Prevention of Treatment Related Adverse Effects in Cystic Fibrosis

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ABSTRACT

Cystic fibrosis (CF) is one of the commonest life-limiting genetic disorders in the Caucasian population. Management involves frequent administration of antibiotics including aminoglycosides. With improving survival, it is time to focus on various age-related and treatment-associated adverse influences. The objective of this research was to evaluate renal function in CF, determine the effects of cumulative antibiotic exposure and to identify ways to reduce associated comorbidity.

A cross-sectional study showed that a small number of adults and children with CF had low glomerular filtration rate (GFR), and there was no association between GFR and cumulative antibiotic exposure. An above normal GFR was identified in one in four children with CF. Estimated GFR calculated by creatinine-based equations did not accurately predict the GFR measured by the gold standard ^{51}Cr-EDTA (^{51}chromium-ethylenediamine tetraacetic Acid).

Pure tone audiograms identified a raised hearing threshold in one in four people with CF, which did not correlate with increasing aminoglycoside exposure. A randomised controlled study established that there is no difference in the pharmacokinetics of tobramycin when administered intravenously in the morning or evening. A Cochrane systematic review concluded that there was insufficient evidence to support a routine use of bronchoalveolar lavage in the management of pulmonary infections with *Pseudomonas aeruginosa* in children with CF below 5 years old.

CF gene (Cystic Fibrosis Transmembrane Conductance Regulator, CFTR) is expressed in pig kidneys. Histological and molecular experiments established that there is no difference between the newborn pigs with genotypes CFTR -/- (knockout) and CFTR +/-

(heterozygous) or CFTR +/+ (wild-type) pig kidneys in the renal morphology and in the expression of various renal endocytic receptor proteins.

The vascular haemodynamic parameter, augmentation index ascertained in a small group of children with CF suggests a possibility that the vascular age may be advanced in people with CF right from their childhood.

In summary, these studies have established a low prevalence of renal disease in CF and a lack of association between cumulative antibiotic exposure and GFR. Further research is needed to evaluate the natural history of high GFR in paediatric CF population. Kidneys from pig model of CF may provide an alternative model to investigate the renal disease in CF.

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ABBREVIATIONS

Abbreviation	Term
^{51}Cr-EDTA	^{51}Chromium-Ethylenediamine Tetraacetic Acid
^{99m}Tc-DTPA	^{99m}Technitium-Diethylenetriamine Pentaacetic Acid
^{125} I-Iothalamate	^{125}Iodine-Iothalamate
18S	18S ribosomal RNA
A. xylosoxidans	*Achromobacter xylosoxidans*
ABC	ATP-Binding Cassette
ACR	Albumin-Creatinine Ratio
ACTB	Beta-Actin
Adj.	Adjusted for
ADQI Group	Acute Dialysis Quality Initiative Group
AER	Albumin Excretion Rate
AIN	Acute Interstitial Nephritis
AIx	Augmentation Index
AIx@HR75	Augmentation Index Adjusted to Heart Rate 75 by the Equation
AIx_HR75	Augmentation Index Adjusted to Heart Rate 75 by the Sphygmocor Software
AKI	Acute Kidney Injury
AKIN	Acute kidney injury Network
AMN	Amnionless
AP	Augmentation Pressure
ASL	Airway Surface Liquid
ATP	Adenosine Triphosphate

Abbreviation	Term
AUC	Area Under the (concentration-time) Curve
AVP	Arginine Vasopressin
BAL	Bronchoalveolar Lavage
B. cepacia	*Burkholderia cepacia*
BMI	Body Mass Index
BNMS	British Nuclear Medicine Society
bp	Base Pairs
BPM	Beats Per Minute
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
CEFIT CF	Cumulative Effects of Intravenous Treatment in Cystic Fibrosis
CF	Cystic Fibrosis
CF-CT Score	Cystic Fibrosis Computed Tomography Score
cfPWV	Carotid-Femoral Pulse Wave Velocity
CFQoL	Cystic Fibrosis Quality of Life Questionnaire
CFQ-R	Cystic Fibrosis Questionnaire-Revised version
CFRD	CF-related Diabetes
CFTR	Cystic Fibrosis Transmembrane Regulator
CFU	Colony Forming Units
CI	Confidence Interval
CKD	Chronic Kidney Disease
CKD-Epi	Chronic Kidney Disease Epidemiology Collaboration
CL	Total Drug Clearance

Abbreviation	Term
CLcr	Creatinine Clearance
CoV	Coefficient of Variation
Cp	Crossing point (in quantitative PCR)
CRITIC	Circadian Rhythm In Tobramycin Elimination in Cystic Fibrosis
CRP	C-Reactive Protein
Ct	Cycle Threshold
DAB	3,3'-Diaminobenzidine
DBP	Diastolic Blood Pressure
DLMO	Dim Light Melatonin Onset
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
dsDNA	Double-stranded Deoxyribonucleic acid
DVP	Digital Volume Pulse
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immuno Sorbent Assay
ENaC	Epithelial Sodium Channel
ESRD	End-Stage Renal Disease
FEV_1	Forced Expiratory Volume in One Second
FVC	Forced Vital Capacity
GC	Guanine Cytosine
GFR	Glomerular Filtration Rate
H&E	Haematoxylin and Eosin
H. influenzae	*Haemophilus influenzae*
HIER	Heat Induced Epitope Retrieval

Abbreviation	Term
HMG-CoA reductase	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A Reductase
HR	Heart Rate
HR75	Heart Rate 75 Beats per Minute
HRCT	High-Resolution Computed Tomography
HRP	Horseradish Peroxidase
hsCRP	High Sensitivity C-Reactive Protein
IATA	International Air Transport Association
ICER	Incremental Cost Effectiveness Ratio
IDMS	Isotope Dilution Mass Spectrometry
IgA	Immunoglobulin A
IHC	Immunohistochemistry
IL-6	Interleukin 6
IPO8	Importin 8
IQR	Interquartile Range
ITS2	Internal Transcribed Spacer 2
IV	intravenous
KDIGO	Kidney Disease: Improving Global Outcomes
Kel	Total Elimination Rate Constant
Kelm	Metabolic Elimination Rate Constant
Kelr	Renal Elimination Rate Constant
KIM-1	Kidney Injury Molecule-1
KW Nodules	Kimmelstiel-Wilson Nodules
LCI	Lung Clearance Index
MAP	Mean Arterial Pressure

Abbreviation	Term
MD	Mean Difference
MDRD Equation	Modification of Diet in Renal Disease Equation
mGFR	Measured Glomerular Filtration Rate
MHRA	Medicines and Health Care Products Regulatory Agency
MIC	Minimum Inhibitory Concentration
mRNA	Messenger Ribonucleic Acid
MSD	Membrane-Spanning Domain
MWCO	Molecular Weight Cut-off
NAG	N-Acetyl-beta-D-Glucosaminidase
NBD	Nucleotide-Binding Domain
NHS	National Health Service
NKF KDOQI	National Kidney Foundation Kidney Disease Outcomes Quality Initiative
NTC	No Template Negative Control
OD	Optical Density
OI	Operator Index
OR	Odd's Ratio
ORCC	Outwardly Rectifying Chloride Channels
Oxalobacter formigenes	*O. formigenes*
P. aeruginosa	*Pseudomonas aeruginosa*
PBB	Protected Bronchial Brushing
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PEF	Peak Expiratory Flow

Abbreviation	Term
PK	Pharmacokinetic
PKA	Protein Kinase A
PP	Pulse Pressure
PPI	Proton Pump Inhibitor
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity
QALY	Quality Adjusted Life Years
QoL	Quality of Life
QPCR	Quantitative Polymerase Chain Reaction
RBP	Retinol Binding Protein
RNA	Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
RIFLE Criteria	Risk, Injury, Failure, Loss and End-Stage Renal disease Criteria
pRIFLE Criteria	Paediatric (Risk, Injury, Failure, Loss and End-Stage Renal disease) Criteria
ROMK	Renal Outer Medullary Potassium Channel
ROS	Reactive Oxygen Species
RPO	Ribosomal Phosphoprotein
RR	Risk Ratio
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RWF	Radiation Weighting Factor
S. aureus	*Staphylococcus aureus*
S. maltophilia	*Stenotrophomonas maltophilia*

Abbreviation	Term
SBP	Systolic Blood Pressure
SCNT	Somatic Cell Nuclear Transfer
SEM	Standard Error of Mean
SD	Standard Deviation
SI Unit	System International Unit
SI_{DVP}	Stiffness index
SIR	Standardised Incidence Ratio
SNHL	Sensorineural Hearing Loss
$t_{1/2}$	Half-life
TAE	Tris-Acetate-EDTA
TBC	Total Body Clearance
Tm	Melting Temperature
TNR-CFTR	Truncated-CFTR
TIVAD	Totally Implantable Venous Access Device
UDG	Uracil DNA Glycosylase
UV	Ultraviolet
VBA	Visual Basic Application
V_1	Volume of Distribution per Kg Body Weight
Vd	Volume of Distribution
Vs	Versus
YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta Polypeptide
Z Score	Standard Score

Chapter-1 Introduction

There are currently nearly 10,000 people with cystic fibrosis (CF) in the UK (*1*). For individuals born in 1968-70, the median survival was 15 to 20 years. For those born in the year 2000, predicted median survival is over 50 years (*2*).

With improved life expectancy, new complications have emerged, such as CF-related diabetes (CFRD), CF liver disease and reduced bone mineral density. Furthermore, treatment-related adverse effects could play a significant role in the overall morbidity of CF. Many patients require frequent antibiotic treatment to reduce lung damage caused by chronic pulmonary infection, particularly with *Pseudomonas aeruginosa (P. aeruginosa)* – an organism, which responds to a limited number of antibiotics. One class of antibiotics, the aminoglycosides, includes several molecules, which are active against *P. aeruginosa*, and so these drugs are frequently used in CF. Unfortunately, the aminoglycosides have some serious side effects, including nephrotoxicity.

Although there is no known renal phenotype of CF, there are studies to suggest increased risk of acute renal failure (now called acute kidney injury, AKI) (*3*) and chronic kidney disease (CKD) (*4*) in these individuals. In addition to aminoglycoside exposure, there are a number of other risk factors for renal damage which include other antibiotics (such as the polymixin antibiotic colistin) (*5*) and CFRD (*4*).

Renal dysfunction is one of the commonest complications in the post-lung transplant period (*6*). People with CF have a more rapid decline in renal function compared to other groups of people receiving lung transplant possibly related to their higher age at transplant (*7*). Altered renal reserve in the pre-transplant period increases the risk even further (*8*), which may affect suitability for receiving a lung

transplant and the post-transplant outlook for people with CF. In addition, impaired renal function may contribute towards accelerated ageing of the vasculature and haemodynamic changes, such as increased arterial stiffness - an independent marker of poor cardiovascular outcome (*9*).

Given the impact which renal impairment can have on the quality of life and survival of individuals with CF and their suitability for transplant, it is important to explore the full extent of renal complications further and develop monitoring and prevention strategies. This research was conducted with an objective to determine the mechanisms of renal impairment in CF (including the role of drug-related adverse effects) and identify ways to minimise these, through optimising antibiotic use and exploring the role of circadian rhythms in drug toxicity. As pigs closely mimic many features of human CF disease (*10,11*), we studied kidneys from CF model of pigs to understand the presentation and pathophysiology of renal disease in CF.

This text is an introduction to this research project carried out with an aim to prevent treatment-related adverse effects in CF with particular emphasis on renal complications.

1.1 Overview of CF

CF is a multisystem genetic disorder related to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Morbidity in CF is related to multiple organ systems that express the CFTR gene. The complex clinical picture of CF includes progressively worsening lung disease, pancreatic insufficiency and associated malnutrition, sweat electrolyte defect, liver disorder, infertility (related to the absence of the *vas deferens*) and nasal polyposis.

1.1.1 A short history of CF

It is supposed that CF appeared about 3000 BC (*12*). There was recognition of this entity since medieval times as perceived by some traditions and folklore, "a child whose forehead tastes like salt when kissed is bewitched and will die soon" (*12,13*). However, it would not be an exaggeration to state that CF is a disease of the 20th century. Many scientists and physicians have seen its course in their lifetime from the first description to discovery of the gene and attempts at gene therapy. This journey has witnessed improving survival from less than 4 years to more than 40 years in about 40 years (Figure 1-1)**.**

DH Anderson, a pathologist, first used the term "Cystic Fibrosis of the Pancreas" in 1938 and gave the first clinical and pathological description of a large series of patients (*14*). It was considered to be related to vitamin A deficiency due to pancreatic insufficiency (*15*), however, very soon after, it was identified that CF is a genetic disease and results from an autosomal recessive mutation (*16*).

Before the availability of antibiotics and any early diagnostic measures, only a few infants with CF survived infancy and early childhood (*17*). During the decade of the Forties, the introduction of penicillin was a breakthrough. As shown in Figure 1-1, penicillin was first used in CF in 1943. Heat prostration in children with CF during a heat wave in 1951 led to the recognition of increased salt content of

sweat in CF and formed a basis of introduction of sweat test as a diagnostic modality for CF (*18*).

A new outlook for management was described including early diagnosis, active early treatment and proper nutrition by Shwachman and Kulczycki. Survival beyond childhood was a remarkable achievement (*19*). At this time a treatment-related adverse effect was noticed in the form of discolouration of teeth present in 80% of CF children receiving long-term tetracycline prophylaxis (*20*).

During the next two decades (1960-1980, please refer to Figure 1-1), there was an extensive search for the primary defect, with numerous explanations but little success (*13*). Several organisations specialising in CF research were formed including the "UK CF Research Trust". Though the outlook was still poor for most patients in the Sixties, with aggressive and more comprehensive treatment approaches, more patients were surviving for longer. There were some encouraging reports including an estimated survival rate of 72% at 12 years and 45% at 20 years at a specialist paediatric CF centre in London (*17*)

With the improving survival, there was a realisation that good nutritional status was associated with better prognosis (*21*) and increasing interest in this field led to the involvement of dietitians in the management. From the early Eighties, acid resistant pancreatic enzymes were used, which later developed as commercial pancreatic enzyme supplements such as "Creon®" that is available as a prescription medicine. At the same time, it was established that dried blood spot immunoreactive trypsin was a reliable and convenient neonatal screening test (*22*). In the UK, newborn CF screening began in regions such as East Anglia (*23*) and expanded to become the national programme, which has been in place since 2007.

One major clinical feature of CF is impaired mucociliary clearance, which leads to retention of mucus, airway obstruction, atelectasis and

hyperinflation. Airway clearance techniques (chest physiotherapy) form an essential important aspect of management of CF. Role of airway clearance measures was recognised very early and has been described in literature from the early 1950s as an important aspect of the treatment regimen (*17*). There was, however, very limited research to find evidence of benefit from chest physiotherapy techniques mainly owing to its already perceived benefits and ethical issues of providing no physiotherapy. A small number of early studies, mostly conducted over short periods, compared outcomes including sputum clearance (with or without radioactive tracers) and lung function with or without physiotherapy showed better mucus clearance with chest physiotherapy compared to control intervention (mostly active coughing) (*24-29*).

Conventional physiotherapy regimen included assisted techniques including manual percussion and postural drainage (*26*). These led the way to techniques that patients could administer themselves including mechanically assisted methods (e.g. high-frequency chest oscillation method, positive expiratory pressure mask etc.) and to different breathing techniques to aid airway clearance (e.g. forced expiratory technique, autogenic drainage etc.) (*26*). Following concerns regarding gastro-oesophageal reflux associated with the head down tilt position during postural drainage, the practice has mostly changed to no head down tilt position for physiotherapy of infants (*30,31*). Most comparative studies including evidence from a systematic review have shown the equal efficacy of different airway clearance techniques (including conventional airway clearance methods) (*25,27,32,33*). Currently, the choice of airway clearance techniques is mainly led by local treatment trends and therapist' personal experiences rather than based on scientific evidence (*32*). The technique of high-frequency chest wall oscillations (vest) is in common practice in the USA; however, other airway clearance techniques are more commonly used

in the UK. This difference in practice is partly contributed by the high cost of the equipment for the vest and is probably somewhat related to local expertise and lack of evidence of the superiority of vest over other methods (*34,35*). Role of exercise is complementary to chest clearance methods (*25*). However, there is insufficient evidence to suggest that physical activity can be an alternative to airway clearance techniques (*27*). In recent years, airway clearance techniques have been combined with inhalation therapies, e.g. bronchodilators, mucolytic agents, inhaled antibiotics and inhaled steroids. In 1989, the CF gene was identified and was named the cystic fibrosis transmembrane conductance regulator (CFTR) (*36*). This was followed by an era of considerable improvement in knowledge and clinical care of CF (Figure 1-1). Many new therapies were introduced with steady improvement in survival. The concept of specialist CF centres was accepted, and comprehensive care was delivered with multidisciplinary input. Earlier, more frequent and intensive courses of intravenous (IV) antibiotics were added to management or treatment of exacerbations (*37*). Another significant development was the success of heart-lung transplant in CF (*38*).

A significant addition to CF treatment was the use of mucolytic, rhDNase (recombinant human deoxyribonuclease, proprietary name Pulmozyme®) which produces sustained improvement in lung function (*39-41*). Furthermore, evidence for the efficacy of eradication treatment for early *P. aeruginosa* infection was found (*42,43*); currently, this is a standard clinical practice in many countries. Following the reports of cross-infection between patients with CF (*44*), segregation of patients, according to their infection status, e.g. *P. aeruginosa* infection, is practised at many centres and recommended in national guidelines (*45*),(*46*).

Since the Nineties through the next two decades, a critical research area has been the development of gene replacement therapy and

understanding of the role of CFTR to help develop novel therapies. Many animal models have been created to help understand the pathophysiology. The development of the pig model of CF in 2008 is a recent, significant innovation (*11*).

Survival data for patients with CF born from 1968 up to 2003 have been published (*2*). Figure 1-2 (a and b) depict a continuous improvement in the life expectancy of 3-year cohorts of male and female patients respectively, who were born in the period 1968-1994.

The UK CF Registry is a secure, centralised database recording demographic, health and treatment data of consenting people (or parents for children) with CF in the UK; about 99% patients with CF are registered with it, and 89% of patients (*47,48*) have complete data (defined as data required to produce key clinical outcomes). Reports from the UK CF Registry suggest that currently there are more adults than children with CF (*1*). Median predicted survival of the patients with CF is nearly 40 years (*1*). Results are similar to those published in the 2014 annual report of the US CF Foundation (*49*).

According to a survey of adults with CF in the UK (*50*), including nearly 1000 patients, a high proportion of individuals were living full and productive lives: 26% of men and 44% of women were married or cohabiting (compare from 63% of women from total population who were married or cohabiting (*51*)), and 55% people with CF were in paid employment (compare from 70% of the general population aged 16 to 64 years (*52*) who were in employment). This survey was conducted in the early nineties. More recent data come from the UK CF Registry reports, which show nearly 70% of adults with CF are either employed or are in education (*1,49*) (compare from 79% of the UK population between 16-64 years who were in employment or education during October to December 2014 (*52,53*).

Given the continuing improvement in survival of CF patients in successive cohorts, the previous prediction of median survival of >50 years of age for individuals born in the year 2000 continues to look realistic (*2*). Recent survival analysis based on the Canadian CF Registry showed a median survival age of 49.7 years in a 5 year period ending in 2012 (*54*). Emerging therapies aimed at correcting the underlying CF defect make the prospects even more promising.

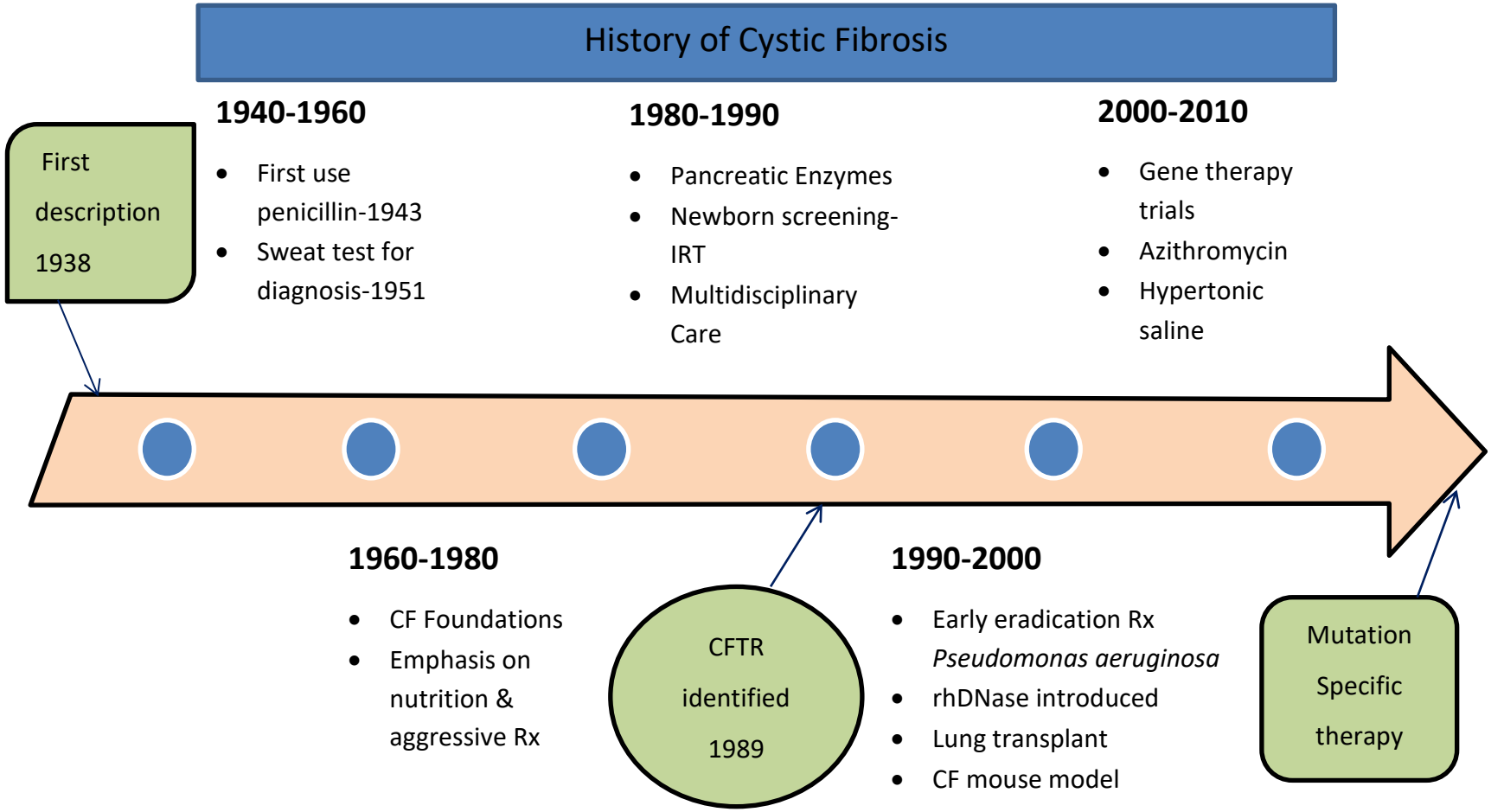


Figure 1-1: The History of CF

CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator; IRT: immune reactive trypsin; Rx: treatment; rhDNase: recombinant human deoxyribonuclease

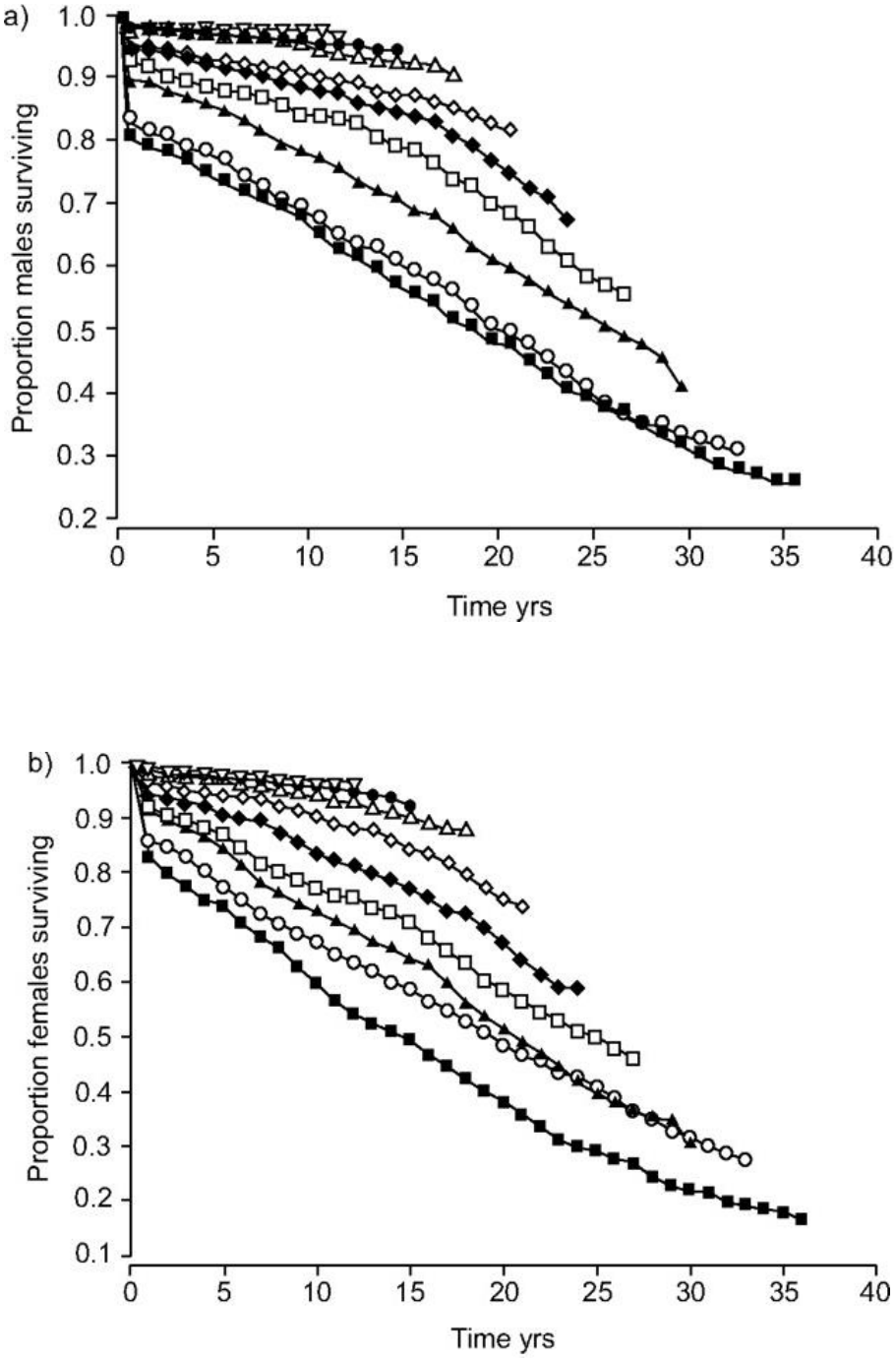


Figure 1-2: Proportion of persons of each 3-yr cohort surviving until 2003.a: males; b: females(Reference: Dodge et al., 2007 (2));▪:1968–1970; ○: 1971–1973; ▲: 1974–1976; □: 1977–1979; ♦: 1980–1982; ◇: 1983–1985; △: 1986–1988; ●: 1989–1991; ▽: 1992–1994.

