (ANOVA) was used to compare the mean values of plasma ACE activity between individuals reaching CRF before or after age 40 years.

Decline in renal function was assessed by linear regression analysis using the least-squares method, with fitting individual regression lines of time vs  $1/\text{creatinine}$  values and extrapolating age at CRF. Kaplan-Meier survival curves were used to calculate cumulative survival to CRF and ESRF. Individuals were grouped according to *ACE* genotype and compared by means of a two-sided log-rank test.

Differences were considered statistically significant at  $\alpha$  of 0.05 ( $P < 0.05$ ). All analyses were performed using SPSS 9.0 (SPSS Inc, Chicago III).

## Results

### The I/D polymorphism and plasma ACE activity

In the overall group, the allele and genotype frequencies of the I/D polymorphism (Table 2) fell within the range reported previously in Caucasian populations [19,10]. A difference between the three populations was observed, with a lower frequency of the D allele in the Polish sample ( $P = 0.02$ ). No deviation from HWE

Table 1. Characteristics of 307 ADPKD patients

	Country			Overall
	Australia	Bulgaria	Poland	
Total #	116	124	67	307
Male/female	53:63	65:59	36:31	154:153
Genetic linkage				
PKD1	58 (50%)	81 (65%)	45 (65%)	184 (60%)
PKD2	3 (3%)	27 (22%)	8 (12%)	38 (12%)
Undefined	55 (47%)	16 (13%)	14 (21%)	85 (28%)
Mean current age	$46.6 \pm 1.4$	$46.7 \pm 1.5$	$39.8 \pm 2.0$	$45.2 \pm 0.9$
Hypertension	85 (73%)	65 (52%)	41 (61%)	192 (63%)
CRF	43 (37%)	50 (40%)	24 (36%)	117 (38%)
ESRF	33 (28%)	26 (21%)	9 (13%)	68 (22%)

**Table 2.** I/D genotypes observed in the three populations

	Country			Total
	Australia	Bulgaria	Poland	
Allele frequencies				
I	0.41	0.41	0.52	0.45
D	0.59	0.59	0.48 <sup>a</sup>	0.55
Genotypes				
I/I	21 (18%)	13 (11%)	18 (27%)	52 (17%)
I/D	54 (47%)	75 (61%)	34 (55%)	163 (53%)
D/D	41 (35%)	36 (29%)	15 (22%)	92 (30%)
Total	116	124	67	307

<sup>a</sup>The frequency of the D allele is significantly lower in the Polish group ( $P = 0.02$ ).

was seen in the overall sample, nor in any individual population.

Plasma ACE activity in all 92 Australian ADPKD patients fell within the reference interval (23–100 U/l). There was a significant correlation between Alu genotypes and enzyme activity, with a mean value of  $41.2 \pm 5.2$  U/l observed in I/I,  $59.0 \pm 4.6$  U/l in I/D, and  $71.2 \pm 5.8$  U/l in D/D subjects ( $P = 0.006$ ). Similar results were obtained after adjustment for the use of ACE inhibitors in the control of hypertension ( $43.5 \pm 6.5$  U/l for I/I,  $56.2 \pm 4.1$  U/l for I/D and  $69.7 \pm 4.5$  U/l for D/D;  $P = 0.004$ ). The fraction of the variance of plasma ACE activity explained by the ACE I/D polymorphism was estimated at 14%.

#### Plasma ACE activity and renal function

The relationship between plasma ACE activity and the development of renal failure was tested in the 92 Australian patients (CRF = 37; ESRF = 27). The mean ACE activity levels were  $61.7 \pm 3.8$  U/l in the group with normal renal function;  $59.8 \pm 6.4$  in the CRF group; and  $57.9 \pm 5.5$  in subjects with ESRF. The difference was not significant ( $P = 0.494$  for CRF vs normal renal function and  $P = 0.872$  for ESRF vs lack of ESRF). Similar results were obtained after adjustment for treatment with ACE inhibitors.

The mean plasma ACE activity was  $62.6 \pm 4.1$  U/l in the group of patients reaching CRF before age 40 years ( $n = 45$ ) and  $56.3 \pm 5.5$  U/l among those reaching CRF after age 40 years. The difference was not significant ( $P = 0.352$ ).

#### The I/D genotype and ADPKD phenotype severity

**Risk of renal failure.** The distribution of I/D genotypes among the 307 ADPKD subjects was examined by comparing subjects with normal renal function to those with CRF and with ESRF (Table 3). No significant differences were found between the three groups:  $P = 0.576$  in the comparison of CRF vs normal renal function and  $P = 0.825$  for ESRF vs lack of ESRF.

**Table 3.** I/D genotypes among ADPKD patients with normal renal function, CRF and ESRF

Renal function	ACE genotypes			Total
	I/I	I/D	D/D	
Normal	31 (16%)	97 (51%)	62 (32%)	190
CRF	21 (18%)	66 (56%)	30 (26%)	117
ESRF	13 (19%)	35 (52%)	20 (30%)	68

$P$  values are 0.576 for normal renal function vs CRF and 0.825 for ESRF vs lack of ESRF.

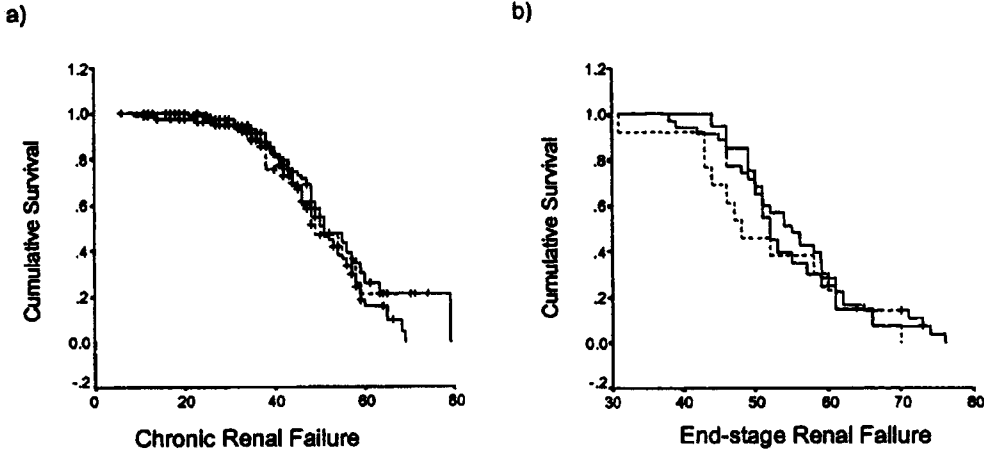
**Age at development of renal failure.** The relationship between the I/D genotype and development of renal failure was examined in a total of 117 subjects with CRF and 68 with ESRF. No significant differences in the mean age to either CRF and ESRF was found. The mean age at CRF was  $40.7 \pm 1.9$  years for I/I,  $39.7 \pm 1.1$  for I/D and  $39.1 \pm 1.5$  for D/D subjects,  $P = 0.810$ . The mean age at ESRF was  $51.5 \pm 3.0$  years for I/I individuals,  $55.5 \pm 1.6$  for I/D and  $54.5 \pm 1.7$  for D/D,  $P = 0.415$ . This analysis was repeated after grouping the patients according to ethnic origin. Again, no statistically significant differences were found, with  $P = 0.739$  and  $P = 0.239$  for the Australian patients,  $P = 0.504$  and  $P = 0.087$  for the Bulgarian and  $P = 0.313$  and  $P = 0.184$  for the Polish.

Lack of significant effect was observed when the different I/D genotypes were compared, within the entire group of patients using Kaplan–Meier cumulative survival curves to CRF ( $P = 0.815$ ) and to ESRF ( $P = 0.478$ ) (Figure 1). Similar results were obtained when Kaplan–Meier survival curves were examined separately for PKD1 and PKD2. Analysis of survival to CRF gave  $P$  values of 0.606 in the PKD1 and 0.529 in the PKD2 group. For ESRF, the  $P$  values were 0.454 for PKD1 and 0.163 for PKD2. No significant difference was revealed between the definite and expanded PKD1 groups. The separate analysis of male and female individuals with ADPKD also failed to detect significant effects of the I/D genotype on survival to CRF or ESRF.

**Hypertension.** The distribution of I/D genotypes was compared between 192 hypertensive and 97 normotensive ADPKD subjects. The I/D distribution for the hypertensive group were: I/I (18%), I/D (51%), D/D (31%), and I/I (12.4%), I/D (57.7%) and D/D (29.9%) for the normotensive group. The analysis failed to reveal a significant difference between the two groups ( $P = 0.384$ ).

## Discussion

The effect of the renin–angiotensin system and specifically of ACE activity on renal disease is an



**Fig. 1.** Analysis of the effect of the I/D polymorphism on survival to CRF and ESRF in the overall group of patients. Kaplan-Meier cumulative survival curves showed no significant differences between I/I, I/D and D/D genotypes as regards progression to CRF ( $P = 0.810$ ) and ESRF ( $P = 0.415$ ).

issue of practical significance, as it relates to the therapeutic potential of ACE inhibitors in the control of deterioration of kidney function. The findings reported to date on ADPKD, as well as other kidney disorders, have been controversial.

We have examined the association between the alleles of the Alu polymorphism in the *ACE* gene and decline in kidney function in 307 ADPKD subjects from three different populations. Inter-population differences did exist in terms of both the frequency of I/D alleles and the ratio of PKD1 to PKD2 patients. However, none of the three populations displayed a significant association between the I/D genotype and ADPKD phenotype severity. Lack of association was also observed for the entire study group. No effect was detected on either the overall risk of renal failure, the age at development of renal failure or the rate of progression from normal function to CRF and to ESRF. The findings were also negative in the separate analysis of patients grouped according to linkage status or sex.