1.1.2 Genetics

CF is the most common, life-limiting, autosomal recessive disorder in Caucasians. It is caused by mutations in CFTR gene, which is a 230 kb gene located on long arm of chromosome 7 and codes for CFTR protein. The CFTR protein functions as cyclic adenosine monophosphate (cAMP)-mediated chloride channel across the cell membrane and also has other cellular functions related to ion and fluid balance (*55*).

1.1.2.1 CFTR mutations

Nearly 2000 mutations have been identified in CFTR (cystic fibrosis mutation database, (http://www.genet.sickkids.on.ca) (*56*)) but a smaller number are associated with a disease phenotype. CFTR mutations are classified according to the mechanism of CFTR dysfunction (*57,58*) (please refer to Table 1-1). Class I mutations, e.g. Gly542X (G542X) affect the synthesis of CFTR. Premature stop codons are created by different mechanisms like nonsense mutations, insertions and deletions that produce frameshift and splice site abnormalities (*59*). There are few or no functioning CFTR chloride channels. Class II mutations involve defective processing of the CFTR protein by the endoplasmic reticulum due to which the protein fails to reach the site of action. This class includes Phe508del, which is the commonest CF-related mutation worldwide, present in nearly 70% of defective alleles. With class III mutations, e.g. Gly551Asp (previously termed G551D), CFTR protein is synthesised and trafficked to the apical membrane, but it does not react to activation signals by cAMP or ATP (adenosine triphosphate). Class IV mutations, e.g. R117H reduce the chloride current and alter the conductance of the chloride channels of the CFTR protein in the apical membrane. Previously described class V mutations (e.g. A455E, now merged with class I) (*58*) were associated with less severe phenotypes and were related to splicing defects leading to reduced production of normal CFTR. A new

class of mutations (Class VI), e.g. Q1412X results in inherently less stable CFTR protein despite normal function (*60*).

Class I-III mutations are associated with pancreatic insufficiency, which is, in turn, a marker for more severe disease (*58*). Furthermore, the correlation of CF mutation and phenotypic expression varies between different clinical components. There is a high correlation of CFTR genotype with pancreatic status whereas there is a weak correlation with severity of lung disease. This factor strongly suggests an influence of environmental and secondary genetic factors on lung disease (*57,58,61*). Some recent studies have shown an association between different severe genotypes and CF pulmonary phenotypes (*62,63*). CF patients carrying two class I mutations had lower lung function (forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) compared to the group with either a combination of class I and class II mutations or two class II mutations (*62,63*).

There may be overlap between different types of mutations, e.g. Phe508del while belonging to class II mutations, also presents reduced sensitivity to activating signals (*64*). A modified, expanded classification has been proposed recently including the original VI classes and their 26 combinations representing the complex phenotypes of CF alleles (*65*).

Table 1-1 CFTR mutation classes & molecular basis			
CFTR mutation Class	**Mechanism of CFTR dysfunction**		**Common examples**
	Molecular mechanism	**Cell surface CFTR protein defect**	
Class I	**CFTR synthesis defect** Premature stop codons or nonsense mutations stop the CFTR protein synthesis.	No protein / unstable truncated protein	G542X
Class II	**CFTR processing defect** Misfolding of CFTR protein causes premature degradation by the endoplasmic reticulum / Golgi bodies	No / small amount of protein	Phe508del
Class III	**Chloride channel regulation defect** Failure of CFTR protein to respond to activation signals by ATP/ cAMP	Normal amount of CFTR protein, but unresponsive with no functional chloride channels	G551D
Class IV	**Conductance defect of chloride channels** Decrease in amplitude of a single chloride channel current or reduced duration of channel opening	Normally responsive CFTR that generates reduced chloride current	R117H
Class V	**Splicing defect** Aberrant splicing of the mRNA	Reduced normal functional CFTR protein	A455E
Class VI	**Protein stability defect** Reduced biological stability of CFTR protein due to lack of 70-98 residues of the CFTR C-terminus	Reduced half-life of the CFTR protein	Q1412X

CFTR: cystic fibrosis transmembrane conductance regulator; mRNA: messenger ribonucleic acid; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate

1.1.2.2 Mutation specific therapies for CF

An understanding of molecular mechanisms of mutations has provided a scientific basis for the development of mutation specific therapy (*66*) including agents that enhance the mutant CFTR protein function (called as CFTR modulators). A number of CFTR modulators have been studied that address specific mutations (*67*). Read-through agents target class I mutations and allow ribosomes to continue translation beyond nonsense mutations allowing the formation of full-length CFTR protein. CFTR corrector agents facilitate the processing (folding/trafficking) of class II mutant CFTR protein to increase the quantity of functioning protein at the plasma membrane. CFTR potentiator agents increase the function of mutant type III/IV CFTR proteins at the plasma membrane by enhancing the open probability (class III) or single conductance (class IV) of CFTR channels. Splicing agents modulate the splicing to restore normal full-length CFTR. Stabilisers are the agents that affect protein degradation rate and correct instability of class VI mutants.

CFTR potentiator drug ivacaftor (formerly called VX-770) acts on the cell surface and potentiates effective opening of CFTR chloride channels. Ivacaftor (marketed as Kalydeco® by Vertex Pharmaceuticals Inc.) has been approved for use in >6-year-old patients (*68*) and more recently for >2-year-old children with CF having a G551D mutation or 8 other non G551D gating mutations (*69,70*). There is good quality evidence suggesting a significant increase in FEV_1 (about 10%), decrease in sweat chloride concentration and increase in weight in patients with named mutations receiving treatment with ivacaftor (*71-75*). Furthermore, recent evidence suggests that rate of decline of lung function was slowed by about half over a 3 year period of receiving ivacaftor when compared to homozygous Phe508del control patients who were not eligible to receive the drug (*76*). However, it is described as one of the world's most expensive drugs, and the ethics of such

high cost has been questioned (*77*). Cost-effectiveness analysis for the use of ivacaftor suggested that incremental cost-effectiveness ratio, ICER (difference in cost between two interventions divided by the difference in their effects or cost per Quality Adjusted Life Year, QALY) varies between £335,000 and £1,274,000; total additional lifetime cost for all eligible people with CF in England ranged from £438M to £479M compared to the lifetime cost for standard care which is £72M (*78*).

Another drug that has received marketing authorisation is a combination (proprietary name Orkambi®, from Vertex Pharmaceuticals Inc.) of corrector and potentiator drugs (lumacaftor and ivacaftor respectively). Phase III trials using this combination therapy in Phe508del homozygous CF patients showed a modest increment in lung function (between 2.6-4% increase in percent predicted FEV_1) and a reduction in the rate of pulmonary exacerbations (30-40% lower than placebo) (*79*). However, the drug has been challenged profoundly, mainly related to cost-effectiveness of its use (due to its only modest clinical effect and a very high cost: increase in ICER of 349,000 per QALY), and uncertainty about effectiveness in long-term (*80,81*). In vitro studies have shown that chronic use of potentiators (e.g. ivacaftor) reduces the efficacy of correctors (e.g. lumacaftor) by decreasing the folding efficiency and metabolic stability of Phe508del CFTR during processing within the cells thus reducing the cell surface CFTR density and function (*82,83*).

Monotherapy with corrector drug, lumacaftor (formerly VX-809), studied in a phase II trial with Phe508del homozygous patients did not result in any improvement in FEV_1 (*84*). Ataluren (formerly PTC-124) is a read-through agent that allows ribosomal read-through of the premature stop codons in messenger ribonucleic acid (mRNA) to improve production of full-length CFTR. Phase II studies comparing ataluren against placebo showed significant improvement in total chloride transport assessed by nasal potential difference and a slight

increase (though non-significant) in lung function (*85-87*) with ataluren. However, phase III study (*88*) on a larger group of class I mutant patients with CF did not demonstrate any clinical benefit of ataluren. As aminoglycosides (*89*) can also improve read-through of premature stop codons, a possible interaction of ataluren with aminoglycosides could have contributed to the lack of effect found in the phase III ataluren study, which was confirmed by an in vitro study (*88*). A post hoc analysis on a subgroup of patients not receiving chronic inhaled aminoglycoside therapy showed a 5.7% increase (from baseline) of percent predicted FEV_1 and 40% fewer exacerbations in the group using ataluren compared to placebo. The safety profile of ataluren suggested an increased frequency of raised serum creatinine levels compared to placebo when used in combination with nephrotoxic systemic antibiotics (e.g. aminoglycosides/vancomycin).

A recently published, phase III randomised controlled study evaluating combination of CFTR modulators tezacaftor (VX-661) and ivacaftor in CF patients, >12years age and homozygous for CFTR mutation Phe508del, showed a 4% increase in FEV1 and 35% reduction in rate of pulmonary exacerbations in the people receiving the combination drug compared to the group receiving placebo (*90*). Many more studies are underway assessing alternative pharmacological agents or combinations/doses etc. (*67*). CFTR modulators, though promising, still have a long way to go before personalised mutation based treatment can become a norm to be available to most people with CF.

1.1.3 Lung Disease in CF

Pulmonary insufficiency is responsible for at least 80% of CF-related deaths (*64*),(*91*), the salient feature being unrelenting lower respiratory infections which, over the course of time, worsen to cause bronchiectasis and other structural changes leading to respiratory failure.

1.1.3.1 Pathophysiology of lung disease in CF

Babies with CF are born with essentially healthy lungs (*92-94*) and normal lung function (*94*) but very soon after, there is a continuous cycle of infection and an exaggerated inflammatory response (*95*), leading to airway structural changes referred to as airway remodelling. Furthermore, it has been speculated that, in addition to infection and inflammation, CFTR dysfunction may cause other changes that lead to airway remodelling. Evidence supporting this concept includes the finding of subtle prenatal changes in CF foetuses and airflow obstruction in very young children with CF who have not had any respiratory complications. Furthermore, histopathological changes have been observed in endobronchial biopsies from children with CF, which appear to be independent of infection and inflammation, e.g. abnormal epithelial regeneration, epithelial thickening, increased reticular basement membrane thickness and mucous cell numbers (*96*). In addition, studies on newborn pig model of CF have suggested that after intrapulmonary bacterial challenge, they failed to eradicate bacteria as efficiently as wild-type pigs, thus indicating a possibility of bacterial host defect soon after birth (*10*).

CF lung disease appears to reflect a failure of airway defence mechanisms to prevent infection. How the CFTR abnormality leads to clinical features (specifically lung disease) is not clear, and several hypotheses have been proposed. Until recently the “low airway surface liquid (ASL)” or “dehydration” hypothesis was most widely

accepted. This has been superseded by low airway surface pH hypothesis, which was proposed after experiments on pig model of CF.

1.1.3.1.1 Low Airway Surface Liquid/Dehydration theory

Normal airway mucosa is a two-phase liquid system that interfaces with the ciliary beating. The upper phase (mucous layer produced by submucosal glands and goblet cells) is composed of high molecular weight mucin molecules (MUC5A and MUC5B) (*97*). The lower phase is the periciliary fluid layer. This 2-layer system allows smooth ciliary beating and is essential for mucociliary clearance of microorganisms. The proficiency of this system heavily depends on the hydration status of airway surfaces, which is maintained by CFTR-dependent regulation of the relative rates of sodium absorption and chloride secretion across airway epithelia (*97-99*). As CFTR negatively regulates the epithelial sodium channels (ENaC), lack of CFTR function leads to unrestricted absorption of sodium and failure of chloride secretion (CFTR itself is a chloride channel). Consequently, there is absorption of water molecules following the osmotic gradient. The resultant dehydration of ASL affects both layers leading to highly viscoelastic mucous layer and a collapsed periciliary layer with lack of lubricant activity that causes adhesion of mucus to the airway surface (*100*). This results in an inefficient ciliary beating and reduced mucociliary clearance. It may also influence secondary defence mechanisms, e.g. the ability of neutrophils to migrate in the mucin layer for bacterial killing or diffusion of antimicrobial substances (*101*).

It has been suggested that CF airway epithelia persistently remain in a modestly dehydrated state, which leads to mucus concentration and ciliary adhesion to airway surfaces. This is worsened by intercurrent insults e.g. viral infections, leading to further ASL depletion (*98*).

1.1.3.1.2 Reduced ASL pH hypothesis:

Contrary to the ASL dehydration hypothesis, studies on airway epithelia from newborn pig model of CF showed that lack of CFTR did not increase trans epithelial sodium or liquid absorption or reduce ASL depth (i.e. dehydration) (*102*). Similarly, study on human CF airway (*103*) did not show increased absorption of sodium. Two other human tissues that express CFTR and ENaC, salivary glands and sweat gland ducts and submucosal glands, do not express increased absorption of sodium in CF (*104*). It is possible that the increase in sodium absorption is not a primary phenomenon directly related to loss of CFTR but reflects secondary changes in airways with disease progression (*104*).

Recent studies suggest that CFTR secretes bicarbonate (*105*) and lack of CFTR prevents bicarbonate secretion in porcine airway epithelium (*102*). Humans (*106*) and pigs (*102*) lacking CFTR have a bicarbonate (HCO_3) secretion defect along with unchecked H+ secretion by the non-gastric H^+/K^+ adenosine triphosphatase (ATP12A) (*107*). This causes abnormally low pH of ASL, which inhibits antimicrobial activity (*108*). In addition, lack of CFTR in submucosal glands causes mucus to develop abnormal biophysical properties, which lead to defective mucus release (*104,109*) and diminished mucociliary clearance, eventually leading to defective bacterial killing. Studies on newborn pig model of CF showed markedly decreased chloride and bicarbonate transport (*102*). Reducing the ASL pH resulted in decreased bacterial killing in the wild-type pig airways whereas increasing ASL pH restored bacterial killing in the airways of pig model of CF (*108,110*).

1.1.3.2 Microbes causing pulmonary infections in CF

Traditionally, it has been believed that a limited number of organisms cause CF lung infection. *Staphylococcus aureus* (*S. aureus*) and *Haemophilus influenzae* (*H. influenzae*) usually colonise patients at an early age. This is often followed by infection with *P. aeruginosa*. This

organism initially infects the airway in planktonic form, when it may be vulnerable to host defence, and robust antibiotic treatment can help achieve eradication. However, *P. aeruginosa* frequently progresses to a mucoid phenotype, which can form biofilms. This form has increased resistance to antimicrobials (*111*) and establishes chronic infection. Acquisition of chronic infection with *P. aeruginosa* is associated with progressive worsening of lung function (*112*).

The cause of vulnerability of CF lungs to infection with *P. aeruginosa* is not completely understood (*113-115*). It has been suggested that the airway epithelia in CF lungs have a defective innate immunity towards some specific bacteria (*S. aureus and P aeruginosa)* (*116*). A failure of the oxidative antimicrobial system involving reactive oxygen species (ROS) has also been implicated (*117*). Cell to cell signalling by intercellular signal molecules has been proposed to be involved in the development of biofilms by *P. aeruginosa* (*118,119*).

There are other pathogens which can lead to infection in CF including *Burkholderia cepacia* (*B. cepacia*) species, Methicillin Resistant *S. aureus*, *Stenotrophomonas maltophilia* (*S. maltophilia*) and atypical mycobacteria (*120*). The role of primary airway infection with *Aspergillus* species is controversial (*121*), though *Aspergillus* can lead to respiratory symptoms and lung damage through allergic bronchopulmonary aspergillosis (*122*). Infection with *B. cepacia* and *P.aeruginosa* has been associated with increased mortality and faster decline in lung function compared to controls colonised with *P.aeruginosa* only (*123*). In some cases, *B.cepacia* can cause a rapid and progressive pneumonic illness called “Cepacia Syndrome” (*124*). *B cepacia* is inherently resistant to multiple antibiotics and is highly transmissible by individual contact.

1.1.3.3 Lung microbiome in CF

Historically lungs were considered sterile in health; however, studies based on culture-independent (e.g. 16S ribosomal ribonucleic acid (rRNA) sequencing) methods have demonstrated that the lower respiratory tract is full of diverse communities of microbes described as the microbiome. Culture-independent methods have similar findings from people with CF.

Compared to the conventional microbiological techniques (direct examination and culture), new molecular methods can lead to the detection of organisms which are difficult to grow or identify in a culture as well as new, yet unknown microorganisms. Pyrosequencing (a method of deoxyribonucleic acid (DNA) sequencing) variable regions of bacterial 16S rDNA (ribosomal DNA) and fungal ITS2 (internal transcribed spacer 2, a universal DNA barcode to identify plant and animal species) genes in eight sputum samples from four people with CF demonstrated several phylogenetically diverse organisms in addition to the previously described pathogens, from both bacterial and fungal taxonomies (*125*). The most represented genera identified in this study were *Pseudomonas*, *Streptococcus*, *Haemophilus* and anaerobes. Other genera isolated included *Prevotella*, *Fusobacterium*, *Veillonella* and *Prophyromonas* (*125*). New bacterial genera including *Gemella* species were found. Furthermore, a highly diverse and complex fungal microbiota was observed with more than 60% of the fungal species identified by pyrosequencing not identified by fungal cultures. Many additional species were identified within aspergillus and candida genera (*125*). Many of these organisms have not traditionally been associated with pulmonary pathology in CF, and some are normal residents of oropharyngeal microflora while others are opportunistic pathogens (*126*).

Fetal lungs are presumed to be sterile, and the initial microbiota is acquired after birth from various sources, e.g. vaginal or intestinal

microbiota in case of vaginal delivery and skin microbiota in case of caesarean section delivery (*127*). The initial microbiota is uniform across various sites of the body, which later differentiates into specific communities (*127*).

Healthy lung microbiome has been characterised in various studies (*127,128*). The most commonly identified phyla include *Bacteroides*, *Firmicutes* and *Proteobacteria (127)*. Predominant genera among healthy individuals include *Prevotella*, *Veillonella*, *Streptococcus, Haemophilus and Neisseria (129)*. Microbiota can shift with external influences, e.g. antibiotics, and it can be different in people from a different geographical area (*128*). Lung microbiome of healthy people shows higher diversity compared to the microbiome of patients with CF (*129*). More microbes identified from healthy subjects correspond to known oral flora compared to those from people with CF.

Microbiome from subjects with CF is highly variable and different from standard oral microbiome; it is dominated by one or few main organisms, e.g. *Pseudomonas*, *Staphylococcus*, *and Stenotrophomonas* etc. (*129*). A study of a large cohort of patients with CF including children showed that the core microbiota consisted of mainly 5 genera: *Streptococcus*, *Prevotella*, *Veillonella* and *Actinomyces* (*130*). CF associated pathogens were less prevalent than core genera, but they had a strong tendency to predominate when present (*130*).

The clinical relevance of this microbiota is not clear yet. There is a considerable heterogeneity in the composition of microbiota obtained from different patients regarding types and relative abundance of different bacterial groups; however, it does not alter much over time within individual subjects (*131*). Cross-sectional and longitudinal studies have shown that density (*131*) and diversity of microbiota is highest in early life up to adolescence and then declines into adulthood, plateauing at approximately age 25 (*130*).

The diversity of microbial communities is lower in patients with poor clinical state and decreased lung function (*125*). However, there is no consistent effect related to pulmonary exacerbations. A longitudinal study of microbiota before, during and following exacerbation in people with CF showed no discernible changes and no correlation with changes in respiratory symptoms (*132*). It is suggested that instead of the mere presence of microbes, their relative abundance, dominance over other taxa and temporal stability determine clinical changes in lung disease. Carmody et al. (*133*) showed that there was no difference in bacterial community diversity and bacterial density between baseline and pulmonary exacerbation samples; however, a subset of patients showed considerable changes in community structures during an exacerbation. Communities that were *Pseudomonas*-dominant became more diverse compared to communities with no or other dominant species. Relative abundance of G*emella* species increased in 83% samples at exacerbations.

Currently, lung microbiota in people with CF is actively studied (*134*), and there is an expectation that this will provide new opportunities for treatment of lung disease in CF.

1.2 New Challenges in the New Era

In the current era, with the increasing life expectancy of CF, new management challenges have emerged. These include consequences of long-standing illness and persistent infections, age-related influences on other organ systems and the cumulative effect of treatment (e.g. antibiotic treatment) on morbidity.

The ageing CF population is more vulnerable to an array of diseases almost as multifaceted as CF itself. This cohort is quite heterogeneous, for instance, individuals diagnosed at different times, people with different severity of impairment of lung function, and patients who have received transplants (e.g. lung or liver). Many of these complications are shared by all the patients whereas others are unique to a particular sub-group (*135*).

Several extrapulmonary comorbidities are to be anticipated with CF, which range from various psychosocial issues to an increased risk of malignancies. Besides, a wide array of complications can be related to different organ transplants and subsequent immunosuppressive therapies. The following sections provide an overview of these various extrapulmonary morbidities with CF. Renal disease in CF is described in Section 1.3.

1.2.1 Metabolic Bone Disease

Low bone density caused by an accelerated bone loss in the form of osteopenia or osteoporosis and osteomalacia (caused by vitamin D deficiency) are common in CF resulting in an increased risk of bone fragility and fractures (*136*). According to a systematic review and meta-analysis, the pooled prevalence of osteoporosis in adults with CF was 23% (95% confidence interval, CI 16.6-31.0) and pooled prevalence of osteopenia was 38% (CI 28.2-48.3). In addition, a high prevalence of vertebral and nonvertebral fractures was also documented (*137*). The pathogenesis of the metabolic bone disease is not clear, and a multifactorial origin has been suggested. Intestinal factors include malnutrition (which may lead to low body mass index, BMI) and impaired absorption of vitamins (D & K). Pulmonary factors include severity of lung disease (which can affect the level of physical activity), the extent of chronic inflammation (affecting cytokine levels, amongst other factors) and the effects of corticosteroids (*138*).

1.2.2 CF-related Diabetes and Complications

CFRD is the most common comorbidity of CF affecting between 15-20% of adolescents, and its prevalence increases with age to about 30% of adults with CF (*139*). According to the UK CF Registry report from 2014, 12% of children (10-16 years) and 32% of adults (>16 years) with CF have a diagnosis of CFRD (*1*). CFRD has features of both type I and type II diabetes mellitus but has been described as a distinct entity (*140,141*). Its pathogenesis has been related to progressive fibrosis and fatty infiltration of exocrine pancreas leading to disruption of islet cell architecture and loss of beta cells (insulin secreting cells). In addition, there may be a varying degree of insulin resistance associated with multiple factors, e.g. pulmonary exacerbations, liver dysfunction, steroid therapy, etc. (*142*).

CFRD is associated with worse pulmonary outcomes and higher mortality compared to non-diabetic CF group, but the difference has decreased substantially over time with early diagnosis and aggressive treatment (*143*). An annual complication screen is recommended as microvascular complications occur in CFRD. A comparison of type I diabetes mellitus with CFRD showed similar microvascular complications (29%); however, CFRD patients showed significantly more microalbuminuria (21% versus 4.1%; P=0.003) whereas retinopathy was more common in patients with type I diabetes mellitus (24% versus 10%; P=0.44). This might be a reflection of other CF-related factors on renal function (*144*). According to a study based on the CF Foundation Patient Registry data, CFRD requiring insulin therapy was shown to substantially increase the risk of CKD (≥ 5years of CFRD requiring insulin versus no CFRD: hazard ratio 4.56, 95% CI 2.84-7.31) (*4*).

Macrovascular complications of diabetes have not been reported so far in CFRD; it is likely that large vessel disease will follow a pattern similar to type I diabetes mellitus where these changes present more than 20 years after the diagnosis. With longer duration of diabetes, the risk of macrovascular complications may be greater.

1.2.3 Malignancies in CF

An association between CF and increased risk of malignancies has been suggested. Several studies have consistently shown an increased risk of gastrointestinal tract malignancies with CF (*145-148*). In a retrospective cohort study, thirteen digestive tract tumours were observed in CF patients as compared to an expected number of two in the general population (observed to expected ratio of 6.5, 95% CI, 3.5-11) (*147*). There are conflicting reports of the overall risk of cancer in patients with CF, being reported as average (*145,147,149*) or high (*146*). A study on patients with CF identified from Swedish population register (*146*), encompassing a 21 year follow-up period, showed an elevated overall increased risk of cancer for patients with CF with the standardised incidence ratio (SIR) of 3.2 (95% CI 2.1-4.6). This study also reported a significantly increased risk of the digestive tract, kidney, thyroid and endocrine cancer, lymphoma and nonmelanoma skin cancer. There was no significant change in the overall cancer risk among the heterozygous gene mutation carriers (parents and siblings) (*146*).

The mechanism behind the increased cancer risk is not clear so far. Various hypotheses have been proposed. High dietary intake of omega 3 fatty acids, particularly docosahexaenoic acid, is associated with reduced risk of colonic cancer (*150*). Maissonneuve et al. postulated that increased risk of intestinal cancers in people with CF is related to low epithelial cell membrane level of docosahexaenoic acid (*145*). Low serum levels of essential fatty acid (docosahexaenoic acid) in patients with CF compared to controls is not related to malnutrition but is a result of abnormal fatty acid metabolism in the epithelial cell membranes linked to CFTR deficiency (*151*). Failure to thrive or malnutrition at the time of diagnosis of CF was associated with a higher risk of developing digestive tract cancer (SIR=7.9, 95% CI=3.8-14.5) (*145*). Influence of genotype has been suggested; the probability

of digestive tract cancers was highest for the patients homozygous for the Phe508del mutation (SIR=5.2, 95% CI=1.9-11.3), intermediate for patients heterozygous for Phe508del mutation (SIR=3.4, 95% CI=1.1-7.8) and lowest for patients carrying other mutations (SIR=2.5, 95% CI=0.1-13.9) (*145*). Role of other genetic modifiers, e.g. mucin genes has been suggested. Mucins are a family of glycosylated proteins produced by epithelial cells; aberrant expression of several mucin genes has been associated with various neoplastic and preneoplastic conditions (*152,153*). CFTR is located (chromosome band 7q31) in proximity to MUC3, MUC11 and MUC12 (chromosome band 7q22) genes (*145*); MUC11 and MUC12 are downregulated in colorectal cancers (*154*). In addition, CFTR itself has been shown to have a tumour suppressing effect (*155*); mice carrying intestinal specific knockout of CFTR developed tumours in colon and small intestines of >60% of mice (*155*); Loss of CFTR expression in patients with early stage colorectal carcinoma was associated with significantly poor disease-free survival (*155*). Other consequences of CFTR deficiency, e.g. predisposition to recurrent infections, chronic gut inflammation (*156*), altered immune response, frequent antibiotic use leading to modification of gut microbiome may also play a role in enhancing the risk of cancers in CF (*157*). The long-term effect of use of pancreatic enzyme supplements on risk of colorectal carcinoma is difficult to assess as 85% of patients with CF (pancreatic sufficient) are on enzyme supplementation; no association has been described in the literature.