In discussing the results of such association studies, one has to keep in mind their oversimplified design, where a DNA polymorphism in one gene is used to represent the overall effect of an entire pathway which is likely to be under complex control. The approach is simplistic even with the regard to ACE itself. The Alu polymorphism in the *ACE* gene is considered to be a neutral marker in disequilibrium with another, biologically relevant polymorphism directly affecting ACE activity levels. Strong evidence coming from recent studies suggests that the *ACE*-linked quantitative trait loci (QTL) are probably located in the 3' region of the gene, where a complexity of intragenic haplotypes can be observed, with the I or D allele of the Alu polymorphism occurring on diverse haplotype backgrounds [20]. The diversity of intragenic *ACE* haplotypes, their complex additive effects on ACE activity, and the observed inter-population differences [20] could account for the discrepant estimates of the proportion of the variance in ACE activity explained

by the Alu polymorphism, where figures range from nil to 47% [9,11,12].

In agreement with previous studies, ACE activity measured in 92 Australian ADPKD patients was related to the I/D genotype, but the proportion of variance due to the Alu polymorphism was only 14%. A similar proportion can be assumed for the patients studied by Baboolal *et al.* [7], since they share the same ethnic background. The striking effect of the D allele on earlier development of renal failure reported in that study [7] is difficult to interpret: firstly, in view of the modest contribution of the Alu polymorphism to the control of ACE expression and secondly because of the lack of expected dosage effect of the D allele observed by Baboolal *et al.* [7] and Perez-Oller *et al.* [8]. Additionally our analysis of plasma ACE activity did not reveal any effect on the development of renal failure, in agreement with Uemaso *et al.* [9] and Van Dijk *et al.* [10].

In conclusion, the results from this study suggest that the ACE is not likely to play a role as a determinant of ADPKD phenotype severity. Unless information on the relation between the Alu polymorphism I/D genotypes and ACE activity is available, association studies are neither conclusive nor comparable.

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## RESEARCH ARTICLE

# Screening the 3' Region of the Polycystic Kidney Disease 1 (PKD1) Gene in 41 Bulgarian and Australian Kindreds Reveals a Prevalence of Protein Truncating Mutations

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Screening for disease-causing mutations in the unique region of the polycystic kidney disease 1 (PKD1) gene was performed in 41 unrelated individuals with autosomal dominant polycystic kidney disease. Exons 34–41 and 43–46 were assayed using PCR amplification and SSCP analysis followed by direct sequencing of amplicons presenting variant SSCP patterns. We have identified seven disease-causing mutations of which five are novel [c.10634–10656del; c.11587delG; IVS37–10C>A; c.11669–11674del; c.13069–13070ins39] and two have been reported previously [Q4010X; Q4041X]. Defects in this part of the gene thus account for 17% of our group of patients. Five of the seven sequence alterations detected are protein-truncating which is in agreement with mutation screening data for this part of the gene by other groups. The two other mutations are in-frame deletions or insertions which could destroy important functional properties of polycystin 1. These findings suggest that the first step toward cyst formation in PKD1 patients is the loss of one functional copy of polycystin 1, which indirectly supports the “two-hit” model of cystogenesis where a second somatic mutation inactivating the normal allele is necessary to occur for development of the disease condition. *Hum Mutat* 16:166–174, 2000. © 2000 Wiley-Liss, Inc.

**KEY WORDS:** PKD1; polycystic kidney disease; autosomal dominant; polycystin 1; genotype–phenotype correlation

## DATABASES:

PKD1 – OMIM:601313, 173900 (PKD); GDB:120293; GenBank:L39891; HGMD:PKD1

## INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common Mendelian inherited disorders (MIM# 173900) with an incidence of approximately one in 1,000 [Dalgard, 1957]. The main clinical manifestation of the disease is the development of fluid-filled cysts in both kidneys which, in most affected individuals, results in kidney failure. Eight to 10% of all patients requiring haemodialysis or renal transplantation have ADPKD [Gabow, 1993]. Additional symptoms may include hypertension, urinary tract infections,

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haematuria, proteinuria, extra-renal cysts, cerebral aneurysms, herniae, cardiac valve defects, renal calculi, gastrointestinal diverticuli [Roscoe et al., 1993], and spinal meningeal diverticula [Schievink and Torres, 1997]. The clinical course of ADPKD varies in terms of presenting symptoms and the rate of progression towards kidney failure. In some patients end-stage renal failure (ESRF) occurs in adolescence while in others ESRF does not occur at all [Choukroun et al., 1995; Parfrey et al., 1990]. Among those who develop ESRF the mean age at onset is approximately 50 years [Milutinovic et al., 1984]. The wide range of clinical manifestations and the variation in severity are most probably a compound product of the genetic heterogeneity, locus as well as allelic, within ADPKD itself and of the effects of modifying genes and environment.

At least three different genes are currently implicated in autosomal dominant polycystic kidney disease: PKD1 (MIM# 601313), located on chromosome 16p13.3 [The European Polycystic Kidney Disease Consortium, 1994], PKD2 (MIM# 173910) on chromosome 4q21-23 [Mochizuki et al., 1996], and at least one more gene which is yet to be identified [Bogdanova et al., 1995; de Almeida et al., 1995; Daoust et al., 1995].

The participation of additional environmental and genetic factors in the course of the disease is supported by the demonstration of a "two-hit" mechanism in renal- and hepatic-cyst formation. Germline and somatic inactivating mutations of the two characterized genes for ADPKD, PKD1, and PKD2 have been identified in cyst-lining epithelial cells [Qian et al., 1996; Watnick et al., 1998; Koptides et al., 1999]. To study the genotype-phenotype correlation and address the functional importance of predicted protein domains further efforts have been made to screen PKD1 and PKD2 for disease-causing alterations of the nucleotide and amino acid sequence.

In affected individuals of European descent, mutations in PKD1 are the most common cause for the disease and account for up to 85% of cases [Peters and Sandkuijl, 1992]. The PKD1 gene consists of 46 exons spanning ~52 kb of genomic DNA [The International Polycystic Kidney Disease Consortium, 1995; The American PKD1 Consortium, 1995; Hughes et al., 1995]. A large part of the gene is duplicated in at least three homologous genes, HG-A, HG-B, and HG-C, which are located on chromosome 16p13.1 and share approximately 95-97% homology with the PKD1 gene [Hughes et al., 1995]. Only 3.5 kb of the PKD1 transcript (ap-

proximately 14 kb in length), located at the 3' end of the gene (exons 34 to 46), is unique to PKD1. The PKD1 gene product, polycystin 1, consists of 4,302 amino acids and is predicted to contain a large N-terminal extracellular domain of ~2,500 residues, several transmembrane domains, and a short cytoplasmic C-terminal region [The International Polycystic Kidney Disease Consortium, 1995; Hughes et al., 1995; Sandford et al., 1997]. It has been demonstrated recently that polycystin 1 and the gene product of PKD2, polycystin 2, associate in vivo in a heterodimeric interaction [Qian et al., 1997] involving the carboxy-termini of the two proteins. In polycystin 1, the proposed region of interaction is positioned in exon 46, the final exon of the gene (nucleotide positions 12854-12955 of the cDNA, acc. L33243). To date, a total of 40 mutations in the 3' end of PKD1 have been reported, mostly detected in patients from western European countries.

Here we present the results of a collaborative study of mutations in the unique region of PKD1 in 41 Bulgarian and Australian ADPKD individuals, which indicate predominant appearance of protein-truncating sequence alterations in this part of the gene.

## MATERIALS AND METHODS

### Patients

Twenty-six of the affected subjects are from Bulgaria and represent a mixed southeastern European population. The Australian group consists of 15 affected individuals predominantly of British descent with one family of Spanish origin. In all patients, the diagnosis of ADPKD was based on standard ultrasound criteria [Bear et al., 1992]. Linkage studies have been conducted in 39 of the families. Two affected individuals, one from Australia (A72.1) and one Bulgarian (B25.1), were included in the study without prior linkage analysis, based on the assumption that PKD1 accounts for most cases of ADPKD.

### DNA Isolation and PCR Amplification

DNA was extracted from peripheral whole blood using standard procedures [Miller et al., 1988]. Polymerase chain reaction (PCR) was performed on 50 ng of genomic DNA in a final volume of 25 µl using 1.5 units of Taq polymerase (Gibco BRL Life Technologies) and 1.5 mM MgCl<sub>2</sub>. The optimised conditions for the PCR amplification of individual exons are shown in Table 1. Prior to SSCP, the PCR products were electrophoresed in 1% agarose gels and visualized with ethidium

TABLE 1. Amplification Conditions for SSCP Analysis of the Unique 3' Region of PKD1

Amplification primers	Amplicon size (bp)	Annealing and cycling conditions	Additives	Nucleotide position*
Exon 34	240	30 cycles at 65°C	BSA (0.5%)	
Forward 5' gtgagctggggtgagaggag 3'			gelatine (2%)	44276-295
Reverse 5' acggctgcctggcctgagtc 3'				44496-515
Exon 35	220	2-step PCR of 30 cycles at 68°C	Non	
Forward 5' ctgcaactgcctctggagg 3'				47330-349
Reverse 5' ctagccctccctgtgagct 3'				47530-549
Exon 36	286	30 cycles at 63°C	Non	
Forward 5' ctgtgagctgcctctcacag 3'				47541-560
Reverse 5' ctacaggcctccatcacggg 3'				47807-826
Exon 37	299	2-step PCR of 35 cycles at 68°C	Non	
Forward 5' ggtaggctacaggcctccat 3'				47801-820
Reverse 5' caccctcgctctgtgtctcc 3'				48080-099
Exon 38	272	30 cycles at 64°C	Non	
Forward 5' caaagcctgtgtcactgt 3'				48439-458
Reverse 5' tagtcagccagaccctagg 3'				48691-710
Exon 39	259	2-step PCR of 35 cycles at 68°C	Non	
Forward 5' gtctctggtggcgcctcact 3'				48929-948
Reverse 5' gcagccttttagcggagctct 3'				49167-187
Exon 40	264	2-step PCR of 35 cycles at 68°C	Non	
Forward 5' gagctggccacaggaaaca 3'				49330-349
Reverse 5' tctacgccaaggacaaggag 3'				49573-593
Exon 41	355	2-step PCR of 35 cycles at 68°C	Non	
Forward 5' cgtctacccaaggacaagg 3'				49571-590
Reverse 5' cttcacagccctcagcca 3'				49906-925
Exon 43	383	40 cycles at 60°C	DMSO (2%)	
Forward 5' cagcgtccctcccgccctctga 3'				50381-403
Reverse 5' gcgcacacccagggtgcaagc 3'				50741-763
Exon 44	292	2-step PCR of 35 cycles at 68°C	Non	
Forward 5' caccacagggtgcaagcag 3'				50746-765
Reverse 5' gtggactccctctggagcgt 3'				51018-037
Exon 45	396	30 cycles at 68°C	Non	
Forward 5' cgtcttagctcagctcagct 3'				50946-965
Reverse 5' agtggggggagaggacac 3'				51322-341
Exon 46 (fragment a)	318	2-step PCR of 35 cycles at 68°C	Non	
Forward 5' gtgcagccggactgactgag 3'				51361-380
Reverse 5' cgtctaccagctggagcagc 3'				51659-678
Exon 46 (fragment b)	336	35 cycles at 66°C	DMSO (2%)	
Forward 5' gaccgactcaaccaggccac 3'				51633-652
Reverse 5' caggcagaatggctgcacg 3'				51950-968

\*According to Genbank accession number L39891.

bromide staining in order to confirm amplification and rule out large deletions or insertions.