A more pronounced risk was shown in those CF patients who had had an organ transplant (*145,146*),(*149,156,158*). This has mainly been attributed to intense immune suppressive regimen required post-transplant (*158*). An Increased incidence of certain cancers, where there is an association with exposure to ionising radiations, has been found in CF. A role of repeated X-ray examinations has been questioned (*146*).

1.2.4 Macrovascular disease in CF

Coronary artery disease has only rarely been reported so far in CF. The blood cholesterol levels are low, and hypertension is usually mild. However, both long-standing diabetes and renal impairment can potentially affect the cardiovascular health of patients with CF who survive longer and further exploration of the cardiovascular disease in ageing CF community is needed.

Elevated triglyceride and cholesterol levels are associated with atherosclerotic cardiovascular disease in general population. Though the cholesterol levels were low, 16% of CF patients showed isolated hypertriglyceridaemia (*159*), which was not related to other characteristics like glucose tolerance status, insulin secretion, age, sex, weight, blood pressure, genotype, liver dysfunction, C-reactive protein (CRP) concentration, steroid use, or pulmonary function. Hypertriglyceridaemia may be related to the presence of chronic low-grade inflammation or could be related to dietary fat malabsorption together with enhanced glucose absorption from the gut (*160*).

Increased large artery stiffness is an indicator of ageing of the vasculature and is an independent risk factor for cardiovascular events (*160*). With increasing stiffness, the resistance offered by peripheral vessels causes backwards deflection of the pressure wave and consecutively, augments the forward pressure wave. This pressure augmentation as a ratio of central pulse pressure (PP) is described as augmentation index (AIx) (*161*), which increases physiologically with ageing. A study on 50 adult CF patients showed significantly elevated AIx suggesting premature vascular ageing with the large artery haemodynamics behaving almost a decade in advance of the chronological age (*160*). Increased AIx was documented in the presence of normal blood pressure. The subgroup with CFRD showed the highest AIx suggesting a high-risk group. A positive relationship was found between systemic inflammatory marker CRP and AIx,

whereas glomerular filtration rate (GFR) showed an inverse relationship. This has been recognised as an expression of 'target organ damage' subsequent to the loss of buffering of pressure pulsations.

1.2.5 Treatment Related Adverse Effects

With increasing age of people with CF, treatment burden as well as overall cumulative treatment exposure increases. Recurrent or prolonged exposure to various treatments may have adverse effects and can increase the burden of comorbidities. Aminoglycosides, one of the most commonly used antibiotic agents in people with CF, have nephrotoxic potential, and recurrent exposure to aminoglycosides may be linked to cumulative toxicity (*5*). Various other antibiotics and anti-inflammatory agents have also been reported to have affect renal function, e.g. ciprofloxacin (*162*), azithromycin (*163*), ibuprofen (*164,165*), etc. More details are given in the next section. Aminoglycosides are also known to cause ototoxicity (*166*), and a high proportion of individuals with CF are shown to have a hearing deficit (*167*). Effects of organ transplant and subsequent immunosuppressive therapy need to be considered (*168*). Having a chronic disease and high treatment burden can have an impact on the mental health of the patients and their families (*169*). Long-term adverse effects of new therapies, e.g. disease-modifying agents, if any, are yet to be identified.

1.3 Renal Disease in CF

Renal impairment has recently been recognised as a problem in people with CF. Various case reports describe AKI in patients with CF, and there are studies that demonstrate an increased prevalence of CKD in CF. A survey of the CF population in the UK revealed an incidence of AKI between 4.6 to 10.5/10,000 CF patients per year. This is significantly higher than the risk in general population (about 100 times increased risk in paediatric population) (*3*). A study from CF Foundation Patient Registry data in adult patients with CF showed a prevalence of CKD of 2.3% annually, which doubled with every decade increase in age (*4*). Renal impairment in this patient population has been linked with various nephrotoxic influences like recurrent treatment with antibiotics including aminoglycosides (*5*) and comorbidities like CFRD (*4*); In addition, a primary renal phenotype related to CFTR has also been speculated by studies based on animal models of CF (*170*).

The following sections summarise the current literature of acute and chronic kidney disease in CF and describe the role of CFTR protein in renal physiology and ion transport within the renal epithelial cells. This is followed by an account of various factors predisposing to renal impairment in CF with emphasis on the recurrent use of antibiotics, which is one of the main treatment strategies in CF. As aminoglycosides can have both nephrotoxic and ototoxic effects, literature about hearing abnormality in CF and any correlation with antibiotic use has been described. In addition, there is an explanation of the techniques used in this study for measurement of GFR as a marker for renal function.

1.3.1 Definition and staging of AKI

AKI (formerly acute renal failure) is a clinical syndrome that covers a spectrum of renal injury extending from less severe forms to more advanced damage, which may require renal replacement therapy. Clinically AKI is characterised by a rapid reduction in kidney function resulting in failure to maintain fluid, electrolyte and acid-base homeostasis. AKI is seen in about 13-18% of all hospitalised patients (*171*) and is associated with increased mortality and increased length of hospital stay (*171-173*). It adds a substantial cost to National Health Service (NHS) budget (in the range of 434 million to 620 million per year) (*171*). The incidence of AKI in the UK population ranges between 140 per million population per year to 486-630 per million population per year (according to more recent data) (*174*). The incidence of AKI in the paediatric population in the UK was estimated to be 8 per million population per year (*175*).

Until very recently, there was no consensus definition for AKI available in the literature, and multiple definitions were being used by various papers. A consensus definition (RIFLE criteria, acronym described in the next sentence) was developed by a group of experts (Acute Dialysis Quality Initiative Group, ADQI group) (*176*), which is based on the rise in serum creatinine levels or reduction in GFR, and urine output. RIFLE criteria also include the outcome in their definition: acronym RIFLE stands for 3 classes of increasing severity of acute renal impairment/injury- Risk, Injury and Failure and 2 categories of outcomes-Loss and End-stage renal disease (ESRD).

These criteria cover a range of patients, also including patients with functional impairment of kidneys, i.e. the group, which may benefit from early interventions.

As children may not have enough muscle mass to achieve high creatinine values, RIFLE criteria were modified for paediatric patients

as pRIFLE criteria (*177*) based on a decrease in estimated creatinine clearance (Schwartz formula) and urine output (*172*). Another modification of the RIFLE criteria was in the form of Acute Kidney Injury Network (AKIN) criteria (*178*), which was proposed to be able to capture small changes in serum creatinine that was not possible in the RIFLE criteria. A combination of RIFLE and AKIN criteria has been used to develop combined recommendations for definition (described below), and the staging of AKI is outlined in Table 1-2.

The renal association clinical practice guidelines for AKI developed by International Kidney Disease: Improving Global Outcomes (KDIGO) Group (*172,174*) suggests diagnosing AKI based on any of the following:

- Increase in serum creatinine by ≥26.5 µmol/L* in last 48 hours;
- Increase in serum Creatinine to ≥1.5 times* within last 7 days;
- Urine output <0.5ml/kg/hr for >6 consecutive hours or >8 hours for children.

(*Above the reference value: the lowest serum creatinine value recorded during hospitalisation)

There are different methods of defining the baseline serum creatinine values, and the best method has been described as using the lowest creatinine from 7 to 365 days before admission. In the absence of such values, imputation from estimated GFR (eGFR, Modification of Diet in Renal Disease Equation (MDRD equation) for adults and Bedside Schwartz formula for children: described in Section 2.3.3.5) has been recommended which may not be reliable in some patients (*179,180*).

Table 1-2 Staging of Acute Kidney Injury		
Stage	**Serum Creatinine**	**Urine Output**
1	1.5-1.9 times baseline Or ≥26.5 µmol/L* increase**	<0.5 ml/kg/h for 6–12 hours (>8 hours for<18 years)
2	2.0-2.9 times baseline**	<0.5 ml/kg/h for ≥12 hours (>16 hours for <18 years)
3	3.0 times baseline ** Or ≥353.6 µmol/L Or Initiation of renal replacement therapy Or Decrease in eGFR to <35 ml/min/1.73m^2 (patients <18 years)	<0.3 ml/kg/h for ≥24 hours Or Anuria for ≥12 hours

**** Equivalent to 0.3 mg/dl, with the SI (System International) units rounded down to the nearest integer, ** Where the rise is known (based on a previous blood test) or presumed (based on the patient history) to have occurred within 7 days. Table adapted from Thomas et al. (180) and Clinical Practice Guideline for Acute Kidney Injury (172); eGFR: estimated glomerular filtration rate***

1.3.2 Definition and staging of CKD

CKD is a serious problem, which incorporates abnormality of renal function and/or structure. Early stages of CKD can often be asymptomatic. However, there is a potential to progress to higher stages of CKD or ESRD with worsening renal function. Reduced GFR has been associated with increased risk of death, cardiovascular events and hospitalisation independent of other known risk factors or history of cardiovascular disease (*181-184*).

In a population-based study, reduction in eGFR of 10 ml/min/1.73m^2 (among people with eGFR of <60 ml/min/1.73m^2) and doubling of albuminuria increased the rate of cardiovascular mortality by 1.29 (95% CI 1.06 to 1.55) times and 1.06 (95% CI 1.04 to 1.08) times respectively (*184*). In addition, CKD has an enormous financial impact modelled to cost the NHS England about 1.44 to 1.45 billion for the year 2009-10, which amounts to about 1.3% of all NHS spending that year (*185*). Adverse outcomes of CKD can be prevented or delayed through early detection and treatment (*186*).

Definition and staging of CKD have evolved with time. In 2002, National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) clinical practice guidelines, defined CKD as the presence of kidney damage or GFR of <60 ml/min/1.73m^2 for 3 months or more, irrespective of the diagnosis. Kidney damage was characterised by pathological abnormalities or presence of markers of kidney damage present in blood, urine or imaging studies (*187,188*). The staging of the CKD was based on the level of GFR (please refer to Table 1-3).

The guidelines from 2002 have been extended in 2012 (*172*), and CKD is now defined as an abnormality of structure or function of kidneys present for >3 months with implications for health. The revised staging system (CGA) includes **C**ause (non-graded) and grades of **G**FR and

Albuminuria. The cause should be based on the presence or absence of systemic disease and the location of observed or presumed pathologic - anatomic findings within the kidneys. Grading of GFR and albuminuria are described separately (please refer to Table 1-4 and Table 1-5) (*172*).

Table 1-3 2002 NKF KDOQI Classification of the Stages of CKD			
Stage	**GFR (ml/min/1.73 m^2)**	**Description**	**Action Plan***
1	≥90	Kidney damage with normal or increased GFR	Diagnosis & Treatment of primary and comorbid conditions
			Slowing CKD progression, cardiovascular disease risk reduction
2	60–89	Kidney damage with mild reduction of GFR	Estimating rate of progression
3	30–59	Moderate reduction of GFR	Evaluating and treating complications
4	15–29	Severe reduction of GFR	Preparation for kidney replacement therapy
5	<15 (or dialysis)	Kidney failure	Kidney replacement therapy

**** The actions listed in the higher stages of CKD also include actions from less severe stages (187,188); NKF KDOQI: National Kidney Foundation Kidney Disease Outcomes Quality Initiative; CKD: chronic kidney disease; GFR: glomerular filtration rate***

Table 1-4 GFR categories in CKD		
GFR category	**GFR (ml/min/1.73 m^2)**	**Description**
G1	≥90	Normal or high
G2	60–89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	<15	Kidney failure

Table adapted from International Kidney Disease: Improving Global Outcomes (KDIGO) Clinical Practice Guidelines, 2012 (189): GFR: glomerular filtration rate; CKD: chronic kidney disease

Table 1-5 Albuminuria categories in CKD			
Albuminuria category	**AER (mg/24hrs)**	**ACR (mg/mol)**	**Description**
A1	<30	<3	Normal to mildly increased
A2	30-300	3-30	Moderately increased
A3	>300	>30	Severely increased

Table adapted from International Kidney Disease: Improving Global Outcomes (KDIGO) Clinical Practice Guidelines, 2012 (189); AER: albumin excretion rate; ACR: albumin-creatinine ratio (nephrotic range ACR≥220 mg/mol); CKD: chronic kidney disease

1.3.3 Renal phenotype in CF

Since the Seventies, there were sporadic studies that explored renal involvement in CF. The first report of an attempt to investigate renal involvement in CF comes from Robson et al., 1971. The study was aimed to determine whether CF was associated with abnormal renal handling of sodium and measurement of free water clearance (*190*).

Following this several studies were conducted which measured kidney function in CF with the focus on identifying any defect in the fluid and electrolyte transport, mainly sodium reabsorption, and responses to hypotonicity and volume expansion. Some reports suggested increased sodium reabsorption in proximal renal tubules and defective renal diluting capacity (*191-193*) whereas others did not (*194*). It was not clear whether these changes were secondary to decreased extracellular fluid volume caused by excessive losses of sodium chloride (salt) in the sweat and faeces or if they were related to a primary defect in renal function caused by mutations in CFTR (*195*).

Other studies have tried to identify renal phenotype in CF using various mouse models of CF, and have been based mainly on assessing the role of CFTR in renal physiology. These studies are described in Section 1.3.4 and Chapter 3, Section 3.1.3 and Section 3.1.4**.**

1.3.3.1 AKI in CF

Until the late Nineties, there were no reports of AKI in CF. In 1996; a case of CF was first reported to develop AKI after a 3-week course of tobramycin (*196*). Following this, there were several isolated cases and case series reported involving CF patients receiving aminoglycosides (*197,198*), aminoglycosides with simultaneous ibuprofen, and aminoglycosides with a cephalosporin (*165*). Other antibiotics including inhaled tobramycin (*199*), and ciprofloxacin (*162*) were also implicated as causative factors.

The first systematic study of AKI in CF was in the form of a national survey conducted by Bertenshaw et al. (2007), which found that the incidence of AKI was 4.6-10.5/10,000 CF patients/year (*3*). Considering the overall incidence of AKI in children, this suggested a 100 time greater risk of AKI in CF compared to the background rate. In 88% of cases, an aminoglycoside was prescribed at the onset of AKI or in the preceding week.

A subsequent case-control study by Smyth et al., 2008, with an aim to determine the causative factors related to AKI in CF, established that aminoglycoside therapy in the previous week increased the risk of AKI by >80 fold (*200*). A significantly higher number of patients had received gentamicin (but not tobramycin) in the previous year compared to controls (P<0.001) and, a known risk factor for renal impairment (prior renal disease, acute dehydration or long-term treatment with a nephrotoxic drug) was present in most cases (Odd's Ratio (OR) 24.0, 95% CI 3.1 to 186.6).

Similarly, a retrospective cohort study in children with CF receiving IV aminoglycosides (≥3 days) for management of pulmonary exacerbation showed incident AKI in 20% children by daily measurement of serum creatinine (*201*). Another retrospective, matched, case-control study showed incident AKI in 14% children with CF receiving aminoglycoside for ≥3 days (*202*). Glass et al. (*203*) investigated tubular damage caused by IV tobramycin administration (thrice daily) for 2 weeks and showed that levels of urinary N-acetyl-beta-D-glucosaminidase (NAG) and urinary retinol binding protein (RBP) (indices of renal tubular damage) increased significantly during the course of treatment, thus indicating acute tubular injury which recovers almost completely after 4 weeks. Similar results have been shown by other studies in the past in smaller group of patients (*204-206*). A larger study by Etherington et al., measuring NAG level in 88 adult CF patients during and after tobramycin and colistin

administration showed similar results with the return of NAG to baseline levels after receiving tobramycin but not after colistin. In addition, the baseline NAG levels were shown to be higher in CFRD group (*207*).

Some studies suggest that AKI may affect the renal function in a longer term or predispose to CKD in future (*208-211*). Following several case reports of aminoglycoside-induced AKI in CF, CKD has become a concern, particularly in the context of repeated acute renal insult caused by several aminoglycoside courses.

1.3.3.2 CKD in CF

As there are no apparent clinical features of renal disease present in CF, it took a long time for the research to focus on chronic kidney damage in CF. Several attempts were made to investigate any renal functional defect in CF, which indicated defects in renal diluting capacity and sodium handling; nevertheless, there was minimal evidence of any manifest CKD. There were sparse numbers of studies on this issue until last decade. Since then, with various case reports and heightened awareness of acute renal failure in CF, chronic renal damage was being explored particularly in the context of the effect of recurrent exposure to various drugs including aminoglycosides.

The first report of ESRD in a CF patient is from Roteller et al. in 1989 that described a 24-year-old patient with CF, without any associated diabetes and liver disorder, who required dialysis. The histological changes included both glomerular and tubular interstitial changes (*212*).

Pederson and co-workers studied renal function by measurement of 24-hour creatinine clearance (a measure of renal function) in children and adults (N=46) who had average 20 courses of IV tobramycin (mean cumulative dose 2,947 mg/kg ranging from 632 to 7,644 mg/kg) in the

past. They found that 39% of their patients had a GFR below normal, i.e. <96 ml/min/1.73m^2 (*213*).

Similar results were found by Al-Aloul et al. (2005) who measured creatinine clearance in 80 adult subjects with CF, which is the largest number so far. This study showed that 42% patients had low creatinine clearance (<80 ml/min/1.73 m^2) in a group of patients with chronic *P. aeruginosa* and no evidence of previous renal failure.

Two studies have measured renal function in CF using ^{99m} Technetium-diethylenetriamine pentaacetic acid (^{99m}Tc-DTPA) method. Prestidge et al. studied a group of 67 patients aged 2.1 to 18.5 years (mean age 9.6 ± 5 years) and suggested that 4/67 (6%) patients had evidence of renal dysfunction in the form of reduced GFR in one patient and persistent microscopic haematuria in 3 patients (*214*). Soulsby et al. measured GFR by ^{99m}Tc-DTPA in 53 patients including both adults and children (mean age 18 years, range 2-54 years). The mean GFR was normal in both the groups: 113 ± 25 (range 68-149) ml/min/1.73m^2 in adults and 142 ± 27 (range 108-211) ml/min/1.73m^2 in children; however, 4/20 (20%) adult patients (age 26.8 ± 9.2) had a GFR value below 90 ml/min/1.73m^2 (*215*).

Andrieux et al. assessed GFR of children with CF using the Schwartz formula at different ages between 1 to 15 years (N=112) and measured creatinine clearance (N=24) by 24hour urine collection (*216*). There was no child with an eGFR of <90 ml/min/1.73m^2; however, 14 of 24 children (58%) had a measured creatinine clearance GFR of less than 90 ml/min/1.73m^2.

A study based on the CF Foundation Patient Registry data from adults with CF showed an annual prevalence rate of CKD in CF as 2.3% which doubled with every 10-year increase in age (*4*).

It is not clear what drives the changes in renal function in people with CF. A number of research studies have reached conflicting conclusions about the effect of increasing number of courses and cumulative dose of IV antibiotics, particularly the aminoglycosides.

Al-Aloul et al. (*5*) showed that creatinine clearance declined with increasing numbers of aminoglycosides courses (r=-0.32, P=0.0055). This inverse relationship was potentiated by coadministration of colistin (r=-0.42, P=0.002). This effect was independent of age, CFRD, BMI, percent predicted FEV_1 and use of other drugs like azithromycin and non-steroidal anti-inflammatory drugs.

Etherington et al. demonstrated that following a single course of IV tobramycin or colistin in non-diabetic adult CF patients, the urinary NAG levels returned to baseline after treatment with tobramycin but not after colistin, where there was an incomplete resolution of the tubular damage. This study also showed a positive correlation of previous exposure to IV antibiotics (tobramycin and colistin) with elevated baseline NAG levels (r=0.375, P<0.001). This association was significant for colistin only (r=0.389, P<0.001) when analysed separately to tobramycin (*207*). This lack of significant correlation of baseline NAG with cumulative tobramycin dose could have been due to their practice of once-daily dosing of tobramycin. The TOPIC study showed that the rise in NAG level was 33% less after once-daily dose of tobramycin than after three-times-daily dose (*217*). If the toxicity of a single course of an aminoglycoside is reduced, through once daily administration, then chronic toxicity through repeated courses may also be reduced.

Pederson et al. did not find a correlation of creatinine clearance with cumulative tobramycin dose (*213*) Similarly, Prestidge et al. found no evidence of a relationship between GFR and lifetime days of aminoglycoside antibiotics in their studies (although both these

studies used a thrice-daily dose of tobramycin). Andrieux et al. (*216*) (paediatric study) did not find a significant correlation between eGFR, (Schwartz formula) or 24h creatinine clearance and the cumulative number of days of aminoglycosides received (once-daily aminoglycosides and other antibiotics). Interestingly, they found a strong association of cumulative dose of netilmicin with creatinine clearance though in a very small group of four patients. Glass et al. (2005) showed that the urinary RBP levels were above normal range in children who had received at least one course of tobramycin; however, they did not find any significant difference between the markers of tubular damage (urinary NAG and urinary RBP levels) in the groups of CF children with different cumulative exposure to tobramycin (*203*). The comparison was, however, limited by small groups and relatively few courses of tobramycin (exposure of 0-2 (N=9), 3-5 (N=8) and >5 courses (N=5).

It is not clear whether, the difference of Al-Aloul et al. study (*5*) from other studies was driven by higher number of courses of antibiotics (median 40 (Interquartile range (IQR) 51.1) courses of aminoglycoside and/or colistin), or this difference was related to some other factor.

A relationship between CFRD and CKD in CF has been implicated in some studies but not confirmed by others. Etherington et al. showed that the patients with CFRD had a higher baseline (before starting IV tobramycin) urinary NAG levels (*207*); this level remained higher than people without CFRD at all time points including follow-up at about median 48 days after IV antibiotics. This may suggest that this group is at the greatest risk of renal tubular damage and cumulative toxicity of antibiotics. However, the study by Al-Aloul et al. did not show any difference in the prevalence of CFRD in the groups with and without renal impairment (*5*). Association of CFRD with the development of microvascular complications is discussed in detail in Section 1.2.2.

It is evident that the true prevalence of CKD at different ages in CF is still not known. The role of cumulative aminoglycoside toxicity and coexisting CFRD or any other causative factors is not clear.

1.3.3.3 Histological changes

There were several attempts at identifying any histological changes in association with CF in different settings, suggesting both tubular and glomerular changes and evidence of nephrocalcinosis.

In an autopsy study in 1972, Oppenheimer examined the kidneys of CF patients for possible glomerular lesions; although the best survival was up to adolescence, changes were demonstrated in 4 of 5 diabetic children with CF. None of the non-diabetic CF patients had any changes; however, the age of these patients was only up to infancy (*218*).

In a post-mortem study of 34 adult and paediatric CF patients (range 4 months to 35 years) in 1982, Abramowsky and Swinehart showed renal histological changes including both glomerular and tubulointerstitial changes. Glomerular enlargement up to 1.5 to 2 times normal size was observed in all the patients. Additional mesangiopathic changes were frequently seen. Evidence of acute and chronic tubular injury was seen, and the latter was characterised by abundant tubular lysosomal proliferation and tubular atrophy suggestive of chronic aminoglycoside injury. A simultaneous immunofluorescence study mainly revealed deposits of immunoglubulin M or complement C3 or both in the glomeruli and arterioles thus suggesting a role for immune complex-mediated injury. Remarkably, in this study, no diabetic lesions were seen, and no correlation was found between the severity of renal histological lesion and age of patient or duration of CF (*219*).

Other histopathological changes have been observed including amyloidosis, immunoglobulin A nephropathy (IgA nephropathy) and tubulointerstitial nephritis (*220*).

Coexistence of IgA nephropathy and CF has been reported (*221-223*), and has been shown to be associated with haematuria, proteinuria

and reduced renal function (*222*). This could be coincidental due to both the disorders being relatively common, or there could be a real association because patients with CF have high circulating levels of IgA (*224,225*) due to recurrent bacterial infections which might be deposited in the kidneys (*220*).

In long-term inflammatory states, there is an imbalance in the production and degradation of acute phase inflammatory proteins such as serum amyloid A protein; this leads to accumulation of amyloid in body organs. In a retrospective review of autopsies, systemic amyloidosis was reported in several organs in CF including blood vessels, spleen, liver and kidney (*226*) in 11 of 33 patients.

Renal amyloidosis is described as a rare complication of CF, which might be silent clinically or presents with proteinuria. The onset of nephrotic range proteinuria with renal amyloidosis has been reported in several cases (*221,227-229*). Usually, there is a rapid deterioration in clinical condition, and the prognosis is poor as the majority of the reported cases have died (15/19) within one year of diagnosis of amyloid (*230*). Inadequate management in childhood or before the diagnosis of CF has been implicated, and newborn screening may help prevent such cases.

Yahiaoui et al. in 2009, retrospectively looked at renal biopsy in 13/510 (2.5%) adult CF patients and suggested a very heterogeneous renal histopathological picture (*223*). Proteinuria was present in all but two cases and was associated with progressive renal impairment in 4/11 patients. Glomerular lesions were present in 12/13 patients with the commonest pattern being Kimmelstiel-Wilson nodular glomerular sclerosis (3/13) and amyloidosis (3/13). The rest of the lesions were quite varied including focal and segmental glomerular lesions (2/13), minimal change disease (1/13), acute endocapillary glomerulonephritis (1/13), IgA nephropathy (1/13) related to Henoch-

Schonlein purpura and membranous nephropathy (1/13). Other changes included interstitial fibrosis in 7 patients and chronic tubulointerstitial nephropathy in one patient. There were diabetic lesions in one non-diabetic patient and in two others with well-controlled diabetes in the absence of diabetic nephropathy. In addition, four patients with diabetes had non-diabetic nephropathic changes (*223*). In another study, Westall described non-diabetic patients with CF who showed changes of diabetic nephropathy (Kimmelstiel-Wilson Nodules nodules). The authors speculated that pro-inflammatory cytokine profile predisposes CF patients to these lesions (*231*).

The autopsy studies should be treated with caution, as these are old studies, and the health status of people with CF has improved dramatically over this time. This might affect the level of inflammation or the number of infections which individuals with CF are subjected to in the current era. It may not be appropriate to apply these results to current or future generations of CF patients. On the other hand, with improving survival and increasing exposure to nephrotoxic agents, the predominantly subtle changes can become more important.

Alternatively, the morphological changes could have been driven by intrinsic CF related effects due to lack of CFTR function. However, studies may only be feasible in animal models of CF due to ethical reasons. Studies on a mouse model of CF have investigated renal physiological effects related to CFTR loss (*232*), but no morphological comparison has been made so far in any animal model of CF, particularly in the neonatal period where the effect of prolonged inflammation or recurrent infection and treatment courses could be excluded.

Clinicians may need to have a lower threshold for performing a renal biopsy and renal function measurement. Renal biopsy should be

considered in patients with proteinuria, other renal symptoms or diabetes. It may give valuable information to determine the type of nephropathy, help alter the treatment and allow planning for transplant.

1.3.4 CFTR in kidneys

CFTR mRNA is abundantly expressed in the kidneys in all nephron segments but predominantly in the renal cortex and outer medulla (*233*). Immunocytochemical localisation study identified CFTR protein located on the apical aspect of the epithelial cells of proximal and distal tubules and in the membranes of the vesicles occupying the apical region of epithelial cells (*234*). It was absent from the basolateral region of epithelia. The structure of CFTR is shown in Figure 1-3.

CFTR belongs to a large ATP-binding Cassette (ABC) transporter superfamily which is involved in the active transport of substrates against the concentration gradient using ATP hydrolysis to provide energy (*235*). CFTR itself functions as a chloride channel (*235*), and recently it has also been described to function as a bicarbonate channel (*236*). The channel opens with a complex process of interaction of ATP with nucleotide binding domains of CFTR. This process is regulated by phosphorylation of protein kinase A (PKA) in the regulatory domain of CFTR (*235*). In addition, CFTR regulates the function of various other channels like stimulation of outwardly rectifying chloride channels (ORCC) (*237*) and inhibition of ENaC (*238*).

Despite the clear knowledge of abundance of CFTR in the kidneys, its role in the renal function is not entirely understood. It has been described to have a role in the regulation of other channels like inhibition of ENaC channels and activation of renal outer medullary potassium channel (ROMK) (*239*). Studies have been done in the kidneys from a mouse model of CF to explain the renal phenotype of

CF including the role of CFTR in the functioning of endocytic receptor proteins.

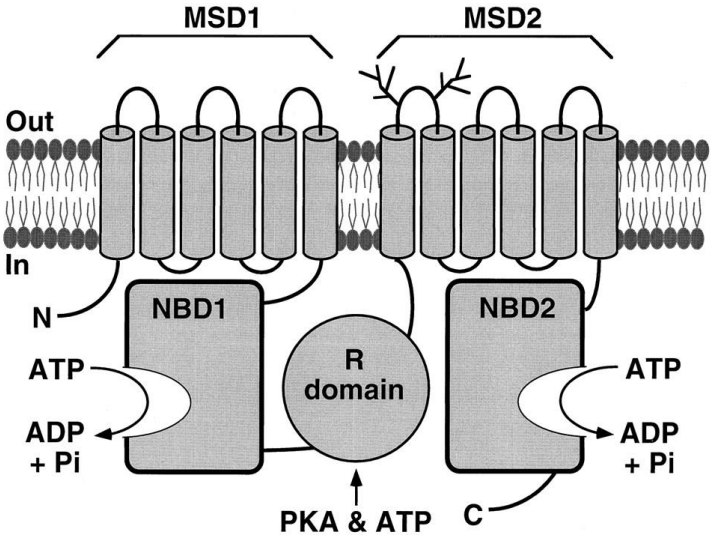


Figure 1-3 Structure of CFTR protein shows two membrane-spanning domains (MSD 1 & 2), two nucleotide-binding domains (NBD 1 & 2) and one regulatory domain (R domain); CFTR: cystic fibrosis transmembrane conductance regulator; PKA: protein kinase A; ATP: adenosine triphosphate; ADP: adenosine diphosphate; Pi: phosphate ion; Reference: Sheppard 1999 (240)

A truncated version of CFTR (TNR-CFTR) is expressed primarily from renal medulla; this is a result of deletion of 145 base pairs (bp) between exon 13 and 14, which causes insertion of a frameshift and a premature stop codon. TNR-CFTR contains only one set of membrane-spanning domain and nucleotide-binding domains (instead of two of each in CFTR) and a regulatory domain. It can perform the same function as CFTR, i.e. cAMP-mediated chloride channelling and regulation of ORCC, though with less efficiency (*233*). TNR-CFTR is

primarily located intracellularly (endoplasmic reticulum) not at cell surface like CFTR (*241*). When exposed to hypertonic shock, total cell protein expression of both types of CFTR increases but the cell surface expression increases only for CFTR. As CFTR regulates the role of ENaC channels and ROMK channels, the finding of increased expression of CFTR with exposure to hypertonicity is probably a reflection that indirectly, CFTR plays a role in the maintenance of renal medullary hypertonicity or helps adapt tubular cells to hypertonic environments (*241*). At the same time, an increase in total cell expression of TNR-CFTR without any increase in its surface expression may be explained by an increase in intracellular expression, again highlighting its mainly intracellular role. TNR-CFTR may provide a (protective) mechanism to account for the paradox of absence of significant primary renal pathology in CF despite abundant presence of CFTR in the nephrons (*239*). Expression of CFTR was modulated by arginine vasopressin (AVP) in rat renal tissue thus suggesting a role of CFTR in ion transport related to regulation of extracellular volume (*242*).

1.3.5 Factors predisposing to renal impairment in CF

1.3.5.1 Predilection to Dehydration

In a study of heat illness in infants and young children in 1962 (*243*), Danks et al. reported, "A child with fibrocystic disease of the pancreas showed severe salt loss even after short exposure to heat."

Various electrolyte abnormalities are associated with CF such as hypochloremia, hypokalemia, hyponatremia, hyperbicarbonataemia (described as chloride depletion hypokalemic alkalosis) which may be related to unrecognised fluid volume depletion (*244,245*). Patients with CF showed excessive loss of sodium and chloride in the sweat leading to low serum chloride concentration and low serum osmolality compared to healthy controls after exercise and heat exposure (*246*). After exercising in temperature of 30-33°C, children with CF had much lower fluid intake compared to controls (*247*), possibly related to having no drive to increase fluid intake (thirst) due to lack of hyperosmolarity induced by sweating (*248*). A recent study showed that with exercise, people with CF had similar thirst perception as control subjects, but their ad libitum fluid intake was 40% lower than that of the control subjects (*249*). Even clinically stable patients with CF, with an apparent normal hydration status, can have low urinary sodium excretion and higher circulating renin and aldosterone levels compared to controls, which may be suggestive of a chronic state of volume depletion in CF (*250*). This predisposition to dehydration can be a potential risk for renal impairment.

1.3.5.2 Tendency for Renal Stones

CF patients are at twice the risk of urolithiasis (prevalence 3-6%) compared to age-matched controls (prevalence 1-3%) or compared to the age-specific prevalence rates (*251-253*). Urolithiasis has been reported within the first year of life of CF patients (*216*). The incidence and prevalence of renal or urinary tract stones have been shown to be

increasing with time (*254*) in the general population, which has been linked to dietary changes (e.g. high fructose consumption) and increasing temperatures (global warming). A study on about 100 adult patients with CF showed a 13% prevalence of renal stones (*253*). Another study described the prevalence of renal stones in adults with CF to be approximately 20% (6/29) compared to 0% (0/30) in a healthy control group (*255*). Although the difference might have been exaggerated in the latter study possibly related to a small sample size of the control group, the prevalence is still approximately double the prevalence described in general population (6-9%) in the same geographical area (*254*). Another interesting observation in the study by Terribile et al. was a 15% prevalence of renal stones in CF heterozygotes and mild urinary hyperoxaluria (*255*). However, this was a small population (n=20) in one centre, mainly including parents of people with CF (who were older than the CF group). This finding will need further confirmation in the wider population.

In an autopsy study, microscopic nephrocalcinosis was observed in 35/38 specimens (92%) and hypercalciuria (>182 mg per gram of Creatinine) in 5/14 patients. Notably, nephrocalcinosis was detected near the time of birth (in six patients under one year old, including two neonates and one stillborn infant). None of the patients with hypercalciuria or nephrocalcinosis had clinical evidence of renal dysfunction (*256*).

In contrast to the above, Bentur et al. 1990, studied histological findings at post-mortem of 14 CF patients and compared the results to those from 12 controls with other chronic diseases; 5/14 CF patients and 6/12 control patients had evidence of nephrocalcinosis; In addition, normal calcium excretion was found in 30/34 cases (*257*).

Various mechanisms have been implicated to explain the propensity for lithogenesis in CF (*251,258*), e.g. altered calcium metabolism,

hypocitraturia, hyperoxaluria, hypercalciuria, renal tubular damage related to repeated antibiotics (co-trimoxazole and ceftazidime) leading to phosphate leak, low urine volume, etc. The most accepted view currently is that increased urinary excretion of lithogenic factors (e.g. hyperoxaluria) and decreased excretion of lithogenic inhibitory factors (e.g. hypocitraturia) in CF patients contribute towards the increased risk of nephrocalcinosis and urolithiasis (*259*) Increased prevalence of hyperoxaluria and hypocitraturia in patients with CF has been confirmed in many studies including both adults and paediatric patients (*253,255,260-263*). A recent study of 112 paediatric CF patients found hyperoxaluria in 70%, hypocitraturia in 75% and urolithiasis in 2/109 (2%) subjects. Hyperoxaluria was also present in 50% of children between 1-3 years old and was associated with severe genotype and pancreatic insufficiency and liver disease (*216*).

Hyperoxaluria has been related to excessive intestinal oxalate absorption due either to malabsorption or to lack of intestinal colonisation with *Oxalobacter formigenes* (*O. formigenes*, an oxalate degrading bacterium) (*259*). *O formigenes* are gram-negative, rod-shaped, obligatory anaerobic bacteria that inhabit the large intestines of humans and many other vertebrate species including pig and sheep (*264*). These bacteria colonise the guts of nearly all healthy 6-8 years old children. They play a symbiotic role, helping in degradation of oxalate, which they appear to use for their metabolism and thus influence the regulation of intestinal absorption and plasma levels of oxalic acid (*265,266*). Prolonged or repeated use of antibiotics may preclude colonisation or destroy established colonies (*267*). Only 16% of CF patients were colonised with *O. formigenes* compared to 71% of healthy volunteers who had their stool specimen investigated by culture and DNA analysis. All the CF patients who had colonisation with *O. formigenes* had normal urinary oxalate levels compared to hyperoxaluria in 53% of non-colonised CF patients, possibly following

repeated courses of antibiotics destroying the bacterial flora (*268*). The aetiology of hypocitraturia is not clear. Dietary factors including high-calorie diet and increased acid load (metabolic acidosis) have been implicated, but no evidence is available so far to establish these (*251,259*).

Nephrolithiasis might affect the kidney function adversely and predispose to the onset of CKD. A population-based cohort study (*269*) showed the risk of incident ESRD, risk of developing stage 3b-5 CKD (eGFR< 45 ml/min/1.73m^2) and having sustained doubling of serum creatinine levels (from baseline) was significantly higher (approximately 1.5 to 2 times) in people who have had renal stones compared to those individuals who did not have kidney stones. The risk was greater in people with multiple episodes of renal stones compared to a single episode or no stones. Increased risk of incident CKD in patients with renal stones has been shown in other studies also (*270-272*). Primarily this association has been linked to obstructive uropathy or pyelonephritis, but crystal plugs at the level of collecting ducts and chronic renal parenchymal injury following chronic shock wave lithotripsy have also been implicated (*273*). It is not known whether renal stones confer the same risks to individuals with CF.

1.3.5.3 CF-related Diabetes (CFRD)

CFRD is a frequent comorbidity of CF, and the prevalence approaches about 40% in patients more than 30 years of age (*143*). As the life expectancy of patients with CF has been such that the microvascular complications and macrovascular complications do not manifest in their lifetime, there are not many studies looking at development of renal disease or CKD in this group of patients; however, more evidence is accumulating with improving care and increasing survival of CF patients. In a CF Foundation Patient Registry-based study, Quon et al. (*4*) showed a 2.4 fold, and a 4.5 fold increase in the hazard ratio for CKD (defined as eGFR of <60 ml/min/1.73m^2) in people with CFRD for

1-4 years and for >5 years duration respectively. This is the first study describing the prevalence of CKD in patients with CFRD. However, some caution should be exercised before generalising the results, as there may be a potential of bias with this epidemiological study. The study defined CFRD by the number of people receiving insulin therapy and thus focussed on a more severe spectrum of CFRD with a potential underestimation of the total number of individuals with CFRD and the risk of CKD in CFRD. On the other hand, there may be a possibility that patients with CFRD are more likely to be screened for CKD and hence a potential for overestimation of the risk of CKD in CFRD than the larger CF population.

As people with CFRD can have multiple, other risk factors for the development of CKD, these can potentiate or contribute to the effect of diabetes on renal dysfunction. A study by Etherington et al. (*274*) assessing urinary NAG excretion in adult CF patients suggested that the patients with CFRD were at a greater risk of antibiotic-associated nephrotoxicity compared to non-CFRD patients, both on the long-term and on a short-term basis (*274*). A higher proportion of patients in the CFRD group compared to non-CFRD patients had elevated baseline NAG levels and elevated levels at follow-up (77% versus (vs) 21 % and 76% vs 30% respectively). In contrast, a study on adult CF patients did not find any difference in the prevalence of CFRD between those with and without renal impairment (*5*). Thus, there may be other factors contributing to the increased baseline urinary NAG levels in the study by Etherington et al. such as an older age group, a female preponderance, lower FEV_1, more severe pulmonary disease and higher number of cumulative days of antibiotic use (*274*).

Microalbuminuria is defined as urinary albumin loss of 30-300 mg over 24 hours or a urinary albumin-creatinine ratio of 3.5-30 in males and 2.5-30 in females measured in a random or first-pass morning urine sample (*275*). Increasing urinary albumin loss has been associated with

progressive diabetic nephropathy, and detection of microalbuminuria has been recommended as a screening tool for diabetic nephropathy (*275*). Microvascular complications have been described in people with CFRD. Anderson *et al*. (*276*) showed retinopathy in 27% patients and microalbuminuria in about 10% of patients with CFRD; Another study found microalbuminuria in 14% and retinopathy in 16% of CFRD patients, with fasting hyperglycaemia, who had been diagnosed for almost a decade (*277*). In a larger study (n=79) by Van den Berg, there were same number of microvascular complications (29%) in patients with CFRD as with type I diabetes mellitus, however, people with CFRD were more likely to have microalbuminuria than retinopathy (21% vs 4%). The reverse was true of the type I diabetes mellitus group (microalbuminuria 10% vs retinopathy 24%) (*144*). This might suggest that in addition to diabetes, there are other mechanisms that drive the nephropathy (*220*).

On intermediate or long-term follow-up, reversal of microalbuminuria to normoalbuminuria has been noted in up to 50% of cases and progression to macroalbuminuria in about 10-20% patients (*278-280*); A persistent predictive factor for regression of microalbuminuria across different studies was low haemoglobin A1C (glycated haemoglobin) level, i.e. tight glycaemic control. Thus, prevention of progression is possible with intervention.

1.3.5.4 Post-Transplant Treatment

Over past two decades, lung transplant has become a viable option for patients with CF having the end-stage lung disease. The number of CF patients receiving lung transplant has increased significantly with the total number of people with CF receiving lung transplant being more than double that 5 years ago (*1*). CF is the commonest indication for a lung transplant in children >6 years of age (*6*). The outcome is improving in terms of survival and quality of life post-transplant with almost 85% survival one-year post-transplant, and many of these

patients return to full-time work or education (*1*). Five-year survival for paediatric lung transplant recipients in the current era is about 50%, which is similar to adults (*6*). Renal dysfunction is one of the commonest complications of lung transplant; by about 7 years post-transplant, 20% of the patients had severe renal dysfunction - defined as creatinine >2.5 mg/dl (221 μmol/L) (*6*). The decline in GFR is highest (about 30%) in the first year post-transplant, and a slow decline continues after that (*7,281,282*).

Multiple factors contribute to the impairment of renal function related to solid organ transplant, which include peri and post-transplant AKI and use of immunosuppressants (*283*). Calcineurin inhibitors (e.g. cyclosporine A and tacrolimus) have been linked to both AKI and CKD (*283*); AKI has been related to reversible vasoconstriction of afferent and efferent glomerular arterioles. Aetiology of CKD is multifactorial, but the direct mechanism is likely to be the effect of oxidative stress, which leads to systemic inflammation and subsequent deleterious effects on endothelial function (*283*). Tacrolimus has been described as less nephrotoxic than cyclosporine (*281,283,284*). Calcineurin inhibitor-sparing therapies have also been attempted using sirolimus and mycophenolate mofetil (*283*).

The risk of CKD after lung transplant provides a further reason to preserve renal function in patients with CF, in the pre-lung transplant period. Compared to other non-renal solid organ transplants, patients receiving lung transplant were 5 times more likely to develop ESRD (*285*). The diagnosis of CF was associated with the worst renal survival after paediatric lung transplant compared to other aetiologies, possibly related to the higher age of children with CF at the receipt of lung transplant (*7*). A low renal reserve at the time of transplant places these patients at an increased risk for having renal function loss post-transplant. A CF Foundation Patient Registry-based study showed the two-year risk of the incidence of renal dysfunction after lung

transplant in people with CF to be 35%, which increased significantly with the age of the recipients (*8*). Diagnosis of CFRD (requiring insulin replacement) and renal function impairment (defined as eGFR 60-90 ml/min/1.73m^2) in the pre-transplant period increased the risk of post-transplant renal dysfunction by 30% and 50% respectively when compared to those without these comorbidities (*8*). Patients with CF are also predisposed to develop serious infections including fungal infection, thus have a potential to be exposed to nephrotoxic antimicrobials. Furthermore, the risk of developing CFRD increases after transplant (*7*).

In addition to increased morbidity, there are also pointers to an adverse influence of CKD on survival in the post-transplant period. Chronic renal failure before the transplant was shown to affect the post-transplant survival adversely in a study on liver transplant recipients (*286*). The occurrence of CKD in the post-transplant period (for solid organs other than the kidneys) increased the risk of death by 4-5 times compared to those without CKD (*287*).

1.3.5.5 Drug-Related Adverse Renal Effects

There is a realisation that with the improving life expectancy, people with CF are receiving an increasing exposure to potentially nephrotoxic drugs during their lifetime.

CF patients may receive multiple courses of antibiotics for several indications including treatment of pulmonary exacerbations, early eradication of *P. aeruginosa* and regular antibiotic courses after the establishment of chronic infection.

1.3.5.5.1 Aminoglycoside-induced nephrotoxicity

Two major adverse effects related to aminoglycosides are nephrotoxicity and ototoxicity. Most data for aminoglycoside toxicity are derived from studies on gentamicin and mainly from animal models. The incidence of aminoglycoside-induced nephrotoxicity

reported in the literature (not specific to CF) varies widely (*288*), mainly due to variation in the criteria used to define nephrotoxicity, administration of different aminoglycosides and patient characteristics (e.g. age, comorbidities, critical care patients). Recent studies are based on standardised definitions of AKI. A retrospective, observational study in adult (non-CF) patients from various hospital specialities receiving gentamicin showed a 24% incidence of AKI (*289*) using RIFLE criteria. A retrospective study in children receiving aminoglycosides (gentamicin in 489 of 557, 88% patients) in non-intensive care setup showed the rate of AKI was 33% using pRIFLE criteria and 20% using AKIN criteria for the definition of AKI (*290*).

1.3.5.5.1.1 *Aminoglycoside nephrotoxicity in CF*

In the last two decades, there are several reports of aminoglycoside-related nephrotoxicity in CF. There is evidence for increased incidence of AKI in this group compared to the general population; details are described in Section 1.3.3.1. A survey across various CF centres in the UK found the incidence of AKI approximately 4.6 to 10.1 cases/10,000 CF patients/year suggesting approximately 100 times greater risk of AKI in children with CF (*3*) compared to the general population of the UK (*175*). In 21/24 cases with AKI in patients with CF, there was a history of aminoglycoside administration in the preceding week compared to 3/42 control patients (OR 81.8, 95% CI 4.7 to 1427) suggesting that IV aminoglycoside use is a risk factor for AKI (*200*). Gentamicin was administered in 15 patients (71%) compared to 5 patients (24%) who had received tobramycin. Another retrospective, matched case-control study showed that, in a multivariate analysis, receipt of IV aminoglycosides within 90 days prior to administration of another IV aminoglycoside course was independently associated with increased odds of incident AKI (OR 2.28, 95% CI 1.10 to 4.72) (*202*). Furthermore, a study on 80 stable adult patients with CF, who were chronically infected with *P. aeruginosa* showed an association

between lifetime IV aminoglycoside exposure and reduced creatinine clearance (*5*). A retrospective analysis of adult patients with CF, followed up for 8.5 years found that 22% patients had ≥1 episodes of AKI during this period (*291*) and use of inhaled colistin was associated with episodes of AKI; however, a particular duration of use above which the risk of AKI increases could not be established. This was the first study suggesting an association of inhaled colistin with AKI, which needs to be assessed in a larger study of prospective design.

Despite the knowledge of the risk of nephrotoxicity, aminoglycosides remain a very commonly administered group of antibiotic in people with CF (*292*), mainly due to their efficacy against *P.aeruginosa*, rapid bactericidal activity (*293*) and synergistic activity with beta-lactams (*294*) and other cell wall active agents. Gentamicin has been shown to be more nephrotoxic than tobramycin and amikacin (*295*); gentamicin is not recommended for routine use in patients with CF (*292,296*). Tobramycin has the highest penetration in the lung tissue compared to other aminoglycosides; it achieves sputum concentration between 20-67% of that in serum (10-30% for amikacin and 20-40% for gentamicin) (*297*). Minimum inhibitory concentration (MIC) is the lowest concentration (mg/L) of an antimicrobial that prevents the appearance of visible growth of a microorganism within a defined period of time (*298*). Tobramycin has the lowest MIC for *P. aeruginosa* in the aminoglycoside group (*299*). Overall tobramycin has highly effective antipseudomonal properties (*293,300*) and has become aminoglycoside of choice in both adult and paediatric patients with CF (*301-303*).

The presentation of aminoglycoside-induced AKI in CF is similar to the non-CF scenario, which is in the form of non-oliguric renal failure and reduction in GFR. Progression to non-oliguric or anuric renal failure is rare with recovery on discontinuation of aminoglycosides being the most common outcome (*207,304,305*). Despite this, there is rising

concern regarding the cumulative toxicity of repeated courses of antibiotics (*207*) which could lead to a reduction of GFR and ultimately to CKD.

1.3.5.5.1.2 *Mechanism of aminoglycoside-Induced Nephrotoxicity*

The primary mechanism of the bactericidal action of aminoglycosides is by impairing the bacterial protein synthesis through binding to prokaryotic ribosomes (*306*). They act synergistically with other cell wall active antimicrobials.

Aminoglycosides are freely filtered, through the glomerulus in the kidney, without any metabolism in the body. Most of the dose is excreted in the urine. Only about 5-10% of the dose is selectively accumulated in the renal cortex, mainly in the proximal tubular cells (*288,307*). The concentration of aminoglycosides is several times higher in the renal cortex than in plasma (*288,307*). The uptake of aminoglycosides has been an area of interest, and different mechanisms have been implicated in this process. Megalin, an endocytic receptor (more details about structure and function in Chapter 3, Section 3.1.4) mediated uptake is the major pathway for aminoglycoside accumulation in tubular cells (*308-310*); in megalin knockout mice, only 0.6% of the intraperitoneally injected gentamicin dose was seen to accumulate in the kidneys 24 hours later compared to 10% accumulation in wild-type mice (*310*). Following the endocytic uptake in proximal tubular cells, aminoglycosides remain there with a long half-life (about 100 hours) leading to renal damage in the form of structural changes, and functional impairment of plasma membrane, lysosomes and mitochondria (*311*).

Aminoglycosides are composed of multiple amino sugars, which are connected to an aminocyclitol nucleus (*288*). The amino groups confer a polycationic character to aminoglycosides, and they bind to negatively charged membrane phospholipids (potentiated in the acidic

pH within lysosomes) (*288*). After endocytic uptake, aminoglycosides accumulate in the lysosomes, Golgi bodies and endoplasmic reticulum where they bind to membrane phospholipids (a process called phospholipidosis) (*288,312*) and alter their turnover and metabolism. After administration of low or clinical doses, these changes are marked by enlargement of lysosomes and progressive deposition of concentric lamellar subcellular structures (called as myeloid bodies). The toxicity of aminoglycosides correlates with the level of phospholipidosis (*288,313*). In experimental animal models, where higher doses of aminoglycosides are administered, the above process progresses. When a certain threshold is reached, the lysosomes rupture (*307*) releasing the aminoglycosides in the cytosol, which then act on the mitochondria to initiate a process of apoptosis and cell death.

These acute tubular changes are associated with signs of tubular dysfunction, e.g. release of brush border or lysosomal enzymes, proteinuria, wasting of $K^{+,}$ Mg^{2+}, Ca^{2+} and glucose, phospholipiduria (*311*). The acute tubular damage is followed by tubular regeneration/repair. When the damage exceeds the repair, renal dysfunction ensues (*288*). Overt renal failure is characteristically non-oliguric in nature. Recovery upon drug discontinuation is most often observed (*311*).

Because of the tubular damage, there is reduced reabsorption of glomerular ultrafiltrate, and increased volume of water and electrolytes are presented to the distal tubules. This initiates tubuloglomerular feedback leading to activation of angiotensin II system and reduction of GFR (*312*). With continued insult, tissue and cellular residue spill to the tubular lumen leading to partial or complete obstruction of the tubule, which in turn exerts a hydrostatic pressure inside Bowman's capsule leading to further reduction in GFR (*312*).

Histologically, tubular necrosis and its effects are supposed to be the primary cause of functional toxicity, but other mechanisms also play a part to explain the ongoing reduction in GFR. There are no gross morphological changes in glomerular structures; however, there is a marked decrease in GFR and ultrafiltration coefficient. This is reportedly due to mesangial cell changes (contraction and proliferation) which lead to a reduction of glomerular ultrafiltration surface and consequent decrease in GFR (*314*). Gentamicin increases the influx of calcium in the mesangial cells and increases the cytosolic free calcium level, which is described to mediate the mesangial contraction and proliferation (*315*). There is a release of various inflammatory markers, which are generated following tubular injury or due to the direct effect of gentamicin on glomeruli (*312*). Various vasoconstrictors are released by mesangial, vascular and tubular cells, e.g. platelet activating factor, endothelin-1 and thromboxane A2. There is activation of the renin-angiotensin-aldosterone system. A large body of evidence suggests a role of gentamicin in increasing oxidative stress and generation of reactive oxygen species (ROS) and reactive nitrogen species. Gentamicin generates superoxide anion and hydrogen peroxide, which, in the presence of a metal catalyst (iron released from mitochondria), produces hydroxyl radical (*316,317*). Gentamicin increases the expression of inducible nitric oxide synthase, which then leads to excessive nitric oxide production (*318*). ROS and nitric oxide interact to mediate the mesangial proliferation and cell apoptosis. All these various factors eventually induce contraction of mesangial cells and constriction of renal blood flow leading to reduced GFR (*312*).

Understanding the mechanism of aminoglycoside nephrotoxicity provides an opportunity to identify and develop strategies for its prevention. One such approach has been extended-interval dosing. This takes into account that the proximal tubular cell uptake of

aminoglycoside is a saturable process (*319,320*); a high peak dose (e.g. once daily dosage) leads to excretion of most of the drug without being bound to the tubular cells thus reduced nephrotoxicity (*217*). This strategy also benefits the bactericidal efficacy of the drug that is dependent on high peak level followed by a long post-antibiotic effect (*321*), which refers to continued suppression of bacterial growth for a period of time when the antibiotic concentration has dropped below MIC. Various other interventions to reduce aminoglycoside nephrotoxicity are in the research stages including approaches to reduce aminoglycoside uptake by megalin by using competitive molecules; inhibition of the receptors; treatment with antioxidants, and anti-inflammatory agents, e.g. platelet activating factor inhibitors and thromboxane A_2 inhibitors (*312,317,322*).