### SSCP Analysis

Single-strand conformation polymorphism (SSCP) analysis was performed using 2 µl of PCR product and an equal volume of formamide loading buffer (98% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue). The mix was heat-denatured and kept on ice prior to loading. We found that the optimal gel composition for electrophoresis was a 12% polyacrylamide gel with a cross-linking ratio (%C) equal to 2.5 [Savov et al., 1992]. The gels (160 × 200 × 0.75 mm) were run at 25°C for varying lengths of time at different constant voltages using a vertical gel apparatus, D Gene™ Sys-

tem (Biorad), in 1 × TAE buffer without glycerol and in 1 × TBE with 5% glycerol [Kukita et al., 1997]. The single strand separation was visualized by a silver-staining procedure [Budowle et al., 1991]. The gels were dried and stored for documentation.

### DNA Sequencing and Restriction Analysis

All samples where an aberrant banding pattern had been detected by SSCP analysis were subjected to manual sequencing using internal labelling with [ $\alpha$ -<sup>32</sup>P]-dATP (Hartmann Analytics). Products for the sequencing reactions were obtained by PCR on genomic DNA with the amplification primers listed in Table 1. Cycle sequencing in forward and reverse directions using the same or internal primers was performed with the SequiTherm™ Cycle Sequenc-



ing Kit according to the manufacturer's protocol (Epicentre Technologies). The products of the sequencing reactions were electrophoresed in 6% denaturing polyacrylamide gels (C% = 5; 6M urea; 350 × 400 × 0.4mm), at 68W constant power for 2–3 hr. Gels were dried under vacuum prior to autoradiographic exposure on Biomax™ films (Kodak).

Restriction digestion of gel purified PCR products was performed in a total volume of 20 µl with 10 units of the appropriate enzyme (Gibco BRL; New England Biolabs), in conditions specified by the manufacturer. Reaction products were separated on a 2.5% agarose gel and visualized by ethidium bromide staining.

All sequence changes were subject to family segregation analysis using SSCP or, where possible, restriction fragment length polymorphism analysis.

## RESULTS

Using the techniques outlined above, we have identified disease-causing mutations in the unique 3' region of the PKD1 gene in seven of the 41 ADPKD patients screened (summarized in Tables 2 and 3). Five novel and two previously reported mutations were detected. The results are presented according to the type of molecular change.

## Protein Truncating Mutations

Two nonsense mutations were detected in this study, both of which have been reported previously.

### Q4041X.

This is a C to T substitution at nucleotide c.12332 (Genbank acc. L33243) which changes a Glutamine to a Stop codon and is predicted to truncate polycystin 1 by 262 amino acids. It was first identified by Turco et al. [1995] in an Italian patient and subsequently reported in one Spanish and two British families [Torra et al., 1998; Daniells et al., 1998].

In our study, Q4041X was found in individual A3.1, the index patient from a large Australian family of British origin, and was also detected in all six affected family members participating in the study. Hepatic cysts have been detected in 3/6 affected individuals. Apart from that, the clinical course of ADPKD, including rate of progression towards renal failure, appears variable, as shown in Table 2.

### Q4010X.

This is a C to T substitution at nucleotide c.12239 which changes a Glutamine to a Stop codon and is predicted to truncate polycystin 1 by 293 amino acids. It has been reported previously in

TABLE 2. Clinical Data of Patients From 5 Bulgarian and 2 Australian PKD1 Families With Disease-Causing Mutations in the Unique Part of the PKD1 Gene Identified in the Present Study

Mutation (exon/intron)	Family & patient ID	Sex	Current age (years)	CRF <sup>a</sup> (years)	ESRF <sup>b</sup> (years)	Hypertension	Liver cysts	Intracranial aneurysms
c.10634-10656del (Exon 34)	B22.1	M	63	42	49	Yes	No	No
	B22.2	F	61	54	57	Yes	No	No
	B22.3	F	26	No	No	No	No	No
	B22.4	F	38	No	No	No	No	No
	B22.5	F	34	No	No	No	No	No
IVS 37-10C>A (Intron 37)	B57.1	F	64	54	55	Yes	Yes	No
	B57.2	F	64	55	No	No	Yes	No
	B57.3	M	42	39	No	Yes	No	No
c.11587delG (Exon 40)	B77.1	F	55	45	No	Yes	Yes	No
	B77.2	F	55	49	No	Yes	Yes	No
	B77.3	M	31	No	No	Yes	Yes	No
	B77.4	M	29	No	No	Yes	No	No
	B77.5	M	28	No	No	No	No	No
c.11669-11674del (Exon 41)	B77.6	M	25	No	No	No	No	No
	B25.1	M	45	44	No	Yes	No	No
Q4010X (Exon 44)	A72.1	M	53	45	49	Yes	No	No
Q4041X (Exon 44)	A3.1	M	60	?	42	Yes	?	?
	A3.11	F	66	50	57	Yes	Yes	Yes
	A3.13	M	45	No	No	Yes	Yes	No
	A3.18	M	41	No	No	No	No	No
	A3.19	F	39	No	No	No	No	No
	A3.33	M	51	No	No	No	No	No
c.51815-5181ins39 (Exon 46)	B41.1	F	35	No	No	Yes	Yes	No
	B41.2	M	39	No	No	No	No	No

<sup>a</sup>CRF, chronic renal failure.

<sup>b</sup>ESRF, end stage renal failure.

TABLE 3. Disease-Causing Mutations in the Unique Part of the PKD1 Gene Identified in the Present Study

Exon/ intron	Site of nucleotide change*	Amino acid change	Restriction site change	Reference
Exon 34	c.10634-10656del	P3457fsX3617	-AvaII	Present study
IVS37	IVS37-10C>A	Splice site	+BsrI	Present study
Exon 40	c.11587delG	R3793fsX3824		Present study
Exon 41	c.11669-11674del	QE3820-3821del	-AluI	Present study
Exon 44	c.12239C>T	Q4010X	-HhaI	Turco et al., 1995
	c.12332C>T	Q4041X	+MaeI	Daniells et al., 1998
Exon 46	c.13069-13070ins39	4286-4287ins13aa		Present study

\*According to Genbank accession number L33243.

an ADPKD patient of British descent [Daniells et al., 1998] and in a Korean patient [Kim et al., 2000].

In our study, the mutation was found in Australian patient A72.1. No other family members were available for this study. The family has lived in Australia for the past three generations and originates from Britain. Patient A72.1 was diagnosed at 15 years of age due to positive family history. He developed hypertension at age 27 and reached ESRF at 49 years (Table 2). His other symptoms include umbilical hernia and gout.

Three of the novel mutations identified in this study are predicted to result in a frameshift and subsequent introduction of a preliminary stop codon. Two of these changes are deletions and one is a possible splice mutation likely to have a frameshift effect.

#### c.11587delG.

A single base pair deletion in exon 40 was found in Bulgarian patient B77.1. The mutation is predicted to result in termination of translation at codon 3824 and to truncate polycystin 1 by 478 amino acids.

The presence of this mutation was tested by the use of SSCP analysis in eight other family members, of whom six are known to have ADPKD. Analysis of the other family members showed that the c.11587delG mutation segregated with the disease. The clinical features of ADPKD in this family are shown in Table 2.

#### c.10634-10656del.

This novel 23 bp deletion in exon 34 results in a stop codon at position 3617 thus truncating the protein by 685 amino acids. The mutation was found in Bulgarian patient B22.1 and in all five affected members of the family. Their phenotypic features are shown in Table 2.

#### Splice-Site Mutation (IVS37-10C>A)

A transversion of C to A at position g.48491 (Genbank accession number L39891) in intron 37

of PKD1 was detected in Bulgarian patient B57.1. This change could create a new acceptor splice site eight nucleotides upstream of the end of IVS37 as shown below.

```

Wild Type  48481 agggccccc cgtccac [cag]
           AGC CTC CTG ....
Mutant     48481 agggccccc [cag] TCC ACC
           AGA GCC TCC TGG ....

```

The new acceptor site produces a frameshift with a translation stop at codon 3684 thus resulting in truncation of polycystin 1 by 618 amino acids.

The nucleotide substitution creates a Bsr I restriction site and restriction fragment length polymorphism analysis was used to screen additional family members and 50 unrelated normal subjects for the presence of the mutation. It was found that the mutation co-segregates with the disease in this family and it is not present in the normal controls (data not shown). Thus, we presume that this could indeed be a disease-causing mutation, although no RNA from this patient was available to verify that. The disease manifestation in the affected family members is summarized in Table 2.

#### In-frame Deletions and Insertions

##### c.11669-11674del.

This 6 bp deletion in exon 41, resulting in the loss of glutamine and glutamic acid at codons 3820-3821, was observed in Bulgarian index patient B25.1. Overview of the disease phenotype in the affected patient is shown in Table 2.