1.3.5.5.1.3 *Factors influencing aminoglycoside nephrotoxicity in CF*

Various factors can influence the nephrotoxicity in people with CF; these may be related to the pharmacokinetics of aminoglycosides, individual parameters, and treatment practices e.g. choice of aminoglycoside, dosing methods, therapeutic drug monitoring, other concurrent treatment, duration of therapy, etc.

Altered pharmacokinetics of aminoglycosides in CF

Terminologies in Pharmacokinetics

In simple terms, pharmacokinetics can be defined as what body does to the drug. Various mechanisms involved in drug disposition include absorption, distribution, metabolism and excretion. Pharmacokinetics is the mathematical description of the rates of these processes and of concentration-time relationships (*323*). A number of parameters are incorporated in pharmacokinetics, some of which are explained in the following paragraph.

Drug clearance is a measure of the ability of the body to eliminate drug (analogous to the concept of creatinine clearance). Clearances by different routes or organs (e.g. liver, kidney and others like saliva, sweat etc.) are added to give total systemic or total body clearance (TBC) (*324*). Clearance is calculated as a fraction of dose available (to systemic circulation) divided by area under concentration-time curve (AUC). AUC represents the total drug exposure integrated over time (*325*) The higher the AUC for a given dose, the lower is the clearance (*325*).

Half-life ($t_{1/2}$) represents the time taken for plasma concentration (or amount of drug in the body) to be reduced by 50%. Elimination rate constant is the constant for the elimination of the drug from a compartment (or when added together represents total elimination rate constant, Kel). It is inversely related to the half-life of the drug (*324*).

The volume of distribution (Vd) relates to the amount of drug in the body to the concentration of drug in blood or plasma (where concentration is measured). It represents (apparent, not real) volume of a pool of body fluids that would be required to account for the total amount of drug in the patient's body if the drug was equally distributed throughout the body in the same concentration as found in plasma. It depends on various factors like binding of the drug to blood cells, plasma proteins and tissue components. Drugs with a higher degree of binding to plasma proteins will have a lower Vd whereas drugs with a higher degree of tissue binding outside plasma (e.g. hydrophobic drug digoxin) will have higher Vd which may exceed total plasma volume. Aminoglycosides are primarily excreted by the kidney (*326*) and are not metabolised in the liver or bound to plasma proteins.

Pharmacokinetics of aminoglycosides in CF

The pharmacokinetics of aminoglycosides in CF has been studied extensively. There is an altered disposition of many drugs in people with CF with differences being more significant for hydrophilic drugs like aminoglycosides than for other drugs like penicillin and cephalosporins (*327-329*). Patients with CF need higher doses of antibiotics compared to people without CF to achieve therapeutic efficacy (*292,327*).

Mann et al. compared the pharmacokinetics of tobramycin and gentamicin in a group of CF patients (N=27) with a (historical) control group (*330*). CF patients had higher TBC and needed much larger doses of aminoglycosides compared to the controls to achieve a set peak level of 8 µg/ml whereas the volume of distribution was similar for the two groups (*330*). A prospective controlled study compared the pharmacokinetics of tobramycin for CF with an age-matched controlled group (*331*) and found higher TBC in CF, however, renal clearance of tobramycin was similar for the two groups. GFR

(iothalamate TBC) was not different in both groups (*331*). These results of high TBC for aminoglycosides in CF were demonstrated by many other studies (*332,333*). One controlled study did not show any difference in body clearance of netilmicin between CF and control groups. However, the groups were small in size, each comprising of total eight children who were age-matched but not weight- or height-matched (*334*).

The underlying mechanism to explain increased aminoglycoside clearance in CF is not clear. There have been speculations about increased extra-renal clearance by an alternate route like gastrointestinal tract or respiratory system (*331,335*). MacDonald et al. (*336*) found that more than 80% of gentamicin was excreted in the urine by 4 hours in individuals with CF. This is similar to the amount excreted in healthy human beings (*337*). There was negligible excretion of gentamicin in sputum, saliva and sweat in this study, which established that kidneys were the major route of elimination of gentamicin in patients with mild CF (*336*). This has also been shown, in CF patients, by other investigators (*326*). Furthermore, the study by MacDonald et al. showed increasing TBC with increasing severity of illness (r^2=-0.73). However, the National Institute of Health score was used to score the severity of illness, which is now outdated given the changing natural history of CF (*338*)). Increased TBC has also been linked to decreased tubular reabsorption or increased tubular secretion by some authors (*339*).

There are conflicting reports of high (*326,331-333*) or normal Vd (*330,334,336,340*) in CF group compared to the non-CF group. The data overall confused the issue as there were methodological flaws in some controlled studies where the control group was only age-matched with CF group and not weight-matched (*331,334*). There was no difference in Vd after normalisation for body surface area (*331*). Similarly, there are conflicting data regarding elimination half-life of

aminoglycosides in CF (*330,331,333,334,340*). Increased Vd in CF has been linked to increased lean body mass per kg body weight (*339*) and decreased adipose tissue. However, this may not be relevant now with the improved nutritional status of many people with CF.

In summary, the overall evidence from the studies investigating aminoglycoside pharmacokinetics in people with CF suggests high TBC; however, there are conflicting results for other parameters. This means that people with CF need higher doses of aminoglycosides than people without CF (*341*). It may be argued that due to higher TBC people with CF are less prone or comparatively resistant to the nephrotoxicity induced by aminoglycosides. A counterargument could be that their renal cortical cells are presented with a higher amount of aminoglycosides and the AUC is higher. Thus the net effect is greater potential for accumulation and subsequent nephrotoxicity.

Other CF specific factors

Considering the severity and nature of the pulmonary illness in CF, high doses of aminoglycosides are also required to achieve effective concentrations (MIC) in respiratory secretions. Tissue penetration of drugs is influenced by physiochemical factors of transfer (*299,342*), e.g., lipid solubility, the degree of ionisation, molecular size etc. Penetration of aminoglycosides in the bronchopulmonary tree is low (*299,342*). Furthermore, different components of sputum like mucin and DNA bind to aminoglycoside molecules, which may reduce the bioactivity of the drug (*343*).

As aminoglycosides have a narrow safety margin, therapeutic drug monitoring is essential. Since there are no large studies on children with CF, owing partially to the risk it carries with suboptimal treatment, most of the practices have been extrapolated from non-CF patients despite the differences in elimination. Although therapeutic drug monitoring is being used for a single course of antibiotics, this

may not diminish the long-term renal damage caused by repeated courses, as most CF patients require several antibiotic courses through their lifetime, which include aminoglycosides.

The presence of other comorbidities, which are risk factors for aminoglycoside-induced renal injury (*344,345*), may pose further risk to influence the overall nephrotoxicity caused by aminoglycosides. These may include poor nutritional status; other infections, e.g. fungal infections requiring the use of other nephrotoxic drugs; CF-associated liver disease; diabetes; cor pulmonale etc. A retrospective, matched case-control study assessed risk factors for AKI (KDIGO criteria) in children with CF while receiving IV aminoglycoside course. The study included 82 admissions (where AKI developed while receiving a course of IV aminoglycosides) as cases and 164 admissions as controls (no AKI developed during a course of IV aminoglycosides) (*202*). The multivariate analysis showed that, in addition to aminoglycoside related factors (receipt of aminoglycosides within 90 days prior to admission and longer duration of aminoglycosides), low serum albumin and coadministration of trimethoprim-sulfamethoxazole were independent risk factors for the development of AKI whereas infection with *staphylococcus* diminished the odds of having AKI. As albumin is transported via the same receptor, i.e. megalin, in proximal tubules (*346*), low albumin levels supposedly may allow more aminoglycoside molecules to reach the proximal tubular epithelial cells thus leading to higher nephrotoxicity. Further studies will be needed to establish whether the effect of trimethoprim-sulfamethoxazole administration on incident AKI is a true effect or is an artefact reflecting the inhibition of tubular secretion of creatinine by it (*347*), without a true change in GFR.

1.3.5.5.1.4 *Strategies for prevention of aminoglycoside-induced nephrotoxicity*

Since the awareness of increased renal impairment in people with CF, various strategies have been put in place with an aim to prevent the nephrotoxicity induced by aminoglycosides. We have become better at therapeutic drug monitoring and dosing calculations. A number of other new interventions are in the research stage.

Use of less nephrotoxic treatment regimen

Choice of antibiotic

Comparing the nephrotoxic potential of different aminoglycosides in a non-CF setting, gentamicin has been demonstrated to pose the highest risk and amikacin the least, whereas netilmicin and tobramycin are quite close to amikacin (*348*). A similar conclusion has been drawn by a quantitative overview of randomised controlled trials comparing aminoglycosides (*349,350*). There is a difference in the renal disposition of different aminoglycosides, and gentamicin has been shown to have the highest degree of tubular reabsorption (despite similar pharmacokinetic (PK) characteristics), which may explain the highest nephrotoxicity of gentamicin in the group (*337*).

Extended-interval dosing

There is evidence that once-daily dose of tobramycin is less nephrotoxic than thrice-daily dosing in people with CF. The TOPIC study, a large randomised controlled trial including 244 people with CF, showed that the once-daily dose of IV tobramycin for pulmonary exacerbations in CF was less nephrotoxic in paediatric age group compared to thrice-daily dose (adjusted mean difference for a change in serum creatinine during treatment was -8%, 95% CI -15.7 to -0.4) (*217*). Furthermore, the increase in urinary NAG concentration (an early marker of renal tubular injury) during treatment was 33% less for once-daily group than thrice-daily group (*217*) A Cochrane systematic review also confirmed similar findings (*351*). Use of once-daily regimen

allows the use of higher doses and helps achieve higher peak levels, i.e. better bactericidal efficacy (*352*). This also means that most of the dose is excreted without being absorbed in the proximal tubules, as absorption of aminoglycosides by proximal tubular cells is a saturable process (*320*). Extended-interval dosing also benefits from continued bactericidal effect (i.e. post-antibiotic effect) and effect on adaptive resistance (*217*).

Surveys across different countries have shown profound changes in the practice of aminoglycoside prescribing (*301,303,353*). Tobramycin is now the most frequently administered aminoglycoside in people with CF, and most courses are prescribed once daily (*301-303*). Therapeutic drug monitoring and weight related dose calculation is recommended (*296*) and is used widely (*302,303*). Gentamicin use is decreasing; one in three centres having stopped using it (*301*). All these changes have a potential to reduce the incidence of nephrotoxicity by aminoglycosides in the CF population. Furthermore, there is a recommendation to avoid the use of other nephrotoxic agents (e.g. ibuprofen, furosemide) while administering IV aminoglycosides (*296,320*).

Therapeutic drug monitoring and target concentration intervention

Aminoglycosides have a narrow therapeutic index; doses above the therapeutic range can cause nephrotoxicity or ototoxicity, whereas those below may lead to suboptimum effect and promote growth of resistant organisms. Therapeutic drug monitoring is recommended (*296*); however, there is no universal recommendation for which method should be used, and there is a wide variation in practice. Measurement of peak and trough levels is recommended for multiple daily doses, and monitoring of trough levels is recommended for once-daily doses (*296*).

As there is a high individual variation in aminoglycoside pharmacokinetics (*335,354*) in patients with CF, individualised dosing regimens have been used in many centres. Various methods are available to guide the individualised dosing regimen for once-daily dose of tobramycin; these include monitoring for trough level concentration of <1 mg/L before next dose, calculation of AUC (*355,356*), and a Bayesian approach (*357,358*). Monitoring for trough concentration of tobramycin does not reflect C_{max} concentration (maximum free drug concentration in serum) or AUC values during the dosing interval, and almost 100% of patients have tobramycin concentration <1 mg/L as trough levels (*357*). Measurement of trough concentration monitoring is not appropriate for once-daily dosing of aminoglycosides (*356*); even in patients where tobramycin clearance is reduced by half, the trough concentration following a once-daily dose will still result in a 24 hour trough of <1 mg/L, albeit the AUC will be significantly higher than the target level (*355*). Daily AUC for aminoglycosides is a significant predictor of nephrotoxicity (*359*). The Bayesian approach ('goal-oriented model-based Bayesian adaptive control') is highly effective (*357,360,361*) for individualisation of dosage; nevertheless, this has not been practiced widely in some parts of the world including the UK, probably related to lack of familiarity and need for a sophisticated software (e.g. MW/Pharm, Mediware a.s., Czech Republic (*358*) used for experiments described in Chapter 4 of this thesis). The initial dose is estimated based on a population model and demographic characteristics such as body weight, serum creatinine. Serum concentration of the drug is measured after the first dose, which is then used to calculate individual PK parameters and individual therapy (*361*). Best results are obtained by using population models based on CF specific population parameters (*361*).

Individualised dosing regimens based on pharmacokinetic-pharmacodynamic interactions are new tools to analyse antibacterial

treatment. 'Target concentration intervention', an extended therapeutic drug monitoring, takes into account individual differences to achieve target effect and associated target concentrations (rather than just aim to achieve a concentration within a chosen range (*362*)). AUC to MIC ratio and C_{max} to MIC ratio are the best predictors of the bactericidal effect of aminoglycosides (*352*). Targets of an AUC: MIC ratio of 80-110 and a C_{max}:MIC ratio of 8-10 are optimal targets to achieve for effective bactericidal action of aminoglycosides against gram-negative infections in adult people (*363*). A paediatric study suggested that a 10 mg/kg dose proves most efficient with an aim to achieve 30 mg/L (24-30 mg/L) C_{max} concentration, and a C_{max}:MIC ratio of 10 is desirable in the paediatric population (*357*). Pharmacodynamic parameters (AUC to MIC ratio and peak to MIC ratio) show significant correlation with efficacy (i.e. increase in FEV_1 and FVC) (*364,365*).

Use of competitive inhibitors to prevent renal tubular uptake of aminoglycosides

As proximal tubular absorption of aminoglycosides is dependent on uptake by an endocytic receptor protein (megalin), competitive inhibition of aminoglycoside uptake by megalin may potentially lead to reduced nephrotoxicity. In an animal study, megalin substrate: cytochrome *c* decreased gentamicin uptake in a dose-dependent manner both in vitro and in vivo. Furthermore, in dehydrated rats, coadministration of cytochrome *c* with gentamicin showed significantly less urinary excretion of NAG compared to administration of gentamicin alone (*366*). However, the dose of cytochrome *c* used may lead to peptide overdose in clinical situations. Unfortunately, the study on peptide fragments of cytochrome *c* did not show an effect on gentamicin accumulation in vivo, possibly related to low stability or extensive binding to various tissues (*366*). Further studies will be needed to evaluate the role of cytochrome *c* and its fragments in the prevention of aminoglycoside-induced nephrotoxicity (*366*).

Another more promising approach has been related to using statins; animal studies have shown that coadministration of aminoglycosides and statins (simvastatin, rosuvastatin, pravastatin) is associated with a reduction in aminoglycoside accumulation in renal proximal tubular cells and reduced cytotoxicity (*367*). Statins are widely used in treatment of hyperlipidaemia and work by inhibition of enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which inhibits the rate-limiting step in the conversion of HMG-CoA to mevalonate. Metabolites derived from mevalonate facilitate activation of guanosine triphosphate binding proteins (required in the process of endocytosis of aminoglycosides). A multicentre, phase IIa randomised controlled clinical trial (The PROteKT study; EudraCT 2014-002387-32, UKCRN ID 16993, ISRCTN26104255 (*368*)) has been undertaken across 13 centres in the UK to investigate the effect of coadministration of rosuvastatin on aminoglycoside-induced nephrotoxicity in children with CF. The study has finished recruitment and results are awaited.

Other strategies

Other approaches to prevent nephrotoxicity of aminoglycosides rely mainly on coadministration of renoprotective drugs and are generally in preclinical research stages (*312*). A number of studies have investigated effects of antioxidants, some of which show beneficial effects; however, the results need to be explored in a clinical setting (*312*). Other strategies include the use of thromboxane A2 inhibitor, calcium antagonists and blockade of immune response (*312*).

A randomised crossover study using fosfomycin (broad spectrum, fosfonic acid antibiotic with antipseudomonal properties) showed that coadministration of fosfomycin with tobramycin reduced the urine levels of renal tubular enzymes elevated by IV tobramycin administration (*369*). Fosfomycin is excreted unchanged through the kidneys (*370*) and is speculated to compete with aminoglycosides for

the binding site within the proximal tubular cells thus preventing cellular apoptosis (*371*).

Another strategy under investigation is the use of nebulised aminoglycosides instead of IV administration for treatment of pulmonary exacerbations in people with CF. A small, randomised crossover study was conducted in 20 adults with CF with promising results showing increased suppression of sputum *P.aeruginosa* and reduced urinary markers of acute renal tubular injury (NAG, β2-microglobulin and alanine aminopeptidase) with inhaled tobramycin compared to IV tobramycin administration (*372*) for a two week period. Larger studies are required to confirm the effect.

1.3.5.5.2 Nephrotoxicity related to other drugs

In addition to aminoglycosides, many other commonly used antimicrobials in CF may lead to nephrotoxicity (*373*), e.g. cephalosporins (*374*), quinolones (*162,375,376*), sulphonamides, colistin and antifungal agents, e.g. amphotericin (*377*), etc.

The nephrotoxicity of colistin has been assessed in people with CF. It was associated with increased damage related to aminoglycosides, but the association was not seen with colistin alone (*5,378*).

Ciprofloxacin is a commonly used antibiotic in patients with CF (*379*). Due to concerns regarding its association with arthropathies in children, several large studies were done to establish its safety in children (N=276, 33% CF (*380*); N=1795, including mainly children with CF (*381*); N=41, children with CF (*382*)). There are three case reports including four patients that suggest an association of ciprofloxacin with renal impairment in isolated cases with CF (*162,375,376*). Two cases of acute renal failure after ciprofloxacin happened in young adults after about 3 weeks treatment with 750 mg BD ciprofloxacin (*162*) and one other patient had received ciprofloxacin at a dose of 750 mg TDS for 3 weeks (*375*). Mechanism of nephrotoxicity was not

clear, and a possibility of allergic interstitial nephritis (related to type III hypersensitivity reaction) was raised (*162*). None of these patients had a renal biopsy and renal function normalised after the drug was withdrawn. One case was a 25-year-old woman with CF and diabetes who had 2 courses of ciprofloxacin (*376*) followed by a decrease in renal function. Renal biopsy showed epithelial vacuoles in distal tubules, which is a finding often associated with gentamicin toxicity. Analysis of data from a French surveillance system for adverse drug reaction to fluoroquinolones showed that acute renal failure is a rare complication: one case to 80,000 patients (all population, not specific to CF) treated with ciprofloxacin (*383*). Considering the efficacy of ciprofloxacin against *P.aeruginosa* and a widespread use in CF population, it is important to emphasise that nephrotoxicity is a very rare complication of ciprofloxacin use, which physicians should be aware of, particularly in the setup of coadministration with other nephrotoxic agents; use of larger doses of ciprofloxacin; presence of other comorbidities like malignancies, HIV infection and heart transplant (*384*).

Azithromycin is a commonly used agent in people with CF for its antimicrobial and anti-inflammatory properties. Only a small amount of ingested azithromycin is excreted unchanged in the urine, and its pharmacokinetics are not affected by mild to moderate renal and hepatic insufficiency (*385*). Azithromycin use in CF has been the subject of both systematic review and meta-analysis (*386,387*). The largest systematic review included about 600 patients where the safety of azithromycin in patients with CF was investigated (*388*). There are three case reports of acute interstitial nephritis (AIN) in non-CF patients (*389*),(*163,390*) who received azithromycin. Two of these patients developed chronic renal failure, one of whom was still on haemodialysis 10 months after the episode of AIN (*389*). There is no case report of kidney injury associated with azithromycin use in CF

population. A PK study on a large group of people with CF receiving azithromycin suggested no significant side effects (*388*). A single centre case series of AKI presented data on adults with CF and chronic multiresistant *P.aeruginosa* infection. Four of eight patients who developed AKI, associated with aminoglycosides, also received azithromycin (*198*). In two cases, azithromycin was considered a possible contributory factor by renal specialists. These data should be treated with caution considering the beneficial effects of azithromycin treatment (*386*) and lack of any other reports of an association between azithromycin and AIN in the CF population.

Immunosuppressive therapies like calcineurin inhibitors are used in the post-transplant care and have been described above in Section 1.3.5.4. Many people with CF are on proton pump inhibitors (PPI) as they potentiate the effect of pancreatic enzymes. Many reports suggest an association of PPI with AIN in the general population (*391*). A population-based study has shown 2.5 times higher risk of development of AKI in elderly (non-CF) patients using PPI (*392*). The association has been speculated to be due to the deposition of PPI in the renal tubular interstitium, which leads to stimulation of T cells to mediate AIN. Recent epidemiological data suggest an association of PPI use in the community with 30-50% higher risk of being diagnosed with CKD (*393,394*). There are no studies in CF population.

Similarly, other drugs like non-steroidal anti-inflammatory drugs, which may be used for their anti-inflammatory properties (*395,396*), have been associated with renal adverse effects (*164,165*).

1.4 Measurement of renal function

GFR is described as the best index of renal function in humans (*397*). It is defined as the sum of the filtration rate of all functioning nephrons in the kidney (*397*). It is measured indirectly, using clearance of filtration markers, which are ideally inert substances of low molecular weight that are not protein bound, filtered only by glomerular filtration, and neither metabolised, secreted nor reabsorbed in the renal tubules (*398*). The filtration markers can be exogenously administered or endogenously produced agents.

1.4.1 Measurement of GFR

Clearance of Inulin, a 5200 D, inert, fructose polymer is described as a gold standard method for measurement of GFR (*397,399*). The classic method of inulin clearance measurement involves a continuous infusion of inulin and collection of urine by bladder catheterisation. The measurement needs to be done under specific conditions, i.e. subjects are studied in lying down position, after an overnight fast, with oral water loading to stimulate diuresis (*399*). Clearly, this is a cumbersome and invasive test, which is less practical to use routinely in both clinical or research settings.

Various radioactive isotopes (radiological IV contrast agents) have been used as alternative filtration markers, and the tests have been validated against inulin clearance. These exogenous markers include ^{125}iodine-iothalamate (^{125}I-iothalamate) (*400*), ^{99m}Tc-DTPA (*401*) and ^{51}Cr-EDTA (*402*). All these alternative methods deviate slightly from the gold standard inulin clearance, mainly related to the differences in the renal and extra-renal handling of the markers and systematic differences in the assays (*403*). Choice of a radioactive substance is mainly based on ease of obtaining, administering and counting the radioisotope (*397*). ^{51}Cr-EDTA clearance is the most commonly used agent in Europe whereas ^{99m}Tc-DTPA and ^{125}I-iothalamate are used in

the USA (^{51}Cr-EDTA is not available in the USA). Comparison of the accuracy of different methods has been presented in a systematic review of studies comparing various tracer clearances to inulin clearances (*404*). There was strong evidence for the accuracy of renal clearance of ^{125}I-iothalamate, renal and plasma clearance of ^{51}Cr-EDTA and plasma clearance of Iohexol. There was not enough evidence that suggested the accuracy of plasma clearance of ^{99m}Tc-DTPA compared to inulin clearance (*404*).

Plasma clearance or urinary clearance of exogenous filtration markers can be used.

1.4.1.1 Urinary clearance methods

After the injection of the exogenous marker (IV or subcutaneous), multiple urine samples are collected (2-4 at 20-30 min intervals) and clearance of the marker is calculated for each of these time periods which is averaged at the end to calculate final clearance (*403*). Urinary clearance methods can be quick to perform but come with a difficulty of the need for water loading to stimulate urine flow to complete the collection of urine before the tracer concentration drops below the detection limit of the assay. Furthermore, incomplete voiding may introduce chances of error.

1.4.1.2 Plasma Clearance methods

Plasma clearance is measured after single IV injection of exogenous filtration marker followed by a collection of multiple blood samples: early blood samples at 2-3 time points until 60 min from the injection and late samples at 1-3 time points from 120 min onwards (*403*). Ideally, a two-compartment model is used with the initial phase of rapid disappearance (distribution phase) followed by slow disappearance (renal excretion phase) (*405*). The clearance is calculated by the amount administered divided by the AUC (of the plasma concentration-time curve) for the filtration marker. For any

exogenous marker that is excreted solely by kidneys, plasma clearance will be equivalent to urinary clearance. However, in the event of extra-renal clearance, GFR will be overestimated. Another limitation of plasma clearance methods is a longer duration of test to determine the disappearance of marker and need for multiple blood sample collection. In addition, results may be inaccurate in people with extensive third spacing, e.g. obesity/oedema or very slow clearance related to low renal function. Simplified methods for measurement of plasma clearance of exogenous markers have been suggested as described in the next paragraph. Radioactivity of the markers has been described as lower than even a chest X-ray. Non-radioactive agents, e.g. minute doses of non-radioactive agents like iohexol are also used followed by High Performance Liquid Chromatography assays.

1.4.1.2.1 Slope-intercept method

This method works on one-compartment characterisation and involves the determination of only later exponential phase of the concentration-time curve. At least two blood samples are taken at 2 & 4 hour after IV injection. The slope of the late exponential phase is calculated by regression. This method introduces a systematic bias leading to overestimation of GFR by ignoring the rapid exponential phase of the curve. Two correction methods have been proposed: Bröchner-Mortensen's method uses a quadratic equation (*406*) whereas Chantler's method (*407*) uses simple proportional correction, i.e. same correction at all levels of GFR. The former equation makes better corrections for early exponential phase; however, it overcorrects the values at the higher end, i.e. underestimates GFR at higher clearance values (*408*). The latter method neglects the increasing weight of the first exponential higher clearance and underestimates the low GFR values (*408*).

1.4.1.2.2 Single-Sample Method

The other technique is based on a single sample collection after injection of the marker (*409,410*). A single blood sample is usually taken after 3-4 hours of the tracer injection. The principle of this method is that at any fixed time point, there is an inverse relationship between GFR and plasma concentration. The calculation is based on apparent tracer volume of distribution, which is derived from the dose administered and the plasma concentration at the sampling time. There is no need for calculation of the AUC and correction factors as above (*411*). However, the accuracy of single-sample GFR depends on marker distribution, accurate estimation of extracellular fluid volume and the GFR itself (*412*). In the case of people with very low renal clearance (<30 ml/min/1.73m^2), if the sampling time is kept the same, there is a likelihood of a significant error (overestimation) in the measured GFR value (*408,413*). Though more convenient, single-sample method has also been criticised as there is no quality control measure because the slope of the late exponential phase cannot be determined (*413*). Various studies have compared one sample technique with multiple sample techniques (*414-416*), and there is some evidence that with improved methods (*417,418*), single-sample GFR measurements perform better than slope-intercept measurement (*416*). However, there continues to be ongoing controversy regarding which method is the preferred (and more accurate) one. The British Nuclear Medicine Society (BNMS) guidelines advise using slope-intercept method (*419*) whereas European Association of Nuclear Medicine guidelines recommends using single-sample method for patients with normal GFR (*410,411*).

GFR measurement using exogenous markers is complicated, expensive and not so readily available in many places; hence, alternative methods such as measurement of clearance of endogenous markers, e.g. creatinine are used. Creatinine clearance is a widely available

method for GFR measurement. There is a need for collection of urine for long periods (6-24 hours) which may be cumbersome and less accurate particularly in the paediatric population. A meta-analysis based on 23 studies demonstrated strong evidence that creatinine clearance is not an accurate marker of GFR (*404*). It overestimates GFR mainly related to secretion of creatinine in the renal tubules. Furthermore, there is a progressive increase in the fractional secretion of creatinine (compared to inulin clearance) by renal tubules with reduction of GFR (*420*), which makes creatinine clearance even more insensitive to changes in GFR .

1.4.1.3 Reference values for ^{51}Cr-EDTA GFR

For the work described in this thesis, GFR measurement by ^{51}Cr-EDTA was selected, as this is the most accurate and widely used method in the UK, and labs have experienced its use. It has been recommended by the BNMS guidelines (*413*). The reference values for ^{51}Cr-EDTA GFR have been established in the non-CF population. The largest study in the paediatric population is from Piepsz et al. who report the GFR values in a group of 623 children, between 0.1 to 15 years age, with unilateral (*421*) well-defined pathology, a normal left to right uptake ratio (relative uptake between 45-55%) and no recent urinary tract infection. GFR increases from after birth up to 2 years of age and then remains constant until 15 years of age. Mean GFR value was 104 ml/min/1.73m^2 with 10th and 90th percentile values being 81 and 135 ml/min/1.73m^2 respectively. Granerus et al. established reference values for ^{51}Cr-EDTA values by compiling 8 different studies including about 500 healthy adult subjects from epidemiological studies and studies in kidney donors (*422*). Half of these studies were based on inulin clearance from which ^{51}Cr-EDTA GFR was calculated by adjusting for extra-renal clearance of EDTA. The normal range for GFR was described to vary with age with a decline at a rate of 4 ml/min/decade below 50 years age and a faster decline of 10 ml/min/decade after 50

years age. The reference values for ^{51}Cr-EDTA GFR were established as following (mean ± 2 standard deviation (SD)): 105 ± 26 ml/min at 30 years, 98 ± 23 ml/min at 50 years and 78 ± 24 ml/min at 70 years. Another large study evaluating reference values for ^{51}Cr-EDTA GFR in 400 healthy live kidney donors (*423*) again showed that GFR remains constant until the age of 40 years at 103.4 (SD 15.5) ml/min/1.73m^2. There is a decline of 9 ml/min/1.73m^2 per decade after this.

1.4.2 Estimation of GFR

Estimation of GFR based on plasma concentrations of endogenous markers, e.g. creatinine and cystatin C are widely described in the literature. Various equations have been developed to help estimate GFR using endogenous markers, mainly based on serum creatinine and recently, based on serum cystatin C. Creatinine is generated in muscles during metabolism of creatine. It is freely filtered across the glomerular membrane and is secreted by renal tubules. Cystatin C is a low molecular weight protein generated by all nuclear cells at a constant rate (*424*). It is freely filtered by glomeruli and then is almost entirely absorbed and metabolised by proximal tubules (hence urinary estimation is not possible).

Plasma levels of endogenous markers are determined by various factors, which include their generation (from cells and dietary sources), extra-renal elimination (from liver or gut, etc.), and renal excretion (filtration, secretion and reabsorption) (*403*). Non-renal determinants of creatinine level are muscle mass, dietary pattern, nutritional status and other factors that can affect the muscle mass, e.g. age, sex, race, ethnicity, etc. (*397,403*). Extra-renal elimination of creatinine has been described particularly at lower levels of GFR mainly related to degradation by intestinal bacteria (*425*). Several creatinine-based equations to estimate GFR adjust for various parameters like age, sex, race, etc. as surrogates to factor in the differences in creatinine generation due to muscle mass. However,

many groups of people are still likely to have large differences between eGFR and mGFR, particularly people at extreme ends of muscle mass, e.g. malnutrition, muscle wasting, chronic illness, amputation; populations different to the group where the equation was developed; people with vegetarian diets, etc. Cystatin C generation is described as more constant than creatinine. However, its serum levels can be affected by age, sex, weight, height, smoking status, the level of CRP and use of corticosteroids (*189,426*). Extra-renal elimination of cystatin C has been described at higher levels.

GFR estimation equations are derived by regression analysis in a specified population using the correlation of mGFR with the level of a particular marker. The more diverse the population from which the equation is developed, the more extensive is its application likely to be (*398*). The most widely used GFR estimating equation in adults is MDRD equation (*427*), and that for paediatric population is (updated/bedside) Schwartz equation (*428*). Recent guidelines (*189*) advise using 2009 Chronic Kidney Disease Epidemiology Collaboration (CKD-Epi) equations for adults. These equations were developed in large, diverse populations. There is evidence that they are more accurate than MDRD equation (*398*). Cystatin C-based equations have been recommended in specific situations, e.g. people with low GFR between 45-59 ml/min/1.73m^2 who do not have markers of kidney damage. The bedside Schwartz equation is still recommended for paediatric population (*189*). There is evidence now to suggest that cystatin C-based equations perform better, and equations using both markers, i.e. creatinine and cystatin C work even better.

Assessment or validation of any equation in a patient group requires estimation of bias and precision. Bias reflects a systematic difference of eGFR to mGFR (or reference method). It is related to differences in the populations, differences in assays and various surrogates affecting non-GFR determinants of the endogenous marker. Precision reflects

random error or variation of difference around mGFR. It can be generated by variation in non-GFR determinants and GFR measurement error or biological variation. Bias and precision can be calculated by creating a Bland-Altman plot and plotting the differences between the two measures against their means. Accuracy includes both precision and bias. One way of assessing accuracy is to calculate the percentile difference and find the proportion of eGFR values lying within 10% (P-10) or 30% of mGFR (P-30).

Although the GFR estimating equations are used widely, the results are limited in several situations due to lack of accuracy. One important source of variation is determined by population or people characteristics; individuals with abnormal habitus and muscle mass at two extremes, chronic illness, malnutrition, different ethnic background, etc. may have a considerable variation of eGFR from mGFR. Estimation equations have been developed in populations in steady state; in unsteady states, e.g. rapidly increasing or decreasing GFR, the change in eGFR follows the same direction but the response lags behind and may be unreliable in identifying acute changes in GFR (*403*). Most of the eGFR estimating equations have been developed in people with CKD or low GFR; the level of accuracy is less in high or normal GFR spectrum. Biological variation in GFR is greater at normal or high GFR levels; inaccuracy conferred by non-creatinine chromogens is higher at lower creatinine levels (i.e. higher GFR). The effect of the difference in analysis method or variation in calibration is higher at the upper end of the spectrum. As part of standardisation of creatinine calibration programme, the current recommendation is for all the laboratories to calibrate their serum creatinine measurement method to single international reference material. This is termed “traceable to IDMS” (Isotope Dilution Mass Spectrometry) reference methodology. Newer equations are based on standardised creatinine values (*189*). Reduced accuracy of eGFR equations at the higher end

precludes the use of these equations for decision-making or management in various situations, e.g. assessment for kidney donation, monitoring kidney transplant recipients, before administration of nephrotoxic agents or chemotherapy, etc.

People with CF are exposed to repeated treatment with nephrotoxic drugs; hence, it is vital to monitor the effect on renal function. Large changes in GFR are detected by eGFR, but due to lack of precision, GFR estimations may not be reliable to monitor small changes in GFR over time. Moreover, GFR estimation may be affected by the level of nutrition or reduced muscle mass in the CF population. There is limited evidence assessing the validity of GFR estimation in people with CF, particularly in the paediatric population, where only one study has evaluated the agreement of mGFR with eGFR (*215*). The evidence is mainly suggestive of high bias and wide limits of agreement (*215,326,429,430*). None of the various equations assessed stood out as highly accurate apart from cystatin C equation in one study (*430*). This was not replicated in another study (*215*); however, the two studies used a different equation and a different tracer for GFR measurement.

1.5 Aminoglycoside-Induced Hearing Loss

As people with CF receive multiple courses of antibiotics, including aminoglycosides, there is also concern regarding ototoxicity including cochleotoxicity and vestibulotoxicity. Cochleotoxicity presents as hearing loss, which may be associated with tinnitus. Vestibulotoxicity presents as dizziness and loss of balance (*166*). Ototoxicity has been described as an independent event, which is not associated with nephrotoxicity when drug levels are adjusted to prevent high serum levels (*431*). Unlike nephrotoxicity, the aminoglycoside-induced hearing loss is usually irreversible. There is a considerable variation in the reported prevalence of hearing loss after aminoglycoside treatment; in most studies, it varies between 2 to 25% (*166*) although up to 50% prevalence has been reported by some authors in individuals with CF (*432*) and in people with *P. aeruginosa* endocarditis (*433*). A 30% prevalence of vestibular toxicity (*434*) after aminoglycoside therapy has been described. In this study, we have focussed on cochleotoxicity, and further mention of ototoxicity will imply cochleotoxicity only.

The large variation in prevalence of aminoglycoside-induced ototoxicity can be attributed to multiple factors including different study methods, different age groups, disease severity, previous exposure to aminoglycosides, genetic predisposition, etc. Part of the variation might have been contributed by differences in drug administration methods, e.g. type of aminoglycoside, duration, dose, plasma levels, etc. A study combining the patients from various randomised controlled trials (not patients with CF) showed a 22% prevalence of ototoxicity after a single course of aminoglycosides. Various risk factors that could help predict ototoxicity were mainly related to higher dose, longer duration of therapy and factors that suggested greater disease severity, e.g. bacteraemia, volume depletion, liver dysfunction, increased temperature, etc. (*435*). These

results were not replicated by another study from the same era, which showed age as an independent factor to influence ototoxicity but not the factors described in the former study. The estimated probability of developing ototoxicity increases with age (*436*). Among different aminoglycosides, netilmicin has been described as least ototoxic (*350*) and amikacin the highest with others, i.e. gentamicin and tobramycin, in between the two (*295,436*).

After administration, aminoglycosides are cleared rapidly from the body by glomerular filtration; however, a proportion of dose accumulates in renal cortex and perilymphatic and endolymphatic fluid of inner ear, which is responsible for nephrotoxic, ototoxic and vestibular toxic effects. The aminoglycosides are cleared very slowly from the inner ear cells due to a very long half-life (*437*). Within the hair cells, aminoglycosides bind to iron and generate ROS (free radicals). The role of impaired calcium influx into the cells is being studied. These complex reactions eventually lead to cell apoptosis.

One important risk factor for aminoglycoside ototoxicity is genetic susceptibility, which contributes to about 17-33% cases of ototoxicity (*437,438*). Mutations in certain mitochondrial genes (12S rRNA), e.g. 1555A>G, 1494C>T may increase susceptibility to aminoglycoside-induced hearing loss and are associated with non-syndromic sensorineural hearing loss (SNHL) too (*437,439*). The hearing loss related to mitochondrial mutations and aminoglycoside ototoxicity is commonly bilaterally symmetrical, involves high frequencies and at times can be associated with progressive SNHL. The hearing loss associated with the 1555A>G mutation is unrelated to age at exposure to aminoglycosides; it can happen after a small dose of aminoglycoside, e.g. after a single injection, and it generally occurs within about 3 months of the treatment (*439*). The penetrance of the mutation can be variable, mainly related to a modifying gene mutation (*440*). Aminoglycosides exert their bactericidal activity by binding to

the ribosomes and inhibiting the protein synthesis. The site of ototoxicity is relevant to this as mitochondrial ribosomes share more similarity to bacterial ribosomes than to mammalian cytosolic ribosomes (*441*). Aminoglycosides bind to mitochondrial ribosomes and interfere with their protein synthesis, which, within the inner ear hair cells, eventually leads to cell death (*441*). These mutations cause about 30% reduction in the mitochondrial translation, and after exposure to aminoglycosides, there is a further 30% reduction in the rate of protein synthesis in the cells carrying the mutation thus causing cell dysfunction and death, leading to high-frequency hearing loss (*440*). Variable prevalence has been reported from different parts of the world (*442*). A study on the UK 1958 birth cohort (age 44-45 year) showed a 0.26% (95% CI 0.14% to 0.38%, or one in 385 chance) prevalence of 1555A>G mutation in the cohort (*443*). There was no significant difference found in the hearing thresholds between those who carried the mutation to those who did not. Thus, the hearing may be preserved until adulthood. Similarly, other studies on people with European descent showed a similar population prevalence for 1555A>G of 0.19% (95% CI 0.10 to 0.28) i.e. 1 in 520 (*444*) and 0.21% (95% CI 0.08 to 0.46) i.e. 1 in 500 (*445*). The hearing thresholds for children with the mutation were within normal range at 9 years age in the British study (*444*), where none of these children had been admitted to neonatal unit (high likelihood of receiving IV aminoglycosides). In the latter study from Australia, after adjusting for confounders like age, sex and other risk factors for hearing loss (e.g. occupational noise exposure, family history of hearing loss), high-frequency hearing loss was identified in three of the six carriers of the mutation (*445*). However, this cohort was different to the British study cohort including older population (more than 50 years old). Moreover, information regarding exposure to ototoxic agents was collected by interview of the patients, which is subject to recall bias.

1.5.1 Hearing loss in CF

A recent report from the UK CF Registry states that prevalence of hearing loss is 0.7% in children and 3.8% in adult CF patients in the UK (*446*). One early study of ototoxicity in children with CF comes from Thomsen et al., who found a low prevalence of hearing loss, i.e. high tone hearing deficit in one of 53 subjects who had received an average of 3.2 courses of tobramycin in the past (*447*). Many case reports, case series and a large number of retrospective studies showed a varied prevalence of hearing loss in people with CF. A systematic review (*167*) including 12 studies and 762 children with CF, between 5 months to 20 years of age, showed a prevalence of SNHL ranging from 0% to 29%. However, people who had received >10 courses of IV aminoglycosides had up to 44% hearing SHNL. The evidence was not high quality, mainly based on retrospective studies, case reports and case series. One randomised controlled study (*448*) was available which showed no measurable hearing loss after a single course of tobramycin given once or thrice daily in short or medium term (6-8 weeks follow-up). There is no unified protocol for hearing screen available for patients with CF. The systematic review by Farzal et al. (*167*) showed that different methods were employed to assess hearing. Pure tone audiometry at standard ± high-frequency thresholds was used by all investigators; other tests like distortion product otoacoustic emissions, transient-evoked otoacoustic emissions and automated auditory brainstem response were also used. Association of hearing loss with previous exposure to aminoglycosides had been assessed, but the results were variable with one study showing no linear relationship between the two (*449*) whereas other studies were showing higher risk of hearing loss with >10 aminoglycoside courses compared to <10 courses (*167,450,451*).

1.6 Aim of the Current Study & Outline of Chapters

The life expectancy of people with CF is increasing, and therefore it is the time that we focus on comorbidities and try, where possible, to prevent any treatment-related adverse effects. There are multiple factors, which can predispose individuals with CF to renal dysfunction. These include both inherent CF-related characteristics (e.g. CFRD) and those contributed by the treatment itself, e.g. recurrent antibiotic courses to treat lung infections. Additionally, there may be a need for an organ transplant in some patients. Pre-transplant renal function impairment increases the risk of post-transplant renal dysfunction by about 50% (*8*). Hence, it is vital to preserve the renal function, and it would be beneficial to take measures in childhood to minimise impairment of renal function later in life. To achieve this, the first step is to define the extent of the problem and associated risk factors so that early monitoring and treatment can be initiated when needed.

The mechanism of renal impairment in CF is not clear. It is uncertain whether the renal function is affected by environmental and iatrogenic factors only or whether CF patients are also inherently more susceptible to develop the renal disease as their age advances. Mouse model of CF has been used to investigate any primary defect related to effects of CFTR function loss. The CFTR deficient mice showed defective receptor-mediated endocytosis and increased low molecular weight proteinuria compared to controls (*232*). The renal phenotype of CF is not entirely clear yet, and further studies are required to explain the role of these diverse factors in the development or progression of CKD in CF.

This study was conducted with the aim of investigating the treatment-related complications of CF with the focus on renal disease. The objectives included evaluating renal function in CF, assessing effects of

antibiotic related toxicity (renal and ototoxicity) and identifying ways to reduce antibiotic associated morbidity by using PK analysis and a systematic review to evaluate evidence for different treatment practices. With increasing survival of people with CF, cardiovascular diseases may become relevant; association of renal function (GFR) with vascular haemodynamic parameters was assessed to evaluate any correlation, which has been identified in adult people with CF.

Renal function was assessed (Chapter 2) in a cohort of CF patients comprising both children and adults, using ^{51}Cr-EDTA method, a highly accurate measure of GFR. Any association of renal function with the cumulative dose of antibiotics received was explored (Chapter 2). In addition, hearing assessment was undertaken, and correlation of hearing defect with cumulative antibiotic exposure was evaluated (Chapter 2).

In the next chapter (Chapter 3), the renal phenotype in CF was investigated by comparing the histology of kidneys from pig model of CF (CFTR-/-) with the control (CFTR+/- or CFTR+/+) pigs. Genetic expression and colocalization of CFTR and endocytic receptor proteins: cubilin, megalin and amnionless (AMN) were studied in the two groups, firstly to establish whether CFTR is expressed in the porcine kidneys and secondly to investigate the expression of renal endocytic receptor proteins both at molecular and protein level to help understand the pathophysiology of renal disease in people with CF.

A randomised controlled study (Chapter 4) was undertaken to investigate the role of circadian rhythm in tobramycin elimination and to explore whether this could be used to enhance excretion of tobramycin (and thus reduced nephrotoxicity).

In order to minimise any antibiotic-related adverse effects, judicious use of antibiotics is essential. To identify pulmonary infections, the standard practice has been microbiological cultures using sputum or

oropharyngeal swabs. Some centres have started using bronchoalveolar lavage (BAL) to establish infection. I conducted a systematic review (Chapter 5) to test if bronchoscopy directed antibiotic therapy (which allows targeted antibiotic treatment of pulmonary pathogens) leads to better outcomes, compared to the standard treatment. This review aimed to establish which investigative approach provided a better clinical outcome to prevent additional investigations and inappropriate antibiotic use (and adverse effects) in some cases.

Finally, I studied large artery stiffness in a group of children with CF to determine if there is early evidence of macrovascular disease. I studied the relationship of arterial stiffness with renal function (GFR) and pulmonary disease (lung function, inflammatory markers and infection) (Chapter 6).

The overall conclusions from work described in this thesis are summarised in Chapter 7.

Chapter-2 Renal function in CF and association with cumulative antibiotic exposure

2.1 Introduction

Renal disease is not a primary problem in CF, and there is no known renal phenotype in the CF despite the knowledge that CFTR is abundantly expressed in renal tubules (*234*). Over the last two decades, there are increasing concerns regarding renal complications in CF and speculation about a potential renal phenotype (*223*).

Multiple factors have contributed to these concerns. In the past, post-mortem studies showed manifest and subclinical renal histopathological changes in patients with CF (*219*), and prevalence of nephrocalcinosis and urolithiasis was found to be higher in CF compared to healthy population (*251*). With prolonged survival, CF patients are exposed to several risk factors for renal impairment. These include comorbidities like CFRD, predisposition to dehydration and iatrogenic factors like drug toxicity related to potentially nephrotoxic drugs (e.g. aminoglycoside antibiotics, post-transplant immunosuppressive therapy). In addition, renal impairment can affect eligibility for receiving a transplant and post-transplant outlook in these patients.

There are many case reports of AKI in patients with CF (*197,198*). A UK based survey showed that children with CF are at 100 times more risk of developing AKI compared to the standard background risk (*3*). A case-control study revealed that aminoglycoside therapy in the previous week increased the risk of developing AKI by 80 fold (*200*).

CKD has been reported in CF patients. A study measuring creatinine clearance in CF patients with chronic *P. aeruginosa* infection (mean age 24.2 years, range 16-56 years.) showed reduced creatinine clearance in 42% patients and that it decreased with increasing cumulative exposure to aminoglycosides (*5*). Pederson et al. found that GFR, measured as 24 hour creatinine clearance, was low in 39% patients with chronic *P. aeruginosa* infection (age range 8-35 years.); however, no correlation between GFR and the cumulative dose of tobramycin was found (*213*). The relationship of CKD with increasing cumulative exposure to aminoglycosides needs to be explored further in view of greater exposure with prolonged survival in CF.

A CF Foundation Patient Registry-based study on adults with CF showed that the annual prevalence rate of CKD in CF is 2.3% and this doubled with every 10 years increase in age (*4*). Creatinine clearance based GFR measured in CF patients between 1-15 years old showed low GFR (<90 ml/min/1.73 m^2) in 14/24 (58%) children (*216*). Caution must be exercised with these data regarding the accuracy of 24-hour urine collection in children.

Measurement of renal function is difficult, especially in young children. Various formulae based on serum creatinine levels have been used to estimate the GFR in both adults and children. Reliability of these equations for use in patients with CF has been questioned. An accurate measurement of renal function is essential for early identification of renal impairment or monitoring of those at risk of developing it. The renal clearance of inulin is considered the gold standard for measurement of GFR; however, it is expensive and time-consuming (*452*). Radioisotope clearance methods have been used to measure GFR. GFR measured by ^{51}Cr-EDTA method correlates well with and is easier to administer compared to the renal clearance of inulin (*452*).

In this chapter, I have presented the CEFIT CF (Acronym for Cumulative Effects of Intravenous Treatment in Cystic Fibrosis) study where I measured renal function by the ^{51}Cr-EDTA method in a cohort of children and adults with CF. The study also assessed the level of agreement of eGFR (based on serum creatinine) with mGFR. As a substudy, the hearing was evaluated in the same cohort using pure tone audiometry. Any association of GFR and hearing deficit with cumulative antibiotic exposure was assessed.

2.2 Aims

2.2.1 Primary Outcome

1. To establish the prevalence of renal dysfunction by measurement of GFR, using ^{51}Cr-EDTA method, in a cohort of paediatric and adult patients with CF
2. To test the agreement between measured (by ^{51}Cr-EDTA) and estimated (by creatinine-based equations) GFR
3. To test the hypothesis that GFR is inversely correlated with cumulative exposure to intravenous antibiotics

2.2.2 Secondary Outcome

1. To assess hearing of patients with CF
2. To test any association between hearing abnormality and level of GFR
3. To evaluate the extent hearing impairment can be explained by previous exposure to intravenous antibiotics
4. To determine the prevalence of proteinuria

2.3 Methods (CEFIT CF study)

2.3.1 Study Design

CEFIT CF study was designed by Professor Alan Smyth and Professor Alan Watson. The study had a cross-sectional, observational design. Initially, it was planned to be conducted at Nottingham University Hospitals NHS Trust (including Nottingham Children and Young People's CF Unit and Nottingham Adult CF Centre). The study was subsequently expanded to recruit patients from other centres, which included University Hospitals Leicester, Royal Derby Hospitals NHS Trust, Sherwood Forest Hospitals NHS Foundation Trust, Chesterfield Royal Hospital, and United Lincolnshire Hospitals. Birmingham Children's Hospital was added as an additional research site. The study was supported by Medicines for Children Research Network who provided nursing and administrative support.

North Nottingham Research Ethics Committee provided ethical approval (Reference number: 09/H0407/23). Research and Development department approval was obtained for each site prior to undertaking study activities. The study was conducted in accordance with the ethical principles that have their origin in the declaration of Helsinki, 1996 (*453*); the principles of Good Clinical Practice (*454*) and Good Laboratory Practice (*455*), the Department of Health Research Governance Framework, 2nd ed., 2005 (*456*), and in accordance with the Human Tissue Act, 2004 (*457*).

2.3.2 Recruitment Methods

2.3.2.1 Inclusion criteria

1. Cystic fibrosis, defined as positive sweat test or genetic result consistent with the diagnosis of CF and clinical features of CF or a positive newborn screening test or history of CF in a sibling (*458*)

2. The participant or participant's legally acceptable representative must be able to give informed consent in accordance with Good Clinical Practice guidelines

3. Age of participant 5 years to 60 years old

2.3.2.2 Exclusion criteria

1. IV antibiotics in the previous 2 weeks

2. Participation in another research project, which excludes the patient from this study

3. Poor patient prognosis where the clinicians feel that patient's clinical condition or other difficult family circumstances would make taking part in research inappropriate during the recruitment period

4. A positive pregnancy test

Participants were recruited from December 2009 until May 2012. Potential participants were identified by the treating physicians and introduced to the 'research team', which comprised of two investigators (Dr Andrew Prayle and myself) and Medicines for Children Research Network nurses). The research team gave information about the study including verbal explanation and information leaflets. The families were given at least a week to consider their decision, and any queries were answered. An informed written consent was obtained from the patients or, in the case of children <16 years age, from their parents/legal guardians on consent forms approved by the ethics committee. Assent was obtained from the participants <16 years old, who were not eligible

to provide consent for themselves. Informed consent and assent were obtained by one of the investigators (Dr Andrew Prayle or myself) prior to the study.

In medical imaging, the biological effect of radiation is described by 'equivalent dose' (or 'effective dose' for tissue or organ-specific irradiation), which takes into account the absorbed dose and 'radiation weighting factor' (RWF) for the type of radiation used. The radiation dose from single ^{51}Cr-EDTA test for adult patients is 0.006 mSv (milliSieverts) (compare from the equivalent dose in an adult patient from a standard chest X-ray of 0.1mSv) (*459,460*). All participants were advised regarding radiation exposure. A pregnancy test was conducted on female participants who were in the childbearing age and agreed to take the test.

2.3.3 Study Procedures

The interested participants were invited by the research team to attend Children's Research Facility at Queen's Medical Centre, Nottingham or Wellcome Trust Children's Research Facility at Birmingham Children's Hospital for half a day. All study related information was recorded on a case report form. Patient's height and weight were measured on the study day. The study procedures including review of medical records, collection of blood samples and pure tone audiometry were done by one of the investigators (Dr Andrew Prayle or myself).

2.3.3.1 Review of medical records

Patient information was collected by direct questioning and retrospective review of medical records. Patient demographics, CF genotype, comorbidities including the history of AKI, CKD and any other significant factors at the time of the study were noted. CFRD or impaired glucose tolerance status, CF liver disease and chronic *P. aeruginosa* infection status were recorded from the recent investigations, e.g. at the annual review (annual assessment of overall health and progress in relation to CF). Recent annual review spirometry results (FEV_1 and FVC, absolute values and percent predicted values) were noted. Review of antibiotic data is described in Section 2.3.3.6. Medical records of adult patients were reviewed to the time where all the antibiotic details were available.