There were no other family members available so no segregation analysis has been performed. However, 50 unrelated normal individuals were tested for the presence of this deletion, using genomic amplification and subsequent separation of the PCR products on a 3% agarose gel. No carrier of the mutation was detected in the control group (data not shown), thus suggesting that this could be a disease-causing mutation.

In order to relate this mutation to an eventual

c.13069-13070ins39.

(c.13067)CCC ^acg cag gga cca ctt cgg gcc  
aag aac aag gtc cac ccc ^ AGC(c.13072).

(4286) Pro ^ Thr Gln Gly Pro Leu Arg Ala Lys  
Asn Lys Val His Pro ^ Ser(4287).

The insertion was detected in the two members of the family who are known to be affected and in two young individuals (born in 1987 and 1986) who are predicted to be carriers of a PKD1

ex. 11 normal sequence

cx 41, c.11669-11674del

**FIGURE 1.** Alignment of polycystin 1, exon 41, and exon 41, c.11669-11674del amino acid sequences and secondary structure prediction using the PHDsec algorithm. The deleted amino acids are underlined in the wild type sequence. E means extended sheet, H means helix and L means loop. The prediction subset in both figures is meaningful for all residues with an expected average correlation greater than 0.69.

ex. 46, normal sequence

**FIGURE 2.** Alignment of the C-terminal 35 amino acids of polycystin 1, ex. 46 and the C-terminal 48 amino acids of ex. 46, c.13069-13070ins39 and secondary structure prediction using the PHDsec algorithm. The 13 inserted residues are underlined. Repeated amino acids are shown in italic case. The symbols for secondary structure are the same as in Figure 1.

## DISCUSSION

Mutation detection in the PKD1 gene is complicated by the high GC content of the gene (around 62%) and, most of all, by the interference of the homologous genes. Predictably, most mutation detection studies have therefore focused on the unique 3' region of PKD1. Of the 82 mutations reported to date [Human Genome Mutation Database], 45 (55% of all mutations detected) are in the 3' unique region which, however, accounts for only 19% of the coding sequence. When these data are viewed as a true proportion of all individuals tested (approx 970 to date) [500 (British, Italian and Spanish) Daniells et al., 1998; 175 (Spanish) Badenas et al., 1999; 15 (Cypriots) Neophytou et al., 1996; 146 (French) Perrichot et al., 1999; 90 (British) Afzal et al., 1999; 41 (Australian and Bulgarian) present paper], only 5–6% of individuals have mutations in this region. In the present study, we have detected mutations in 16% of the ADPKD individuals investigated (12.5% Australian and 18.5% Bulgarian) over the same region. Both sets of data suggest that there is no clustering of mutations in the unique part of the PKD1 gene. Expressed as a percentage of patients investigated, the number of disease-causing mutations in the 3' end of the gene is roughly proportional to its length, therefore the majority of PKD1 mutations should remain located in the duplicated region.

Given the sequence characteristics of PKD1 and its complex genomic structure, designing a mutation screening strategy is not easy. Our experimental protocol, using SSCP as the screening technique, has been successful in identifying seven disease-causing mutations in 41 individuals tested. Thus, the proportion of identified mutations in our study is at least as high as that in other studies of this part of the gene. Five out of the seven sequence alterations detected would lead to a premature termination of translation. They were all identified through SSCP analysis, which is clearly not a method biased towards this type of mutation.

The exons analyzed here in the 3' region did not include exon 42 of the gene. Despite repeated attempts at amplification, using two different sets of oligonucleotide primers, we failed to obtain a specific product. No mutations have been reported to date in this exon, suggesting that other groups may also experience similar difficulties with specific amplification. This could be due to the presence of a non-disease-causing polymorphism in IVS 42. It includes a 34 bp repeat region and produces an intron size variation of ~325 bp to ~130 bp [Peral et al., 1996a]. This variation may explain the problems we encountered in the amplification of this part of the gene.

Out of the total of 50 mutations identified in the unique part of PKD1 so far (including the present study), only five, namely Q4041X [Turco et al., 1995; Daniells et al., 1998; Torra et al., 1998; Badenas et al., 1999], Q4010X [Daniells et al., 1998; Kim et al., 2000], R4020X [Rossetti et al., 1996; Neophytou et al., 1998], R4227X [Peral et al., 1996a; Peral et al., 1997], and R4275W [Badenas et al., 1999] have been reported to occur in more than one family. Two of these, namely Q4041X and Q4010X, were also detected in the present study. Comparison of polymorphic haplotypes of the Q4041X-bearing chromosomes in the Italian, British, and Spanish families with this mutation [Torra et al., 1998], revealed no identity by descent. A slipped-mispairing model explaining the recurrence of this mutation was subsequently proposed by Daniells et al. [1998]. We cannot rule out identity by descent for either Q4041X or Q4010X. The two mutations have been identified previously in British patients [Daniells et al., 1998] and in our study were detected in Australian individuals of British ancestry. Whatever the mechanism of origin, the most common PKD1 mutation known to date, Q4041X, accounts for less than 1% (five out of 877) of disease alleles. The emerging pattern is that of a large

multitude of private mutations confined to individual families, thus complicating further the study of molecular defects and of genotype-phenotype correlations in ADPKD.

Regardless of the variety of mutations detected, preliminary conclusions are already possible on the predominating type of defects. Of the reported mutations in the 3' end of PKD1 [Human Genome Mutation Database, Cardiff], approximately 80% are protein truncating. This is in contrast with the findings in the 5' part of the gene, where 37% (13/35) of the defects so far reported are missense mutations resulting in amino acid substitutions [Human Genome Mutation Database, Cardiff]. The same trend was observed in our study, where five of the seven disease-causing mutations detected in the unique part of the PKD1 gene would lead to a truncated protein. Moreover, missense mutations were identified in our studies but failed to segregate with the disease and were thus considered to be polymorphic variations (manuscript in preparation). It should be noted, however, that our study was performed on multiplex families where the disease penetrance is high and linkage analysis indicating PKD1 as the gene involved in ADPKD was possible. The spectrum of mutations could be different in the whole disease population which contains many isolated families with too few cases to make linkage analysis possible and where the disease penetrance may be low. Nevertheless, the protein-truncating mutations identified in our study cause C-terminal disruption of polycystin 1 prior to the last 113 amino acids, which would render the protein incapable of interaction with polycystin 2 [Tsiokas et al., 1997; Qian et al., 1997]. These findings suggest that the first step toward cyst formation in PKD1 patients is the loss of one functional copy of polycystin 1, which indirectly supports the "two-hit" model of cystogenesis where a second somatic mutation inactivating the normal allele is necessary to occur for development of the disease condition.

The novel in-frame deletion and insertion we found in this study seem to be pathological mutations, not only as judged by their non-occurrence in healthy subjects. The secondary structure prediction data for the relevant segments of the protein do show a potential functional interference caused by these changes. c.11669-11674del is perhaps distorting a possible interaction of polycystin 1 with an extracellular ligand by changing the topology of the second large extracellular loop, according to the protein model proposed by Hughes et al. [1995]. c.13069-13070ins39 is clearly chang-

ing the spatial organization of the very C-terminus of the protein by introducing an additional loop. This mutation causes an early onset of ADPKD in the assayed family. The last 115 amino acids of the cytoplasmic C-terminus of polycystin 1 seem to be essential for modulation of Wnt signaling during renal development [Kim et al., 1999] but the proposed potential ligand(s) for cytoplasmic-signalling cascades have not yet been identified. A conformation change as gross as the one induced by this insertion could definitely influence the interacting properties of this portion of the protein. The inserted repeated stretch of 10 amino acids, on the other hand, could be a consequence of a more general slipped-mispairing mutation generating mechanism.

An assessment of the phenotypic effects of mutations in PKD1 is rendered difficult by the limited clinical data provided in mutation reports. We have been able to find information on the age at onset of ESRF for 21 families with 19 different mutations in the unique region of PKD1, including this study [Peral et al., 1996a, 1996b, 1997; Turco et al., 1995; Neophytou et al., 1996; Torra et al., 1998; The European Polycystic Kidney Disease Consortium, 1994]. The total number of affected individuals in these families was 39. The mean age at onset of ESRF in patients with mutations in the transmembrane domain (N=28) was 53.8 years and for patients with mutations in the cytoplasmic region (N=11) it was 52.7 years. No significant differences could be detected when mean age at onset of ESRF was compared between groups of patients with different types of mutation. It was 53.2 years for patients with truncating mutations (N=30) and 54.5 years for non-truncating mutations (N=9). A meaningful assessment of the clinical effect of mutations in different exons is not possible because of the very small numbers of patients with mutations in the same exon. As already suggested by the comparison between individuals with mutations in the transmembrane domain and those with mutations in the cytoplasmic part of polycystin 1, most reach ESRF about age 55 regardless of the specific exon affected.

Since the causes of clinical variation in ADPKD are of great interest and since allelic variation may be one such cause, it is of interest to compare the phenotype characteristics of individuals with the same molecular defect. We have therefore examined the information on ADPKD patients with Q4041X. Hepatic cysts appear to be commonly associated with this mutation and have been re-

ported to occur in eight of 12 individuals on whom clinical information is available (including this study). The rate of deterioration of kidney function is variable, with 2/5 individuals developing ESRF before age 45 and 3/5 after 55 years. The presence of hypertension has been reported in seven of 10 individuals over the age of 30. This variation points further to the complexity of the disorder and supports again the "two hit" model suggesting that germline mutations in the PKD genes are only one of many determinants in the development and clinical spectrum of ADPKD.

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# Identification of *PKDL*, a Novel Polycystic Kidney Disease 2-Like Gene Whose Murine Homologue Is Deleted in Mice with Kidney and Retinal Defects\*

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Polycystin-1 and polycystin-2 are the products of *PKD1* and *PKD2*, genes that are mutated in most cases of autosomal dominant polycystic kidney disease. Polycystin-2 shares ~46% homology with pore-forming domains of a number of cation channels. It has been suggested that polycystin-2 may function as a subunit of an ion channel whose activity is regulated by polycystin-1. Here we report the identification of a human gene, *PKDL*, which encodes a new member of the polycystin protein family designated polycystin-L. Polycystin-L has 50% amino acid sequence identity and 71% homology to polycystin-2 and has striking sequence and structural resemblance to the pore-forming  $\alpha 1$  subunits of  $\text{Ca}^{2+}$  channels, suggesting that polycystin-L may function as a subunit of an ion channel. The full-length transcript of *PKDL* is expressed at high levels in fetal tissues, including kidney and liver, and down-regulated in adult tissues. *PKDL* was assigned to 10q24 by fluorescence *in situ* hybridization and is linked to D10S603 by radiation hybrid mapping. There is no evidence of linkage to *PKDL* in six ADPKD families that are unlinked to *PKD1* or *PKD2*. The mouse homologue of *PKDL* is deleted in *Krd* mice, a deletion mutant with defects in the kidney and eye. We propose that *PKDL* is an excellent candidate for as yet unmapped cystic diseases in man and animals.

Polycystin-1 and polycystin-2 are the respective gene products of *PKD1* and *PKD2*, mutations in which account for ~95% of cases of ADPKD.<sup>1</sup> ADPKD affects up to 1/1,000 individuals and is associated with a 50% incidence of end-stage renal failure by the sixth decade of life (1). At least one additional gene is known to be mutated in the ADPKD population (2, 3) but has yet to be identified.

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<sup>1</sup> The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; EST, expressed sequence tag; PCR, polymerase chain reaction; kb, kilobase(s).

Polycystin-1 encodes a 4,303-amino acid plasma membrane protein with a large extracellular N-terminal domain that contains leucine-rich repeats, a C-type lectin domain, and an LDL-A-like domain, all three of which are involved in cell-cell or cell-matrix interactions in other proteins (4–6). These domains are followed by 16 repeats of the so-called PKD domain and by an REJ (receptor for egg jelly in sea urchin sperm)-like domain. Polycystin-1 has 7 to 11 transmembrane domains. The short cytoplasmic tail (197 amino acids) of polycystin-1 contains a coiled-coil domain, which appears to interact with other proteins containing similar structures (7, 8).

The predicted amino acid sequence of the *PKD2* gene is homologous to the C terminus of polycystin-1 (9, 10). Polycystin-2 is a 968-amino acid protein with ~46% sequence similarity to each domain of the pore-forming  $\alpha 1$  subunits of  $\text{Ca}^{2+}$  and other cation channels, and like these channel subunits, it is predicted to have six transmembrane domains. Polycystin-2 has a putative  $\text{Ca}^{2+}$  binding structure (EF-hand) in its C-terminal cytoplasmic domain. It interacts biochemically with polycystin-1 and with itself (7, 8).

Here we report the identification, chromosomal localization, and expression of a third gene encoding a protein of the polycystin family. The product of this gene is an excellent candidate for a component of the pore-forming subunit of a polycystin-related channel and is also a candidate for various human and murine cystic diseases.

## EXPERIMENTAL PROCEDURES

**Isolation of *PKDL* cDNAs**—Overlapping EST sequences W27963 and W28231, derived from a retina cDNA library, were identified by their gene product homology to polycystin-2 (gb 189 U50928). A 340-base pair fragment in the overlap region of both ESTs was amplified from human adult kidney and brain poly(A)-selected RNA by reverse transcription-PCR using primers 5'-TCTTCGTGCTCCTGAACATG-3' and 5'-CCT-GTCGATTTTCTCTGTT-3'. 5'- and 3'-rapid amplification of cDNA ends were performed with human skeletal muscle and kidney rapid amplification of cDNA ends kits (CLONTECH, Palo Alto, CA), respectively. Primers were designed based on *PKDL* reverse transcription-PCR products. Nested amplification was performed following manufacturer's instructions. The 5'-rapid amplification of cDNA ends product was random-labeled with [<sup>32</sup>P]dCTP and used to screen a human retina cDNA library (CLONTECH). Hybridization was performed in a buffer containing 5 × SSC (1 × SSC, 0.15 M NaCl and 0.015 M sodium citrate), 50% formamide, 1% SDS, and 5 × Denhardt's solution at 42 °C, overnight. Filters were washed three times in buffer (0.1 × SSC and 0.1% SDS) at 65 °C. Positive signals were purified, and inserts were subcloned into pBluescript II (Stratagene, La Jolla, CA) and sequenced.

**Sequence Analysis**—Clones were sequenced from both strands, and the sequences were aligned to give an overall consensus sequence. The

computer program MOTIFS of GCG (11) was used to identify putative glycosylation and phosphorylation sites (20). Prediction of coiled-coil structure by Lupas' algorithm (12) was performed with the COILS computer program with and without 2.5-fold weighting of positions *a* and *d*, whereas prediction by Berger's algorithm (13) was performed with Paircoil program. Kyte and Doolittle's hydropathy analysis (14) was performed using PEPLOT (GCG). Secondary structure analysis was performed with PEPTIDESTRUCTURE (GCG) computer program and with the protein sequence analysis software using type-2 discrete state-space models (15, 16). Analysis of transmembrane segments was performed with the TMpred computer program (17) using windows of 18–33 residues and with SOSUI<sup>2</sup> and TMAP (19). Alignments with homologous sequences from polycystin-1, polycystin-2, and Ca<sup>2+</sup> channel  $\alpha$ 1 subunits were performed with LINEUP and BESTFIT (GCG) and optimized by visual comparison.

**RNA Dot Blot and Northern Hybridization**—Human adult and fetal RNA blots (CLONTECH) were hybridized with a randomly labeled 5'-most 1.5 kb of the coding sequence of *PKDL* in 5 × SSC, 50% formamide, 1% SDS, 5 × Denhardt's solution at 42 °C overnight. Filters were washed twice in 2 × SSC, 0.1% SDS at room temperature and at 50 °C. Signals were visualized by autoradiography.

**Fluorescent in Situ Hybridization**—A 1.7-kb human *PKDL* genomic fragment between cDNA positions 2,006 and 2,206 was PCR-amplified with exonic primers and subcloned into pCRII (Invitrogen, Carlsbad, CA). One  $\mu$ g of this vector was labeled with digoxigenin-11-dUTP as described previously (20), coprecipitated with 10  $\mu$ g of Cot-1 DNA, and resuspended in 1 × Tris-EDTA at 200  $\mu$ g/ml. Hybridization of metaphase chromosome preparations from peripheral blood lymphocytes of normal human males was performed with *PKDL* at 10  $\mu$ g/ml in Hybrisol VI as described previously (21). Digoxigenin-labeled probe was detected using reagents supplied in the Oncor Kit (Oncor, Gaithersburg, MD) according to the manufacturer's recommendations. Metaphase chromosomes were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride. Map position of *PKDL* was determined by visual inspection of the fluorescent signal on the 4,6-diamidino-2-phenylindole dihydrochloride-stained metaphase chromosomes using a Zeiss AxioPhot microscope. Images were captured and printed using the CytoVision Imaging System (Applied Imaging, Pittsburgh, PA). Twenty-one metaphases were assessed for probe localization.

**Radiation Hybrid Mapping**—An intron between cDNA positions 1,042 and 2,043 was amplified with exonic primers and sequenced. A set of primers was designed to amplify part of this intron. The Stanford G3 panel (22) was screened by PCR with this primer set. Data was processed at the Stanford Human Genome Center RH server.

**Linkage Analysis**—Two families, one Italian and one Spanish (F431 and F432) have been previously described (3, 23, 24). Four other families were studied: TOR1, a three-generation pedigree with 42 members of whom 26 were affected; TOR2, a two-generation pedigree with 8 members of whom 4 are affected<sup>3</sup>; and Singa 1, a two-generation pedigree with 5 members of whom 4 are affected, and Bulga 1, a 3-generation pedigree with 9 members of whom 5 are affected (24).

Génethon polymorphic marker D10S603, which has the same distribution as *PKDL* by radiation hybrid mapping and two flanking markers (D10S198–1.2 cM–D10S603–0.2 cM–D10S192) were selected to test for linkage to ADPKD in six families previously shown to be unlinked to the *PKD1* and *PKD2* loci. Genomic DNA from members of these families were used as templates for PCR. [<sup>32</sup>P]dCTP-labeled PCR products were separated by polyacrylamide gel electrophoresis. Pairwise affected-only linkage analysis was performed using the FASTLINK suite of programs. A fully penetrant dominant model with a disease gene frequency of 0.0001 and equal allele frequencies was assumed. The data was calculated using two-point lod scores.

**Southern Blot Analysis**—The *Krd* mutation arose on strain C3H and is maintained on strain C57BL/6J (27). C57BL/6J-*Krd* mice were crossed with strain SPRET/Ei to generate heterozygous (C57BL/6J-*Krd* × SPRET/Ei) F1 mice, as described previously (27). Aliquots of genomic DNA (10  $\mu$ g) were digested with restriction endonucleases, electrophoresed on agarose gels, and transferred to nylon filters (Zeta-probe GT, Bio-Rad). A 5' 1.5 kb human *PKDL* cDNA probe was gel-purified and radiolabeled as described above. Filters were hybridized and washed according to the manufacturer's instructions. Signals were visualized by autoradiography.

## RESULTS

Through data base searches we identified two EST sequences of ~500 nucleotides, W27963 and W28231, with similarity to polycystin-2. The deduced amino acid sequences of W27963 and W28231 showed 78% homology and 56% identity (over residues 649 to 749) and 65% homology and 39% identity (over residues 678 to 786 with a single three-residue gap) to polycystin-2, respectively. The two EST sequences shared 94% identity over 421 base pairs. We tentatively concluded that these ESTs arose from the same gene.

Using primer sets based on these overlapping EST sequences, we amplified the same reverse transcription-PCR product from adult kidney and brain RNA whose translated amino acid sequence shows 67% homology and 46% identity to residues 670 to 779 of human polycystin-2. We further performed 5'- and 3'-rapid amplification of cDNA ends with skeletal muscle and kidney poly(A) RNA, respectively, and obtained 0.8 kb (5MR1) and 0.9 kb cDNAs (3MR20), respectively. Using 5MR1 as a probe, we screened a human retina library. Three clones, PKDL-6, PKDL-7 and PKDL-8, were obtained and sequenced. The consensus 3,044-base pair sequence revealed an open reading frame of 2,415 base pairs, which encodes a protein of 805 amino acids (Fig. 1). The putative translation start site at cDNA position 384 (5'-TTCCCCATGA-3') is not accompanied by a typical Kozak sequence. A single in-frame stop codon is found in the putative 5'-untranslated region. The open reading frame is followed by several in-frame stop codons, and the 3'-untranslated region contains a consensus polyadenylation signal (5'-AATAAA-3') 10 nucleotides upstream from the poly(A) tail.