2.3.3.2 Measurement of GFR by ^{51}Cr-EDTA method

Patients were advised to refrain from caffeine-containing beverages from the night before the study. They were allowed to eat breakfast and normal lunch. Two IV cannulas were inserted in different limbs. A totally implantable venous access device (TIVAD, e.g. a Port-a-Cath®), where present, was accessed instead of one of the cannulae. The dose of ^{51}Cr-EDTA was calculated by the medical physics department based at the respective research sites and was injected by one of the two investigators (Dr Andrew Prayle or myself) or nuclear medicine technologist, Ravinder Mahandru for patients in Birmingham. A bolus of between 0.28 and 2.8

MBq (megabecquerel, SI Unit of radioactivity (*461*)) of ^{51}Cr-EDTA was injected through IV access site1. The dose of ^{51}Cr-EDTA was scaled from adult dose based on participants' weight (Dose Calculation Guide in the Appendix Chapter II 8.2.1).

Before injecting the tracer, the line was flushed with 10ml 0.9% saline to exclude any extravasation. The tracer injection was immediately followed by 10 ml 0.9% saline. Time of injection was recorded as the midpoint of administration of the tracer. The empty syringe was retained for counting the remaining background-subtracted activity. Timed blood samples were taken after 2 hour, 3 hour and 4 hour from the injection by bleeding back from the second IV access point. A 3-5 ml discard was taken before each sample was withdrawn and time was recorded at the midpoint of sample collection.

As the hospital protocol for ^{51}Cr-EDTA had been adopted for the study protocol, we planned to include ^{51}Cr-EDTA results if done for clinical reasons in any patients (usually done as a part of pre-transplant assessment).

2.3.3.2.1 Sample handling and Lab analysis

The blood samples were labelled with the study code, participant initials and date of birth. All samples were transported to the lab on the same day in a secure manner in sealed containers. The processing of GFR samples was performed in the nuclear medicine department at Nottingham University Hospitals (site: Queen's Medical Centre, Nottingham) and in biochemistry lab at Birmingham Children's hospital, depending on where the patients had their study investigations undertaken. These labs are managed in accordance with all the regulatory and statutory requirements including storage and disposition of samples according to Human Tissue Act, 2004 (*457*) (HTA licence number 11035 for Queen's Medical Centre, Nottingham and HTA licence number 11005 for Birmingham Children's Hospital) . All the organisations

and employers undertaking procedures using ionising radiations are required to comply with Ionising Radiation (Medical Exposure) Regulations 2000 (amended 2006) (*462*). In addition, certification from the Administration of Radioactive Substances Advisory Committee (ARSAC) is required for individuals/ professionals carrying out administration of radioactive substances. ARSAC certification was requested for CEFIT CF study as part of the Integrated Research Application System (ARSAC certificate number: RPC253/3406/24958 (Nottingham University Hospital), RPC552/473/28026 (Birmingham Children's Hospital).

The GFR analysis was done according to slope-intercept method following BNMS guidelines (*413*). A low GFR was defined as a ^{51}Cr-EDTA value of <90 ml/min/1.73m^2 as per the 2012 CKD guidelines (*189*); high GFR cut-off was defined as a value of >135 ml/min/1.73m^2 in line with the >90th centile values of Piepsz reference of ^{51}Cr-EDTA GFR in children, which is the largest cohort so far (*421*). The high GFR cut-off value for adults is age dependent. As most of the adult CF population are young (20-40 years age), the cut-off was described as >130 ml/min/1.73m^2 (*422*).

2.3.3.3 Early morning urine

Participants were advised to collect the first morning urine sample on the day of study visit, which was sent to the laboratory to test for protein to creatinine ratio.

2.3.3.4 Hearing Assessment

Screening questions were asked to exclude hearing loss from other causes, which included the history of premature birth, chronic middle ear disease, ear surgery, familial deafness, head injury and infections like meningitis, mumps and rubella. Any patients with risk of confounding conditions were excluded from analysis.

The UK CF Trust recommends annual pure tone audiogram in patients receiving frequent courses of IV aminoglycosides. The commonest

hearing impairment due to aminoglycosides is high-frequency SNHL. The investigators were trained in conducting pure tone audiometry by Robert Frost at Children's Hearing Assessment Centre, Nottingham. Hearing assessment was done by air conduction pure tone audiometry (Kamplex KD-29 Audiometer, P.C. Werth, London, UK), which was administered in a quiet room (*448,449,463*). Bone conduction pure tone audiometry was not undertaken as it is more complex and is affected more by any ambient noise compared to the air conduction audiometry (*463*). The test procedure was explained to the participants. Supra-aural masks (Telephonics TDH-39P audiometric earphones, P.C. Werth, London, UK) were applied, and participants were asked to sit facing away from the audiometer in a way that investigator was able to see their face. Before starting the procedure, tones of 1000 Hz were presented at 30 dB intensity to help familiarise with the sounds. Tones of 1000 Hz, 2000 Hz, 4000 Hz, 8000 Hz and 500 Hz were presented in that order. At each frequency, various intensities of tones were used. Initial tones were presented at 30 dB. These were reduced by 10 dB on a satisfactory positive response until no further response was noted. At this point, the level of tone was increased in 5 dB steps until a response was observed. After the first positive response, the level was decreased again by 10 dB and then increased by 5 dB until the participant responded at the same level on two out of two or >50% occasions (*463*). Criteria for classification into abnormal hearing category included fulfilling any of the following in either ear (*449*):

a. Presence of at least one threshold of ≥25 dB hearing level
b. Two or more thresholds at ≥20 dB hearing level

2.3.3.5 Calculation of estimated GFR

Single blood samples for serum creatinine were taken while inserting the cannulae and were sent to biochemistry laboratory for analysis. Creatinine-based formulae were used to calculate the eGFR. The bedside Schwartz formula was used for children (<18 year age) (*452*). Abbreviated MDRD (*427*) and CKD-Epi formulas (*464*) were used for adults (>18 year age). The formulas are as follows:

Bedside Schwartz Equation:

$$GFR\ (mL/min/1.73\ m^2) = (36.2 \times Height\ in\ cm)\ /\ Creatinine\ in\ \mu mol/L$$

Equation 2-1

Abbreviated MDRD Equation:

$$GFR\ ((ml/min)/(1.73m^2)) = 175\left(\frac{Scr}{88.4}\right)^{-1.154} X\ (Age)^{-0.203} X\ (0.742\ if\ female) X (1.212\ if\ African\ Am)$$

Equation 2-2

CKD-Epi Equation:

$$GFR\ ((ml/min)/(1.73m^2)) = 141 X min(Scr/k, 1)^{\alpha} X\ max(Scr/k, 1)^{-1.209} X\ 0.993 Age X 1.1018\ (if\ female) X\ 1.159(if\ black)$$

Scr is serum creatinine in µmol/L, κ is 61.9 for females and 79.6 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr /κ or 1, and max means the maximum of Scr /κ or 1.

Equation 2-3

2.3.3.6 Calculation of cumulative antibiotic dose

Lifetime antibiotic courses were recorded from a review of medical records using various sources including medical notes, annual review reports, clinic review reports, transition/transfer letters and consulting the UK CF Registry database. Those patients where details of less than 80% of the courses were available were excluded from the analysis.

Cumulative antibiotic exposure was calculated as the total number of courses of antibiotic received, total days of antibiotic treatment received and total dose of antibiotic per kg. Any course of treatment where antibiotics were administered for ≥7 days was counted towards the calculation of the total number of courses of antibiotics received. Therapies for <7 days duration did not contribute to the cumulative number of courses but were taken into account while calculating the total number of days of treatment and cumulative antibiotic dose in mg/kg. Cumulative antibiotic dose was calculated by the following method:

$$Cumulative\ antibiotic\ dose\ mg/kg = (total\ days * \frac{mg}{kg}/dose * no.of\ times\ administered\ each\ day)$$

Equation 2-4

Example: cumulative dose calculation for 10 mg/kg once daily tobramycin for 28 days=28*10*1=280mg/kg

For the courses, where total dose was given as a standard maximum dose rather than mg/kg dose (e.g. in adult patients), considering the weight has varied over the years, average weight was assumed to be 50 kg, and the following calculation was used:

$$Cumulative\ antibiotic\ dose\ mg/(50)kg = (total\ days * mg/dose * no.of\ times\ administered\ each\ day)/50$$

Equation 2-5

During the study period, if one particular antibiotic was prescribed differently e.g. thrice-daily and once-daily aminoglycoside, the total dose was calculated for each and then added to determine the cumulative dose. To calculate the cumulative dose/kg, the weight of the patient was taken from the prescription sheet or medical records during the admission. When a prescription sheet or medical records for an antibiotic course were not available, the weight from nearest clinic appointment was used, and the dose was calculated from the standard dose of antibiotics as per British National Formulary or the UK CF Trust Guidelines (*296*).

2.3.3.7 Statistical Methods

SPSS for windows version 22 was used for statistical analysis, and graphs were prepared using GraphPad Prism Version 7.03. Data were analysed for normality by viewing the distribution in a histogram and, where in doubt, by Shapiro-Wilk test. Mean and SD are presented for normally distributed data; median and IQR are used for the description of non-normally distributed data. Linear regression analysis was used to assess the associations of normally distributed outcome variables where linear regression assumptions were met. Hearing screening results were defined as normal and abnormal based on criteria defined in the methods section and association with GFR was calculated using the Unianova test.

Bland-Altman plots were constructed to assess the level of agreement of the eGFR values with ^{51}Cr-EDTA values. Further simple linear regression analyses were undertaken between mean GFR values and difference between the measured and the estimated GFR values to test the hypothesis that the coefficient for the mean is equal to or close to zero; a p value of >0.05 allows us to accept the null hypothesis (i.e. there is no proportional bias). In other words, the vertical spread of the difference along different values of mean does not vary along its course.

2.4 Results

Seventy patients were recruited in the study. Please refer to the study flow chart Figure 2-1. The data for adults and children (<18 years old) were analysed separately.

Result for ^{51}Cr-EDTA GFR was available for 68 patients (16 adults and 52 children) with CF. Two children withdrew consent due to fear of needles. One child did not want to participate although their parents had consented, and a second child withdrew consent during the study as their vascular access device stopped bleeding back and the child did not want cannulation.

Hearing assessment was done in 64 participants (15 adults and 49 children). Three children had their GFR assessed for clinical reasons so did not come for hearing assessment. One adult patient had strong family history of hearing impairment and was using hearing aid for a long time, so was excluded from hearing analysis.

Information for some patients could not be traced, as their medical records were not available to be reviewed. Notes were not available for 4 adult patients. For the rest of the adult group, notes review was conducted up to the point where all the antibiotic data was available. Protein to creatinine ratio measurement was added with an amendment to the study protocol during the middle of the study after a grant application for a separate study to assess proteinuria in CF was unsuccessful. The results are, hence, available for a smaller group (N=38) of patients.

2.4.1 Review of medical records

The demographic details of the study participants including results of recent spirometry and the spread of genotypes are shown in Table 2-1 and Table 2-2 respectively. Two children were unable to perform reliable spirometry due to their age (5.8 and 7 years old). CF genotype result is

presented for all paediatric patients and for 6 adult patients. Comorbidities are presented in Table 2-3. Two adults and 4 children had a diagnosis of CFRD, and 4 adults and 2 children had impaired glucose tolerance tests. Four children had a history of AKI in the past; three of these were following a course of gentamicin and one following a course of tobramycin. All these patients were treated with dialysis during the acute phase. Two patients with a history of AKI had a renal biopsy, both of which suggested tubulointerstitial changes.

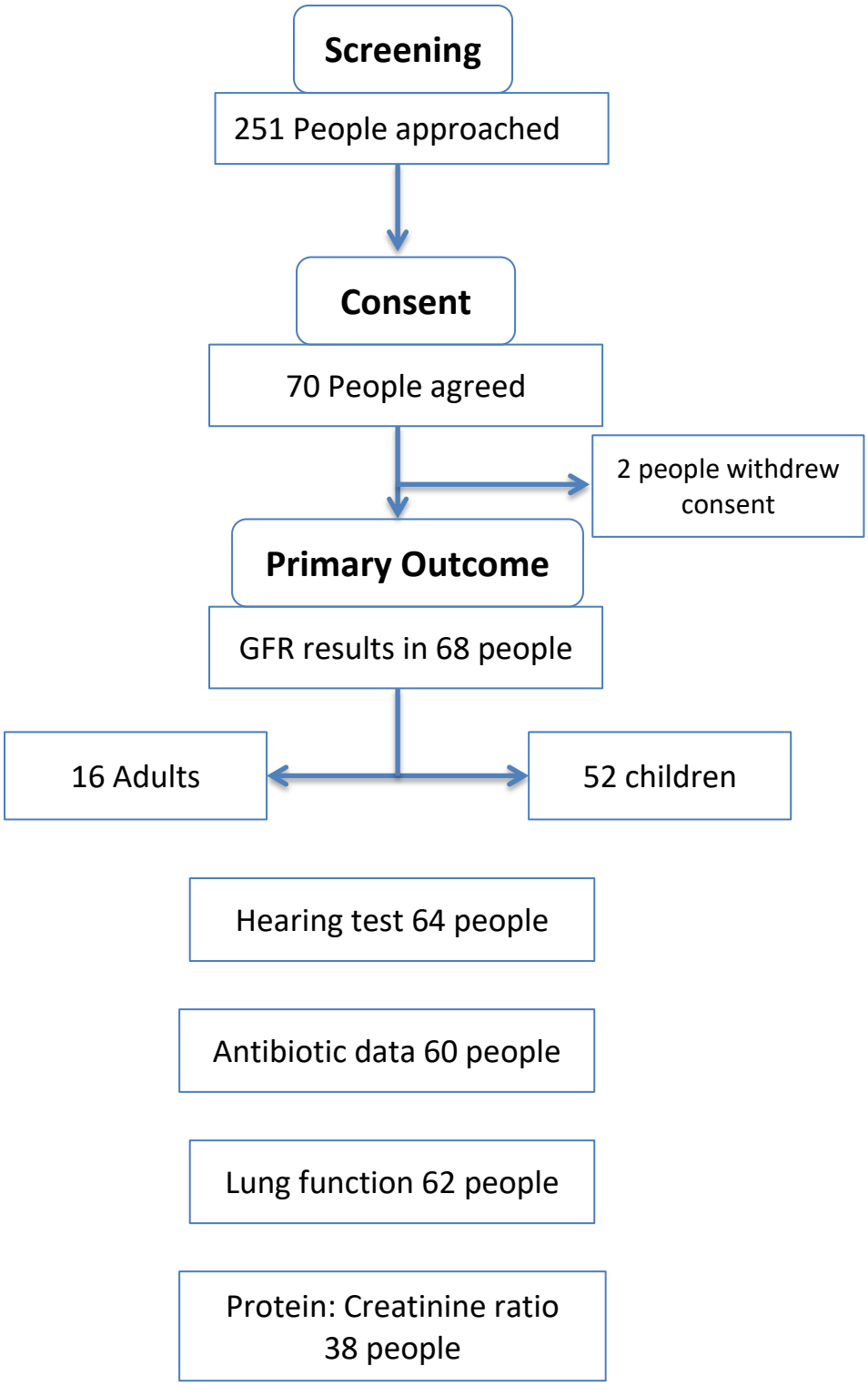


Figure 2-1 CEFIT CF Study Flowchart; CEFIT CF: acronym for Cumulative Effects of Intravenous Antibiotics in CF; GFR: glomerular filtration rate

Table 2-1 Demographic data for study participants				
	Paediatric		**Adult**	
Number (N)	**52**		**16**	
Sex (male N, %)	N=27	52%	N=11	69%
Characteristic	**Median**	**IQR (25-75%)**	**Median**	**IQR (25-75%)**
Age (Years)	10.8	8.1 to 14.1	29.4	25.8 to 35.1
Weight (Kg)	34	24.1 to 45.0	73.4*	65.8 to 81.0**
Height (cm)	137.7	125.6 to 156.0	172.8	167.5 to 180
Serum Creatinine (µmol/L)	45.7*	42.4 to 49.2**	78.0	68.5 to 83.5
FEV_1 % Predicted	82.2* (N=50)	74.9-89.5%**	67.5 (N=12)	53.5 to 92.5

Mean (**95% Confidence Interval); IQR: interquartile range; FEV_1: forced expiratory volume in one second

Table 2-2 CF Genotype of participants				
Genotype	**Paediatric**		**Adult**	
Number n* (N)	52 (52)	Percentage	6 (16)	Percentage
Phe508del Homozygous	41	88.50%	4	66.7%
Phe508del Heterozygous	5	9.60%	2	33.3%
Both Other	1	1.90%	0	
Phe508del-Unknown	5	9.60%	0	

n: number results available; N: number of participants in that group; CF: cystic fibrosis

Table 2-3 Comorbidities of participants				
Comorbidity	**Paediatric**		**Adult**	
	Total Patients (N)	**With Comorbidity (%)**	**Total Patients (N)**	**With Comorbidity (%)**
Acute Kidney Injury in past	52	4 (7.6%)	13	0
Chronic Kidney Disease	52	0	13	1 (7.7%)
CF-related Diabetes/ Impaired Glucose Tolerance	52	6 (11.5%)	13	6 (46.1%)
CF-related liver disease	52	15 (28.9%)	12	3 (25.0%)
***Pseudomonas* colonization**	51	24 (47.1%)	13	9 (69.2%)

CF: cystic fibrosis

2.4.2 Renal Function measurement

2.4.2.1 ^{51}Cr-EDTA GFR values

Mean GFR of the whole group was 118.8 ml/min/11.73m2 (95% CI 112.6 to 125.1. The spread of data is shown in the histogram in Figure 2-2. As the spread of the data appeared bimodal in nature, Shapiro-Wilk test was undertaken, which suggested a normal distribution (P value 0.49). There was only mild skewness of the data (skewness value -0.39) and ratio of skewness value to its standard error was <1.96. Hence GFR was taken as normally distributed for statistical analysis.

Mean (95% CI) GFR of paediatric patients was 124.8 (118.6 to 131) ml/min/1.73m^2 (Table 2-4). The GFR of paediatric patients did not show any association with age (adjusted R^2=-0.09, P=0.523, β=1.6 with 95% CI=-3.410 to 6.6) after adjusting for confounders (height, weight, sex and CFRD/impaired glucose tolerance status).

Mean (95% CI) GFR of adults was 99.6 (85 to 114.3) ml/min/1.73 m^2 (Table 2-4). Two adult patients had had a lung transplant, both of who had a GFR of <90 ml/min/1.73m^2 (61 and 34 ml/min/1.73m^2, 37 and 53 years old respectively). On excluding the results of these two patients who had lung transplants, the mean GFR of the adult group (N=14) was 107.1 (95% CI 96 to 118.1) ml/min/1.73m^2, the range being 79-145 ml/min/1.73m^2. There was no association of GFR (of adult CF patients) with height, weight, sex and CFRD/impaired glucose tolerance status. All the adult patients were between 20-40 years old except three patients who were 47, 53 and 58 years old. There was a variance of GFR with age in the adult patients, (R^2=0.376, P=0.012, β=-1.5 ml/min/1.73m^2, 95% CI -2.6 to -0.4); however, this was not adjusted for confounders due to a small number of patients in the group. A sensitivity analysis was conducted to assess the relationship of GFR with age in adult patients after excluding patients who had received a lung transplant and after restricting the upper age limit to 50 years. The results showed no

significant association of GFR with increasing age (R^2=0.02, β=-0.4 ml/min/1.73m^2, 95% CI -2.2 to 1.4 ml/min/1.73m^2).

2.4.2.1.1 Low GFR values

Two children had a GFR value of <90 ml/min/1.73m^2. One of these children (16 years old) had never received any antibiotics whereas another one (8 years old) had had previous AKI after a course of IV gentamicin. Four adult patients had GFR value <90 ml/min/1.73m^2, which included two patients with lung transplants. Two other adult patients who had GFR <90 ml/min/1.73m^2 (80 and 79 ml/min/1.73m^2) were 27 and 58 years old respectively.

2.4.2.1.2 High GFR values

High GFR values (>90th centile i.e. >135 ml/min/1.73m^2) (*421*) were observed in 15 children (29%); these values ranged from 137 to a maximum of 173 ml/min/1.73m^2. One adult patient showed high GFR (i.e. >130 ml/min/1.73m^2) of 145 ml/min/1.73m^2.

2.4.2.2 Protein to Creatinine ratio

Protein to creatinine ratio was available in 32 children and 6 adults; median and IQR values are presented in Table 2-4. Three paediatric patients showed the protein to creatinine ratio to be >15 mg/mmol; these did not overlap with the ones who had low GFR. Three adult patients had a high protein to creatinine ratio (i.e. >15 mg/mmol); two of these also had low GFR, and one of these had had a lung transplant.

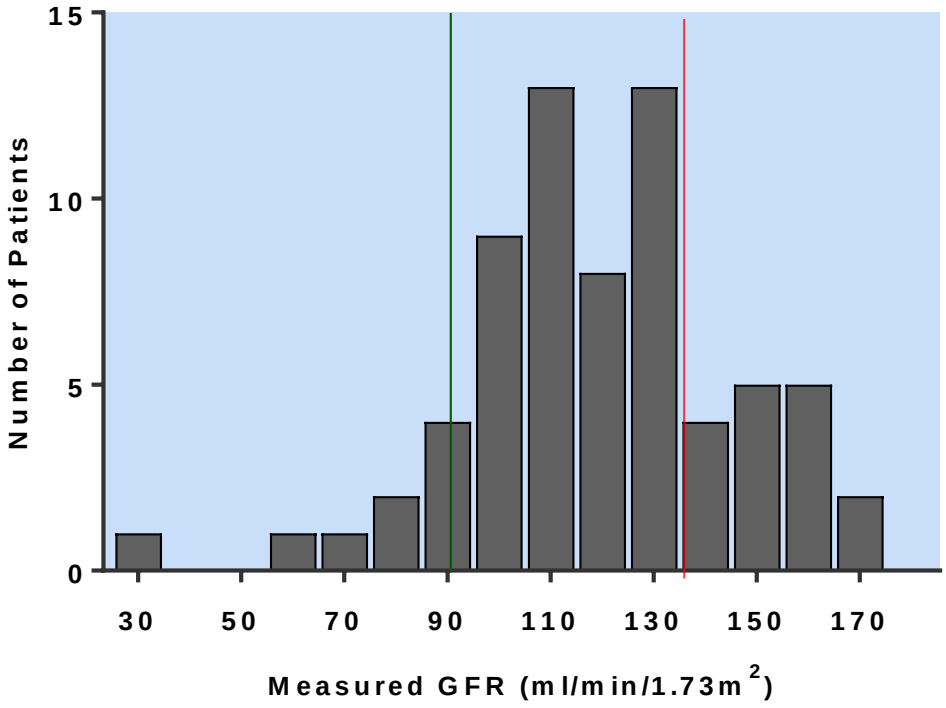


Figure 2-2 Spread of measured GFR (^{51}Cr-EDTA GFR) values for all patients (N=68, Mean=118.8 (standard deviation 25.7) ml/min/1.73m^2), the vertical green line and the red line represent the lower cut-off (90) and the upper cut-off value of normal GFR (135) respectively; GFR: glomerular filtration rate; ^{51}Cr-EDTA: ^{51}chromium ethylenediamine tetraacetic acid

Table 2-4 Renal Function results				
Parameter	**Paediatric**		**Adult**	
	(N)	**Median (IQR) (Range)**	**(N)**	**Median (IQR) (Range)**
^{51}Cr-EDTA GFR ml/min/1.73m^2	52	*124.8 (118.6 to 131.0) (74.0 to 173.0)	16	*99.6 (85.0 to 114.3) (34.0 to 145.0)
eGFR ml/min/1.73m^2	52 Schwartz	110.4 (95.8 to 131.3) (81.5 to 257.2)	16 (MDRD)	90.82 (78.6 to 103.4) (34.8 to 143.0)
			16 (CKD-Epi)	104.0 (89.4 to 117.9) (36.5 to 130.4)
Protein to Creatinine Ratio mg/mol	32	8.0 (2.5 to 11.5) (2.5 to 95.0)	6	9.8 (2.5 to 20) (2.5 to 72.0)

Mean and 95% confidence interval; ^{51}Cr-EDTA: ^{51}chromium-ethylenediamine tetraacetic acid; GFR: glomerular filtration rate; eGFR: estimated GFR (Schwartz formula for children, MDRD & CKD-Epi formula for adults); MDRD: modification of diet in renal disease equation; CKD-Epi: chronic kidney disease epidemiology collaboration equation; IQR: interquartile range

2.4.3 Level of agreement of estimated GFR with measured GFR

The results of the Bland-Altman analysis to assess the level of agreement between eGFR and mGFR are presented in the Table 2-5**.** The Bland-Altman plots are presented in Figure 2-3, Figure 2-4, Figure 2-5. MDRD equation overestimated the mGFR by 6.5 ml/min/1.73m^2, whereas CKD-Epi equation underestimated the mGFR by 2.6 ml/min/1.73m^2. The paediatric equation, Schwartz, also overestimated the mGFR by 7.2 ml/min/1.73m^2 with a wide range of 95% CI for the level of agreement (please refer to Table 2-5)**.**

In a systematic review of various methods of measuring GFR (in non-CF context) (*404*), a GFR measurement method was considered to have sufficient accuracy if all the following criteria were met:
1. Median bias did not exceed 5%.

2. Mean bias did not exceed 10%.

3. At least 80% of index measurements were within ± 30% of reference measurements (P-30 ≥80%)

4. At least 50% of index measurements were within ± 10% (P-10 ≥50%)

NKF KDOQI clinical practice guidelines (2002) recommend a P-30 of >90% in validated population (*465*).

Putting our results in clinical context, although the mean bias with all the three equations is < 10ml/min/1.73m^2 and may not be clinically too significant considering a large normal range of GFR (e.g. 90 to 135 for our study), the wide limits of agreement suggest that the results may vary widely, e.g. in any child with mGFR of 90, the eGFR value measured by Bedside Schwartz equation can range from 35 to 160, thus having significant clinical implications, particularly at lower end. Between the two adult equations, CKD-Epi equation showed lower bias and greater precision than MDRD equation.