The deduced amino acid sequence of *PKDL* is shown in Fig. 1. Hydropathy analysis of the polycystin-L sequence showed five highly hydrophobic regions predicted to be transmembrane segments (Fig. 2A). Three additional relatively hydrophobic peaks were identified. Polycystin-L showed significant homology to polycystin-2 as expected (71% homologous, 50% identical). This homology is generally higher in predicted transmembrane segments and in the loops between transmembrane segments (Fig. 2B). Polycystin-L also showed a moderate similarity (similarity 45%, identity 22%) to polycystin-1 over residues 1 to 797. This similarity is slightly higher in transmembrane segments, but there is one conserved positively charged short amino acid stretch in the first loop between transmembrane segments (Fig. 2B).

Polycystin-L, like polycystin-2, shows homology (similarity ~47%, identity ~21% overall) to each of the four domains of various Ca<sup>2+</sup> channel  $\alpha$ 1 subunits and other cation channels. Regions of homology are clustered in the last four transmembrane segments and the pore region of each domain of the Ca<sup>2+</sup> channel  $\alpha$ 1 subunits (Fig. 2B). In polycystin-L and polycystin-2, the regions corresponding to this pore region include the last of the three relatively hydrophobic peaks. The first two-thirds of this region is predicted to form a helical structure, which is characteristic for various cation channels.

Two algorithms (12, 13) predict that polycystin-L has a coiled-coil domain in its C-terminal cytoplasmic tail (Fig. 1). Polycystin-L also has a putative Ca<sup>2+</sup> binding structure or EF-hand (25) that generally consists of two helices and a loop between them (Fig. 1). The C-terminal helix in the EF-hand of polycystin-L overlaps with the predicted coiled-coil region. Polycystin-L has a putative cAMP phosphorylation site in its C terminus. Putative protein kinase C phosphorylation sites are all in regions predicted to be cytoplasmic. Four of five putative casein kinase II phosphorylation sites with strong motif sequences (positions 249, 563, 674, 703, 719) are also found in the C-terminal cytoplasmic domain (Fig. 1).

<sup>2</sup> Mitaku Group, Department of Biotechnology, Tokyo University of Agriculture and Technology (1998) SOSUI: Classification and Secondary Structure Prediction of Membrane Proteins. <http://www.tuat.ac.jp/~mitaku/adv-sosui/>.

<sup>3</sup> Y. Pei, L. Kalaydjieva, and J. Zhou, manuscript in preparation.



1 MNAVSGPEGQ ELHLKLGSGAW DNPAYSGPPS PHGTLRVCTI SSTGLPQPP KKPEDEPQET AYRTQVSSCC LHICQIGRL 80  
81 WGTTLTENTA ENRELYIKT<sup>+</sup> LRELLVYIVF LVDICLLTYG MTSSSAYYT KVMSEFLHT<sup>+</sup> PSDTVGSFQA ISSMADFDF 160  
161 AQGPLLDSLY WTKWYNQSL GHGSHSFIYY ENMLLGVPR LQLKVRNDSC VVHEDFREDI LSCYDVYSPD KEEQLPFGPF 240  
241 NGTAWTYHSQ DELGGFSHWG RLTSYSGGGY YLDLPGSRQG SAEALRALQE GLWLDGRTRV VFIDFSVYNA NINLFCVLRL 320  
321 VVEFPATGGA IPSWQIRTVK LIRYVSNWDF FIVGCEVIFC VFIFYVVEE ILELHIHLRL YLSSIWNILD LVILLISIVA 400  
401 VGFIHIFRTLE VNRLMGKLLQ QPNTYADFEF LAFWQTQYNN MNAVNLFFAW IKIFYISEN<sup>+</sup> KTMTQLSSTL ARCAKDILGE 480  
481 AVMFFIVFFA YAOLGYLLFG TQVENFSTFI KCIFTQFRII LGDFDYNAID NANRILGPAY FVTYVFFVFF VLNMFALAI 560  
561 NDTYSEVKEE LAGQKDELQL SDLLKQGYNK TLLRLRLRKE RVSDVQKVLQ GGEQEIQFED FTNTLRELGH AEHEITELTA 640  
641 TFTKFD RDGN RILDEKEQEK MRQDLEERV ALNTEIEKLG RSIVSSPQ GK SGPEAARAGG WVSGEFYML TRRVQLQET<sup>+</sup> 720  
721 LEGVVSQIDA VGSKLKMLER KGWLAPSPGV KEQAIWKHPQ PAPA VTPDPW GVQGGQSEV PYKREEALE ERRLSRGEIP 800  
801 TLQRS\*

FIG. 1. Deduced amino acid sequence of *PKDL*. Potential transmembrane segments are underlined. The two-headed arrow indicates the putative EF-hand; the dotted line indicates the putative coiled-coil region. Potential *N*-glycosylation sites are marked with an asterisk. Potential phosphorylation sites are marked as follows: diamond, cAMP phosphorylation site; inverted open triangle, protein kinase C phosphorylation sites; and plus sign, casein kinase II phosphorylation sites.

Multiple tissue RNA dot blot analysis using the 5' 1.5-kb of the coding sequence as a probe revealed highest expression in adult heart and kidney (Fig. 3A). Northern blot analysis showed the presence of 5- and 1.5-kb bands in fetal tissues including kidney, liver, and brain (Fig. 3, B and C). This result suggests the presence of alternatively spliced forms. The abundance of the two splice variants is ~1:1 in fetal tissues. In adult tissues, however, the long transcript is only detected after prolonged autoradiography.

Chromosomal assignment of *PKDL* to 10q24 was achieved by fluorescent *in situ* hybridization on 4,6-diamidino-2-phenylindole-dihydrochloride-stained metaphase human chromosomes using a 1.7-kb genomic probe (Fig. 4). In 17 of 21 metaphase preparations analyzed, a hybridization signal was found to be present on the long arm of chromosome 10 in band q24. In six spreads, both copies of chromosome 10 were labeled, and in 11 metaphase spreads, a signal was detected on a single chromosome 10. No signals were observed on other chromosomes. With the Stanford G3 radiation hybrid panel, *PKDL* was found to have an identical distribution pattern as polymorphic marker D10S603 (lod score greater than 1,000). Linkage analysis of the *PKDL* locus using flanking markers D10S603, D10S198, and D10S192 gave negative lod scores in six ADPKD families previously documented to be unlinked to *PKD1* and *PKD2* loci (Table I).

The human *PKDL* gene is located within a linkage group that is conserved on the distal portion of mouse chromosome 19 (26) (Fig. 5A). A 7-centimorgan deletion of this region has been described in *Krd* mice (27). To determine whether the mouse homologue, *Pkd1*, is located within the *Krd* deletion, we analyzed genomic DNA from F1 animals obtained from a cross of strain C57BL/6J-*Krd* with strain SPRET/Ei. To detect the C57BL/6J-*Krd*-derived allele in the F1 DNA, we utilized restriction fragment-length polymorphisms detected by hybridization with a 1.5-kb human *PKDL* cDNA probe (Fig. 5B). Strain C57BL/6J contains three hybridizing *TaqI* fragments of 5.5, 5.0, and 1.8 kb. Strain C3H, on which the *Krd* mutation originally arose, contains three hybridizing *TaqI* fragments of 5.5, 5.0, and 1.6 kb. Strain SPRET/Ei contains two hybridizing fragments of 8 and 4.5 kb. The (C57BL/6J-*Krd* X SPRET/Ei) F1 mouse inherited the hybridizing fragments contributed by the SPRET/Ei parent but did not inherit the fragments from C57BL/6J or C3H (Fig. 5B). This result indicates that the mouse *Pkd1* locus is located within the region that is deleted by the *Krd* mutation.

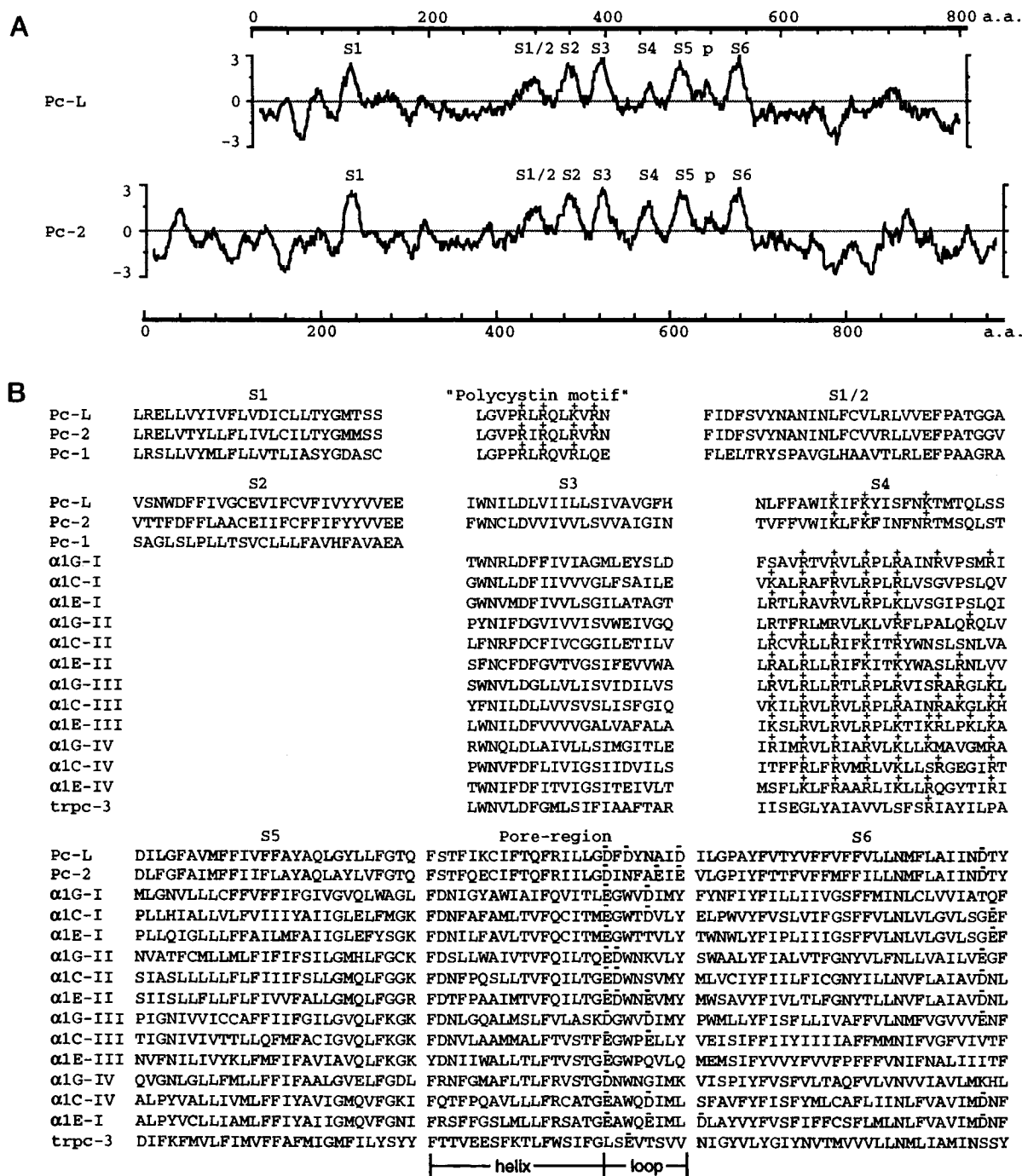
## DISCUSSION

**A Novel *PKD2*-like Gene**—The manifestations of *PKD1*- and *PKD2*-linked ADPKD are generally similar, raising the likelihood that the gene products function in the same or parallel biological pathways. Homology between polycystin-2 and the pore-forming  $\alpha 1$  subunits of voltage-activated  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  channel proteins, combined with evidence of interaction between polycystin-1 and polycystin-2 (7, 8), has led to the proposal that polycystin-2 forms homo- or heteromultimeric complexes with itself, with polycystin-1, or with another protein to function as an ion channel (9). Inasmuch as a small fraction of ADPKD families are not accounted for by *PKD1* and *PKD2* mutations and the function of polycystin family members may be cooperative, we postulated the existence of additional polycystin family members.

Here we report the identification and cloning of a third gene encoding a member of the polycystin superfamily, polycystin-L. Its gene, *PKDL*, is therefore an excellent candidate gene for human and murine cystic diseases. The temporal expression pattern of *PKDL* is similar to that of *PKD1*.

**Sequence Analysis: Implications for Polycystin Function**—The hydropathy patterns of polycystin-L and polycystin-2 are similar except in the region corresponding to the S4 segment of polycystin-2 where polycystin-L has a much lower hydrophobicity score, suggesting that this is a secondary membrane-spanning region. Polycystin-L and polycystin-2 both have putative EF-hand structures in their C-terminal cytoplasmic domains, suggesting that their functions are influenced by cytoplasmic  $\text{Ca}^{2+}$  concentration. In several  $\text{Ca}^{2+}$  channels, binding of  $\text{Ca}^{2+}$  to EF-hand structures inactivates the channels (28).

Polycystin-L and polycystin-2 show moderate but significant sequence similarity to  $\text{Ca}^{2+}$  and other cation channels, especially within their S3-S6 segments and the loop between the S5 and S6 segments. In addition, the last two membrane-spanning segments of polycystin-L, polycystin-2, and  $\text{Ca}^{2+}$  channel  $\alpha 1$  subunits share structural characteristics with the *Streptomyces lividans*  $\text{K}^{+}$  channel (KcsA) whose structure has been determined by crystallography (29). The common structural features include: lining residues of the last membrane-spanning segments that are mostly hydrophobic except for the negatively charged acidic amino acid near the end of these segments; loops between the last two membrane-spanning regions (pore region) that are mildly hydrophobic (Fig. 2A); first  $\frac{2}{3}$  of pore regions



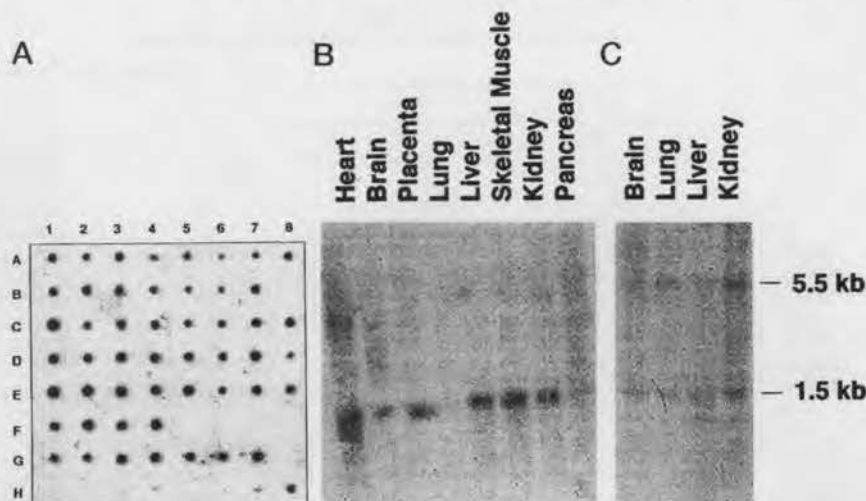
**FIG. 2.** A, hydropathy analysis of polycystin-L (Pc-L) and polycystin-2 (Pc-2). Hydrophobic peaks that are considered to be primary membrane-spanning regions are described as S1, S2, S3, S5, and S6. Mild hydrophobic peaks indicating secondary transmembrane domains are labeled S1/2, S4, and p. a.a., amino acids. B, alignment of polycystin-L with polycystin-2 (gb 189 U50928), polycystin-1 (Pc-1) (gb 189 U24497), voltage-activated  $\text{Ca}^{2+}$  channel  $\alpha 1\text{G}$ ,  $\alpha 1\text{C}$ , and  $\alpha 1\text{E}$  (31), and transient receptor potential related channel 3, trpc3 (EMBL 189 Y13758). Roman numbers indicate domains of voltage-activated  $\text{Ca}^{2+}$  channel  $\alpha 1$  subunits. Positively charged residues in polycystin-shared motif and S4 segment are marked with a plus sign; negatively charged residues in pore-loop and S6 segment are marked with a minus sign.

that are predicted to form short helical structures (pore-helix); and finally, last 1/3 of pore regions that begins with negatively charged residues which have been considered to determine the selectivity to  $\text{Ca}^{2+}$  in known  $\text{Ca}^{2+}$  channels (30).

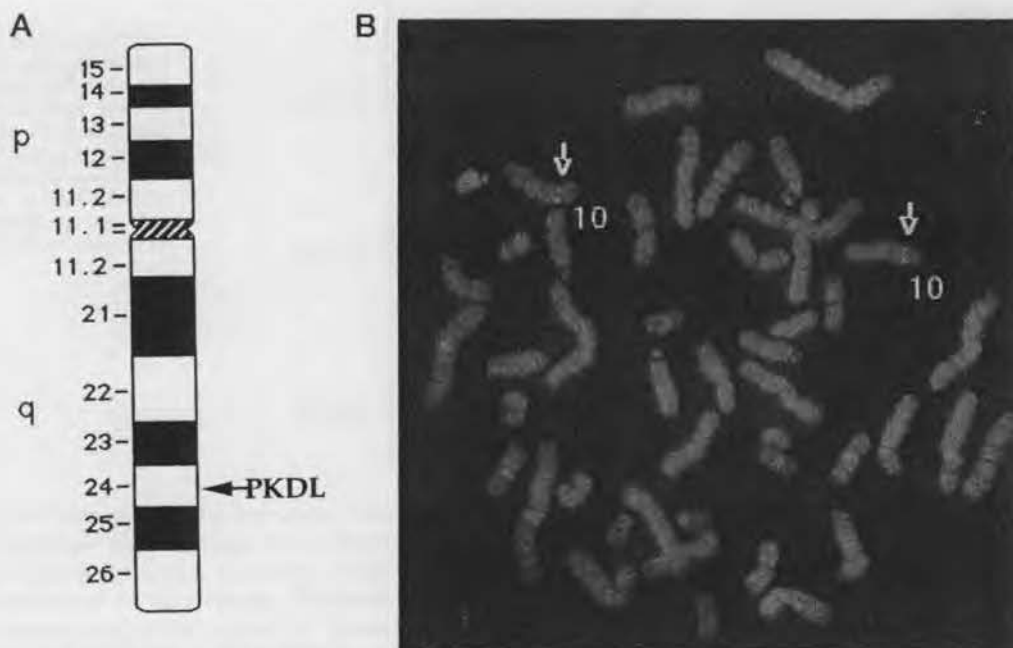
Polycystin-L differs from polycystin-2 most significantly in the N-terminal cytoplasmic domain where it lacks a 100-amino acid segment. In the C-terminal cytoplasmic domain, polycystin-L is strongly predicted to have a coiled-coil structure, which has the potential to tightly interact with molecules with a similar structure like polycystin-1. Lupas' algorithm (12) also

predicts a coiled-coil structure in polycystin-2, but it is not supported by Berger's algorithm (13).

Polycystin-L and polycystin-2 have three positively charged residues in S4 as opposed to five to eight in voltage-gated channels. Whereas the S4 region in voltage-gated  $\text{Ca}^{2+}$  channels is considered to be a voltage sensor (31), it is not clear whether a membrane-spanning region with only three basic residues could act as a voltage sensor. Polycystin-L also has several putative phosphorylation sites: one cyclic nucleotide, two protein kinase C, and four casein kinase II phosphorylation



**FIG. 3. Expression of PKDL gene.** The first 1.5 kb of coding sequence was used as a probe. **A**, RNA dot blot hybridization analysis. Dot A1, whole brain; A2, amygdala; A3, caudate nucleus; A4, cerebellum; A5, cerebral cortex; A6, frontal lobe; A7, hippocampus; A8, medulla oblongata; B1, occipital lobe; B2, putamen; B3, substantia nigra; B4, temporal lobe; B5, thalamus; B6, subthalamic nucleus; B7, spinal cord; C1, heart; C2, aorta; C3, skeletal muscle; C4, colon; C5, bladder; C6, uterus; C7, prostate; C8, stomach; D1, testis; D2, ovary; D3, pancreas; D4, pituitary gland; D5, adrenal gland; D6, thyroid gland; D7, salivary gland; D8, mammary gland; E1, kidney; E2, liver; E3, small intestine; E4, spleen; E5, thymus; E6, peripheral leukocyte; E7, lymph node; E8, bone marrow; F1, appendix; F2, lung; F3, trachea; F4, placenta; G1, fetal brain; G2, fetal heart; G3, fetal kidney; G4, fetal liver; G5, fetal spleen; G6, fetal thymus; G7, fetal lung; H1, yeast total RNA 100 ng; H2, yeast tRNA 100 ng; H3, *Escherichia coli* rRNA 100 ng; H4, *E. coli* DNA 100 ng; H5, poly(rA) 100 ng; H6, human Cot-1 DNA 100 ng; H7, human DNA 100 ng; H8, human DNA 500 ng. Northern analysis with mRNA blots containing human adult (**B**) and human fetal tissues (**C**).



**FIG. 4. Chromosomal localization of the human PKDL gene.** **A**, ideogram of human chromosome 10 showing the map location of human PKDL at 10q24 (arrow). **B**, photograph of human metaphase chromosome counterstained with 4,6-diamidino-2-phenylindole dihydrochloride. The two chromosomes are indicated by numbers. Arrows point to the site of hybridization of the digoxigenin-labeled human PKDL on both chromosomes 10 in band q24.

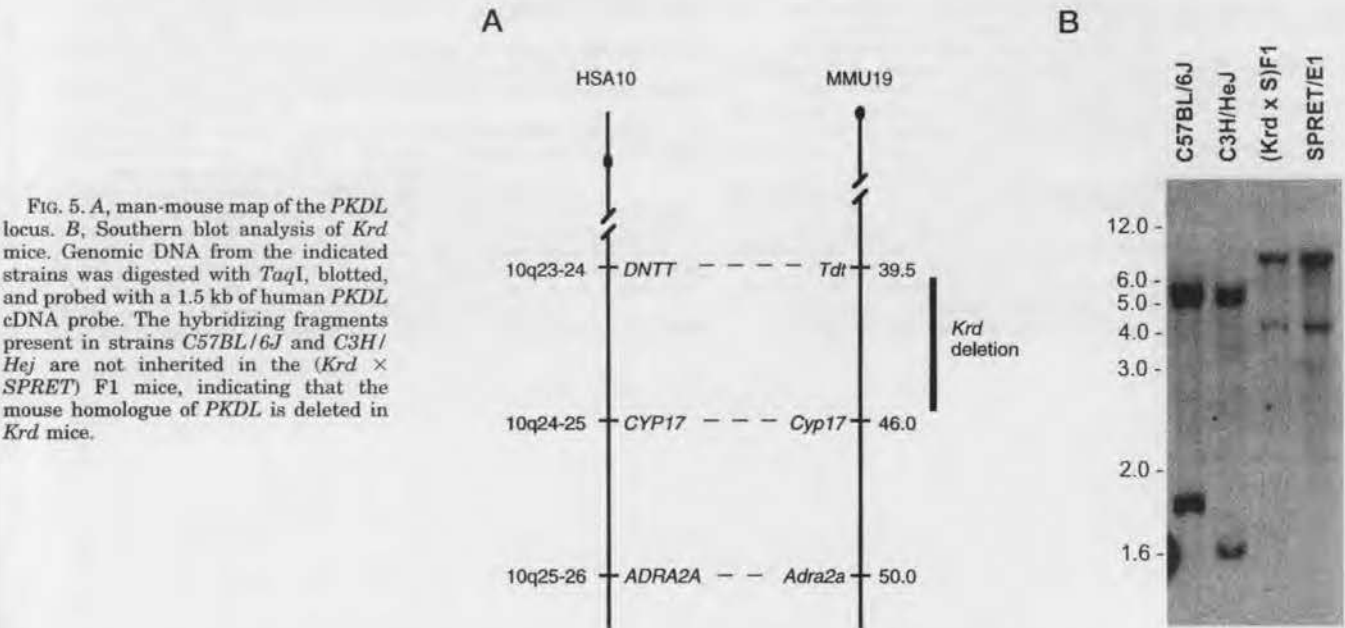
sites with strong motif sequences in the C-terminal cytoplasmic domain. Two other putative protein kinase C phosphorylation sites are also found in the N-terminal cytoplasmic domain. Phosphorylation of these motif sequences may be involved in the gating process of the channel. Another scenario is that the channel is gated by a direct or indirect signal from associating proteins, *e.g.* polycystin-1. Given that polycystin-1 has domains that may be involved in cell-cell or cell-matrix interaction and is known to interact with polycystin-2 (7, 8), we hypothesize that the binding of ligand(s) to polycystin-1 may be associated with the gating of a polycystin-related channel.

Sequence analysis and comparison to other channels support the six or seven membrane-spanning plus one pore-region topology of polycystin-2 and polycystin-L. In addition to the five putative transmembrane segments, the middle of the three relatively hydrophobic peaks, which corresponds to S4 in  $\alpha 1$  subunits of cation channels, is likely to be another transmembrane segment. Whether the N-terminal peak (S1/2) forms a membrane-spanning region is not clear.

One common feature of the polycystin-L/polycystin-2 structure that is rarely observed in known ion channels is that they both have relatively long extracellular loops between the first

TABLE I  
Individual and cumulative lod scores from six families

Family Bulga 1 is not informative for all markers.						
Family	Theta	0	0.05	0.1	0.2	0.3
F431	D10S198	0	0	0	0	0
	D10S603	-0.721	-0.463	-0.317	-0.149	-0.060
	D10S192	-0.721	-0.463	-0.317	-0.149	-0.060
F432	D10S198	-∞	-1.184	-0.761	-0.343	-0.136
	D10S603	0	0	0	0	0
	D10S192	-2.698	-1.376	-0.862	-0.380	-0.148
TOR1	D10S198	0.700	0.599	0.500	0.300	0.160
	D10S603	-0.770	-0.510	-0.390	-0.260	-0.190
	D10S192	-1.110	-0.850	-0.690	-0.430	-0.250
TOR2	D10S198	0.700	0.599	0.500	0.300	0.160
	D10S603	-0.770	-0.510	-0.390	-0.260	-0.190
	D10S192	-1.110	-0.850	-0.690	-0.430	-0.250
Singa 1	D10S198	0	0	0	0	0
	D10S603	0	0	0	0	0
	D10S192	0.602	0.535	0.465	0.318	0.170
Cumulative	D10S198	-∞	0.014	0.239	0.257	0.184
	D10S603	-2.261	-1.483	-1.097	-0.669	-0.440
	D10S192	-5.037	-3.004	-2.094	-1.071	-0.538



and the second putative transmembrane segments. Although this loop region does not show high homology to any known ion channels, polycystin-2 and polycystin-L maintain a high level of homology with each other in this region. Moreover, this region contains a 13-amino acid stretch with 3 to 4 basic residues that is conserved not only between polycystin-2 and polycystin-L but also with polycystin-1. The function of this polycystin-shared motif is not clear.

**Chromosomal Assignment and Linkage Studies**—Studies using D10S603, which maps to the same interval as *PKDL* by radiation hybrid mapping, and two adjacent markers, D10S192 and D10S198, did not reveal linkage in six non-PKD1, non-PKD2 families, making it unlikely that mutations in *PKDL* cause the disease in these families. Among other as yet unexplained human cystic kidney diseases, it is unlikely that *PKDL* plays a role in autosomal recessive polycystic kidney disease, as mutations in most autosomal recessive polycystic kidney disease families have been mapped to chromosome 6 (32). The *PKDL* locus can, however, be considered as a candidate for unmapped human genetic cystic disorders such as dominantly transmitted glomerulocystic kidney disease of postinfantile onset (33), isolated polycystic liver disease (34), and Hajdu-

Cheney syndrome/serpentine fibula syndrome (35, 36).

The region syntenic to the human *PKDL* locus is located on chromosome 19 in mice (26). This region is partially deleted in mice with the mutation *Krd* (Kidney and retinal defects) (27). The 7 centimorgans *Krd* deletion is located between *Tdt* and *Cyp17* and includes the paired box gene *Pax2*. Mice heterozygous for a null mutation of *Pax2* frequently demonstrate reduction in kidney weight, which ranges from 10 to 100% normal (37). The reduced size is due mainly to calyceal and proximal ureteral diminution as well as cortical thinning, with a reduced number of developing nephrons (37). In contrast, the phenotype of *Krd*/+ heterozygotes includes aplastic, hypoplastic, and cystic kidneys, as well as reduced viability on strain C57BL/6J (27). Our Southern analysis demonstrates that the mouse ortholog of *PKDL* is deleted in *Krd* mice. Further study is needed to clarify the contribution of *Pkd* to the *Krd* phenotype.

Several other congenital murine and rat models with polycystic kidney disease are also known to exist, although the genetic defects in these models are as yet to be identified (38, 39). Among mouse PKD models, loci for *cpk*, *bpk*, *pcy*, *jck*, *jeprk*, *kd* have been mapped to mouse chromosomes 12, 10, 9, 11, 10, and 10, respectively, and are unlikely to involve the mouse

homologue of *PKDL*. In Han:SPRD cy/+ rat, the disease gene was mapped to rat chromosome 5, whose human syntenic region resides on human chromosome 8 (18).

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