Table 2-5 Bland-Altman Analysis of eGFR values against mGFR						
eGFR Equation (Subjects)	**Bias**		**Precision**	**Width of 95% LoA**	**P-10 %**	**P-30 %**
	Mean	**Median**				
Bedside Schwartz (Children)	7.2	10.3	40.6	124.7	36.5	82.7
MDRD (Adults)	6.5	8.6	27.2	73.3	31.3	93.8
CKD-Epi (Adults)	-2.6	-0.98	20.3	73	56.3	87.5

Mean/Median bias: mean/median of differences between mGFR and eGFR; precision: interquartile range of differences between mGFR and eGFR (189); P-10 and P-30: the proportion of eGFR values within 10% and 30% of mGFR; mGFR: measured glomerular filtration rate (^{51}Cr-EDTA: ^{51}chromium ethylenediamine tetraacetic acid); eGFR: estimated GFR; LoA-:level of agreement; MDRD: modification of diet in renal disease equation; CKD-Epi: chronic kidney disease epidemiology collaboration equation

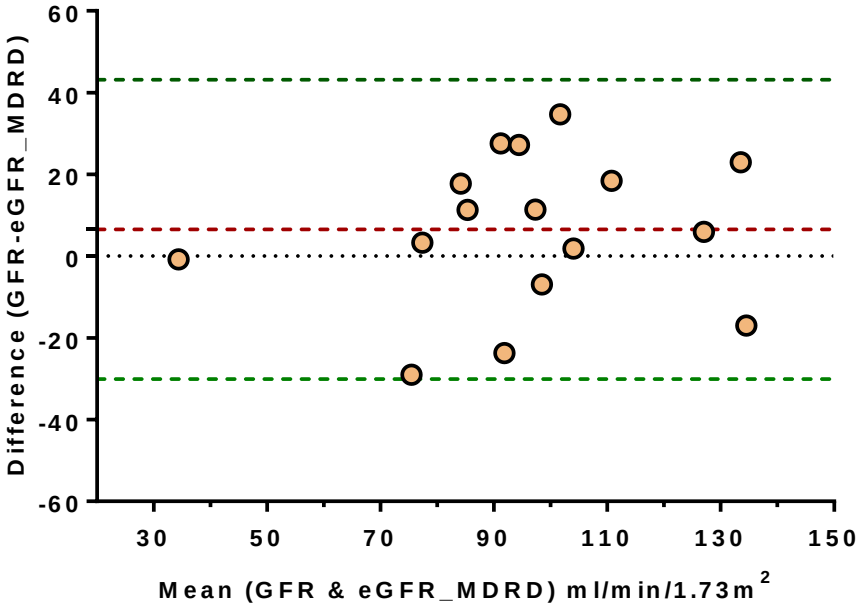


Figure 2-3 Bland-Altman plot to show level of agreement between ^{51}Cr-EDTA GFR and eGFR (MDRD equation) in adult CF patients; eGFR measured by MDRD equation over-estimates ^{51}Cr-EDTA GFR by 6.5 ml/min/1.73m^2 and the values of eGFR may be 30 ml/min/1.73m^2 lower to 43 ml/min/1.73m^2 higher than the mGFR which would not be acceptable for clinical purposes. The broken red line shows the mean difference (6.5 ml/min/1.73m^2); the broken green lines show 95% confidence interval for limits of agreement between measured and estimated GFR (upper=43.2 and lower=-30.07); P value=0.588, suggests no proportional bias; GFR: glomerular filtration rate; eGFR: estimated GFR; MDRD: modification of diet in renal disease equation

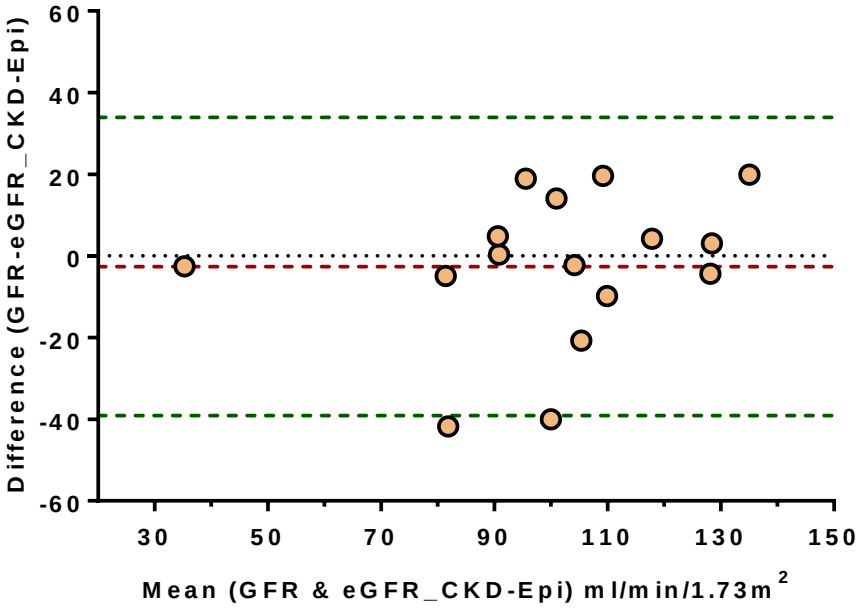


Figure 2-4 Bland-Altman plot to show the level of agreement between ^{51}Cr-EDTA GFR and eGFR (CKD-EPI equation) in adult CF patients; eGFR measured by CKD-Epi equation under-estimates ^{51}Cr-EDTA GFR by 2.6 ml/min/1.73m^2 and the values of eGFR may be 40 ml/min/1.73m^2 lower to 34 ml/min/1.73m^2 higher than the mGFR which would not be acceptable for clinical purposes. The broken red line shows the mean difference (-2.59 ml/min/1.73m^2), the broken green lines show 95% confidence interval for limits of agreement between measured and estimated GFR (upper=33.91 and lower=-39.1), P value=0.358, suggests no proportional bias; GFR: glomerular filtration rate; eGFR: estimated GFR; CKD-Epi: chronic kidney disease epidemiology collaboration equation; ^{51}Cr-EDTA: ^{51}chromium ethylenediamine tetraacetic acid

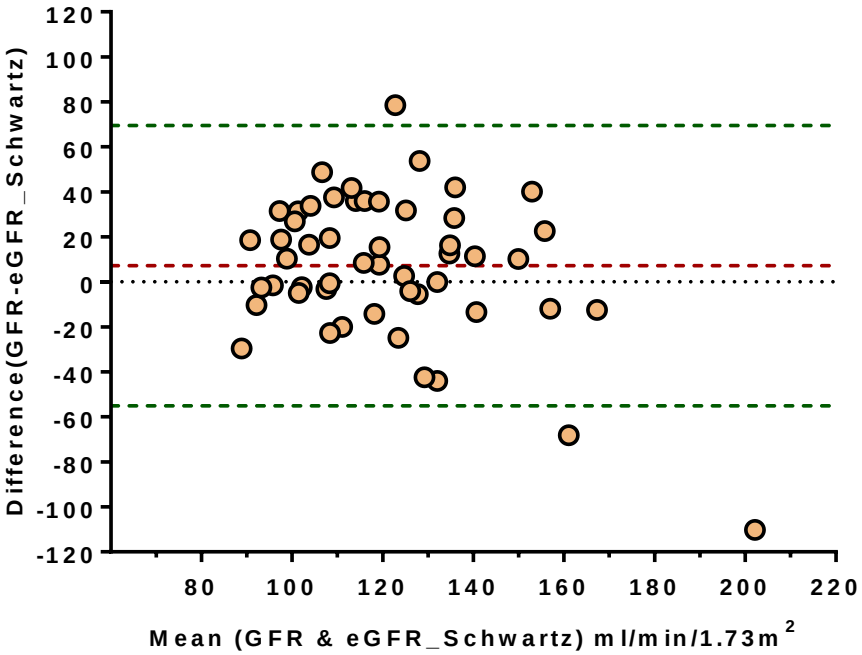


Figure 2-5 Bland-Altman plot to show the level of agreement between ^{51}Cr-EDTA GFR and eGFR (Bedside Schwartz equation) in children with CF, eGFR measured by Bedside Schwartz equation over-estimates ^{51}Cr-EDTA GFR by 7.2 ml/min/1.73m^2 and the values of eGFR may be 55 ml/min/1.73m^2 lower to 69.5 ml/min/1.73m^2 higher than the mGFR which would not be acceptable for clinical purposes. The broken red line shows the mean difference 7.21 ml/min/1.73m^2), the broken green lines show 95% confidence interval for limits of agreement between measured and estimated GFR (upper=69.551 and lower=-55.11), P value=0.358, suggests no proportional bias; GFR: glomerular filtration rate; eGFR: estimated GFR; ^{51}Cr-EDTA: ^{51}chromium ethylenediamine tetraacetic acid

2.4.4 Hearing Test Results

Hearing assessment was done in 64 participants (15 adults and 49 children). Three children had their GFR assessed for clinical reasons (pre-transplant assessment) so did not come for hearing assessment. One adult patient was excluded from hearing analysis due to familial deafness and long-term severe hearing deficit requiring hearing aid.

Results from 15 adults and 45 children were used for statistical analysis. Four children were excluded from final analysis due to confounding factors: two children had middle ear surgery in the past, one child had a history of glue ear, and another child had a history of communicating hydrocephalus and a known moderate to high frequency hearing loss.

One child had a history of deafness in father and a paternal cousin. This child was included as the mitochondrial mutations affecting aminoglycoside ototoxicity are transmitted maternally. This child had a normal hearing threshold. One child was known to have high frequency hearing loss following a previous aminoglycoside-induced AKI. One adult patient complained of persistent tinnitus. Both these patients were included in the analysis, as these were associated with aminoglycoside administration and not related to other confounding factors.

Increased hearing thresholds (as described in methods) were demonstrated in 9 of 45 children (20%), and 6 of 15 adults (40%) (please refer to Figure 2-6). Hearing thresholds at different frequencies are depicted in Figure 2-7**.**

Mean GFR of children with abnormal hearing was 7.3 (95% CI -24.7 to 10.1) ml/min/1.73m^2 lower than the group with normal hearing; the difference was not statistically significant (R^2=0.02, P=0.40). Mean GFR in adult patients with abnormal hearing results was 16.7 ml/min/1.73m^2 lower than the adult patients with normal hearing test (95% CI of the difference -48.2 to 14.8 ml/min/1.72m^2, R^2=0.09); the difference was not statistically significant, P=0.27).

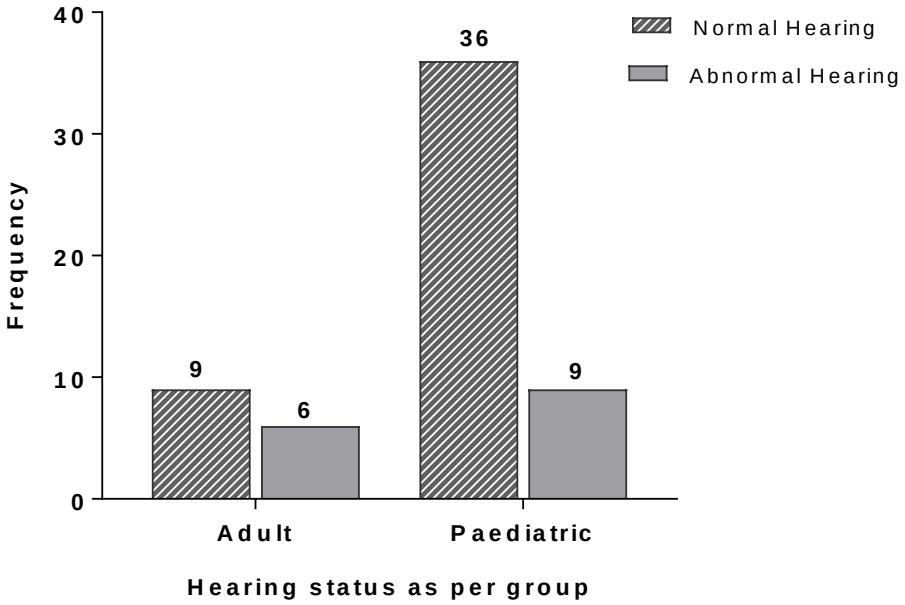


Figure 2-6 Hearing status in adults and children with CF ; The patterned bars represent number of people with normal hearing and plain bars represent number of people with abnormal hearing; 20% of children and 40% of adults had abnormal hearing test result.

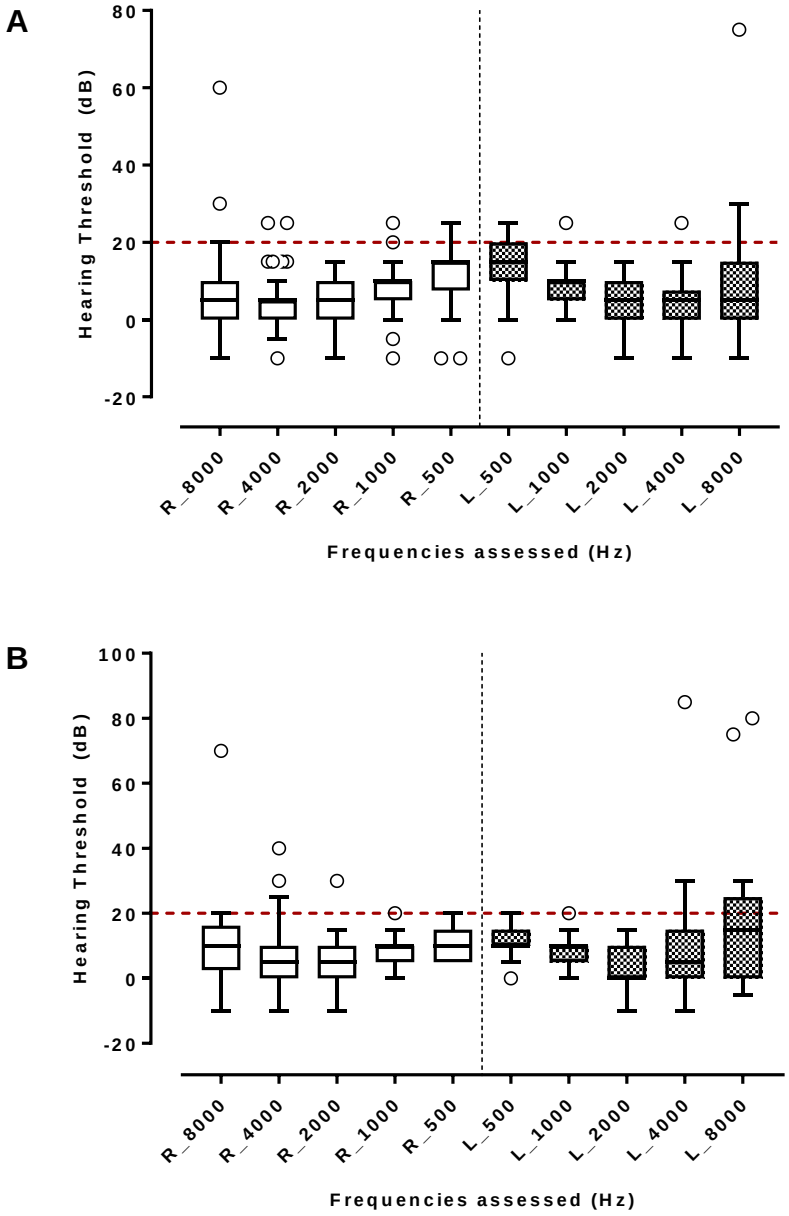


Figure 2-7 Hearing thresholds (dB) at different frequencies (Hz); A: paediatric group; B: adult group; note increased hearing thresholds at higher frequencies for the outliers; boxes show interquartile range (IQR, 75th percentile (Q3) to 25th percentile (Q1)); lines inside the boxes depict median; upper/lower fence of the whiskers stretch from Q3 or Q1 to ((≤1.5 X IQR) +/- Q3 or Q1); circles show outliers (≥1.5 X IQR +/- Q3 or Q1); plain boxes: thresholds from R ear; patterned boxes: values from L ear; red line at 20 dB level shows the threshold for hearing impairment; vertical black line separates values from two sides; R:right ear; L: left ear; R/L_500-8000: said frequency tested in respective ear.

2.4.5 Antibiotic usage and correlation with GFR & Hearing

Total number of antibiotic courses, number of days of antibiotics received and cumulative dose received (per Kg body weight or per 50 Kg) until the day of GFR measurement were available for 60 participants (50 children and 10 adults); data are presented in Table 2-6 and Table 2-7.

Two adult patients who had had lung transplant were excluded from the analysis, as they were likely to have received calcineurin inhibitors. Medical records of 2 adult patients and all paediatric records of other 2 adult patients were not available as they had moved from other areas. Thus, antibiotic data was analysed for 10 adult patients. For 2 paediatric patients, the total number of days and courses of antibiotics received were available from annual review reports, which were included in the analysis. However, any details of the type of antibiotics and doses received could not be reviewed, as they received all their treatment at shared care centres; they were excluded from this part of analysis. Three patients had partial records (of 19, 25 and 44 courses) accessible with details of ≥80% treatment received (rest could not be traced); they were included in the analysis using the data available.

Tobramycin was the most commonly used aminoglycoside with the median use of 42 days (IQR 8 to 104) in children and 28 days (IQR 6 to 126) in adult patients. Eight patients in the paediatric group and one patient in the adult group received gentamicin. Three patients had a history of developing AKI after a dose of gentamicin. Two of these 9 patients were excluded from hearing analysis due to confounding factors; two of the remaining 7 patients who had received gentamicin (total duration of therapy of 14 days and 28 days), had raised hearing thresholds. Only 2 adults and 3 children received amikacin; 6 children and 1 adult patient received colistin. There was no association of GFR or hearing abnormality with total antibiotic/aminoglycoside use including the total number of courses, dose or cumulative dose/kg (please refer to

Table 2-8**,** Table 2-9 and Figure 2-9. Association of hearing with antibiotic exposure was analysed in paediatric group only due to small number of patients in the adult group for a meaningful comparison (Please refer to Table 2-10)**.**

Table 2-6 Cumulative exposure data for all antibiotics and aminoglycosides				
Parameter	**N**	**Paediatrics Median (IQR)**	**N**	**Adults Median (IQR)**
Time since last course of antibiotics (days)	44	144 (60 to 638)	10	71 (44 to 441)
Number of courses of all antibiotics	52	4.5 (1.0 to 13.5)	10	2.0 (2.0 to 14.0)
Number of days of all antibiotics	52	61 (14 to 194)	10	31 (28 to 196)
Number of courses of all aminoglycosides	50	3.0 (1.0 to 8.0)	10	2.0 (0.0 to 8.0)
Number of days of all aminoglycosides	50	42 (8 to 107)	10	28 (12 to 133)
Cumulative dose of all aminoglycosides (mg/kg)[#]	50	417.9 (80.0 to 1130.0)	10	324.8 (93.6 to 1297.8)

[#] Patients where the maximum daily dose (instead of mg/kg dose) was administered, the weight was assumed as 50kg; IQR: interquartile range

Table 2-7 Cumulative exposure data for individual antibiotics		
Parameter	**Paediatrics Median (IQR)**	**Adults Median (IQR)**
	N=50	N=10
Number of courses of tobramycin	3.0 (1.0 to 7.0)	2.0 (0.0 to 7.0)
Number of days of tobramycin	42. (8 to 104)	28 (6 to 126)
Cumulative dose of tobramycin (mg/kg)#	417.9 (80.0 to 1040.0)	324.8 (79.2 to 1192.8)
Number of courses of gentamicin	0.0 (0.0 to 12.0)*	0.0 (0.0 to 1.0)*
Number of days of gentamicin	0 (0 to 165)*	0 (0 to 12)*
Cumulative dose of gentamicin (mg/kg)#	0.0 (0.0 to 1980.0)*	0.0 (0.0 to 94.0)*
Number of courses of amikacin	0.0 (0.0 to 5.0)*	0.0 (0.0 to 3.0)*
Number of days of amikacin	0 (0 to 75)*	0 (0 to 43)*
Cumulative dose of amikacin (mg/kg)#	0.0 (0.0 to 2250.0)*	0.0 (0.0 to 1290.0)*
Number of courses of colistin	0.0 (0.0 to 26.0)*	0.0 (0.0 to 9.0)*
Number of days of colistin	0 (0 to 364)*	0 (0 to 121)*
Cumulative dose of colistin (Units/kg)#	0.0 (0.0 to 27.3 X 10^6)*	0.0 (0.0 to 14.5 X 10^6)*
Number of courses of Ceftazidime	3.0 (1.0 to 9.0)	1.5 (1.0 to 12.0)
Number of days of ceftazidime	42 (13 to 126)	24 (14 to 168)
Cumulative dose of ceftazidime (mg/kg)#	6300.0 (1950.0 to 18900.0)	2880.0 (1680.0 to 24360.0)

 # Patients where the maximum daily dose (instead of mg/kg dose) was administered, the weight was assumed as 50kg; * IQR value was 0; hence the range of values is presented; IQR: interquartile range

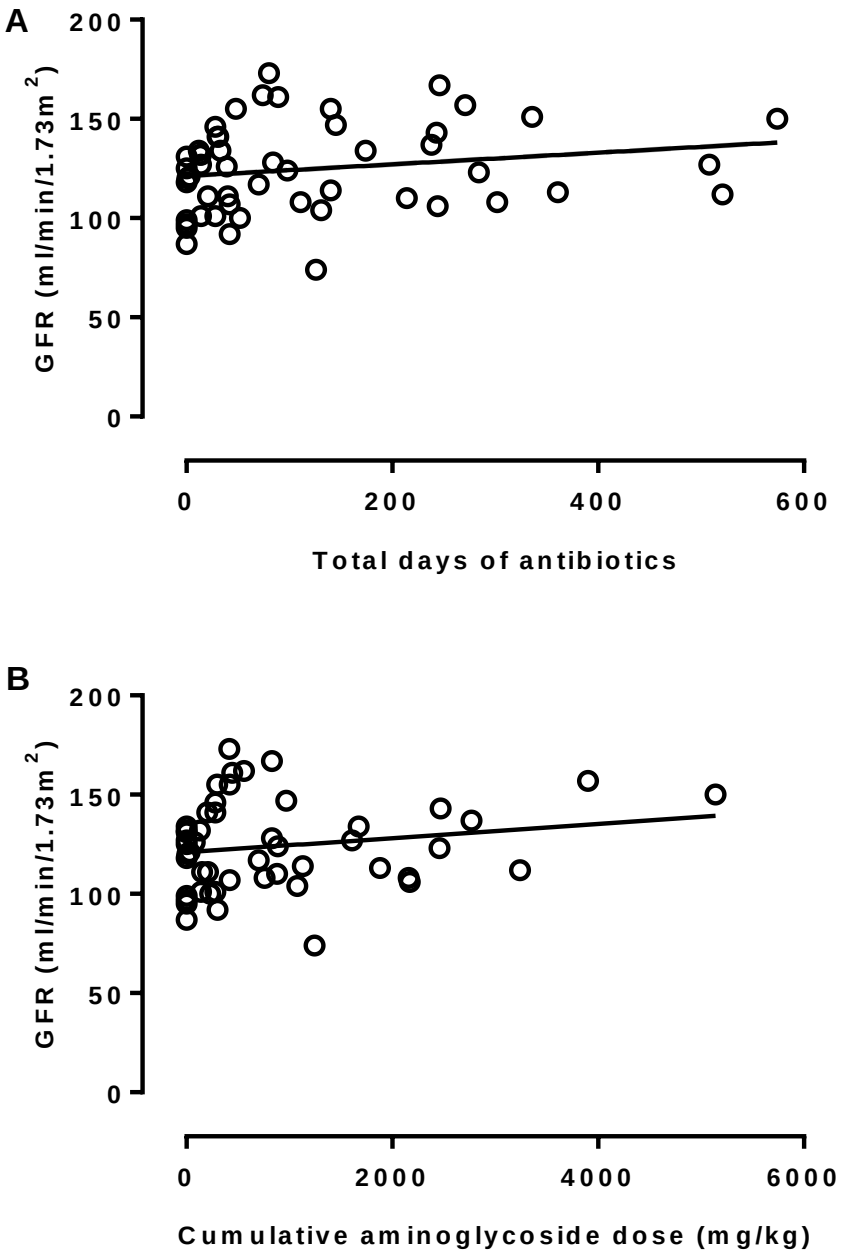


Figure 2-8 GFR against antibiotics received by paediatric CF patients A: total days of all antibiotics received; no association between GFR and total number of days of antibiotics received (P=0.18); B: cumulative dose of all aminoglycosides; no association between GFR and cumulative aminoglycoside dose received (P=0.38); GFR: glomerular filtration rate

Figure 2-9 Total number of courses of aminoglycosides received against GFR and hearing The black empty circles represent children with CF with normal hearing test result; the red solid circles represent children with CF with abnormal hearing rest result; no association of abnormal hearing threshold with abnormal GFR and cumulative exposure to aminoglycosides; GFR: glomerular filtration rate

Table 2-8 Association of cumulative antibiotic exposure with GFR in paediatric group				
Antibiotic usage parameter	**N**	**Linear regression results**		
		P value	**Regression Coefficient (B)**	**R^2 value**
Days since last antibiotic course	55	0.62	0.00	0.01
Total number of days of antibiotics	62	0.18	0.03	0.04
Total Number of courses of antibiotics	62	0.22	0.35	0.03
Total number of days of aminoglycosides	60	0.28	0.04	0.03
Total number of courses of aminoglycosides	60	0.33	0.41	0.02
Cumulative dose of aminoglycosides (mg/kg)#	60	0.21	0.00	0.03
Total number of days tobramycin	60	0.38	0.03	0.02
Total number of courses of tobramycin	60	0.41	0.43	0.01
Cumulative dose of tobramycin (mg/kg)#	60	0.38	0.00	0.02
Total number of days of ceftazidime	60	0.54	0.02	0.01
Total number of courses of ceftazidime	60	0.62	0.20	0.01
Cumulative dose of ceftazidime (mg/kg)#	60	0.57	0.00	0.01
Total number of days of colistin	60	0.87	0.10	-0.20
Total number of courses of colistin	60	0.86	0.15	0.00
Cumulative dose of colistin (units/kg)#	60	0.87	1.318 E-7	0.00

[#] Patients where the maximum daily dose (instead of mg/kg dose) was administered, the weight was assumed as 50 kg; NA: not applicable

Table 2-9 Association of cumulative antibiotic exposure with GFR in Adult group				
Antibiotic usage parameter	**N**	**Linear regression results**		
		P value	**Regression Coefficient (B)**	**R^2 value**
Days since last antibiotic course	9	0.73	0.00	0.02
Total number of days of antibiotics	10	0.06	-0.06	0.39
Total Number of courses of antibiotics	10	0.08	-0.80	0.34
Total number of days of aminoglycosides	10	0.16	-0.08	0.23
Total number of courses of aminoglycosides	10	0.19	-1.07	0.20
Cumulative dose of aminoglycosides# (mg/kg)	10	0.16	-0.01	0.23
Total number of days tobramycin	10	0.18	-0.09	0.22
Total number of courses of tobramycin	10	0.23	-1.11	0.17
Cumulative dose of tobramycin (mg/kg)#	10	0.19	-0.01	0.21
Total number of days of ceftazidime	10	0.08	-0.08	0.34
Total number of courses of ceftazidime	10	0.09	-1.18	0.32
Cumulative dose of ceftazidime (mg/kg)#	10	0.09	0.00	0.32

[#] ***Patients where the maximum daily dose (instead of mg/kg dose) was administered, the weight was assumed as 50 kg; NA: not applicable***

Table 2-10 Association of cumulative antibiotic exposure with hearing in paediatric group			
Antibiotic usage parameter	**N**	**Logistic regression hearing status vs antibiotics**	
		(P value)	**B (exp) Odd's Ratio**
Days since last antibiotic course	37	0.359	1.001
Total number of days of antibiotics	45	0.869	1.000
Total Number of courses of antibiotics	45	0.777	1.011
Total number of days of aminoglycosides	43	0.751	1.002
Total number of courses of aminoglycosides	43	0.731	1.023
Cumulative dose of aminoglycosides (mg/kg)[#]	43	0.908	1.000
Total number of days tobramycin	43	0.477	1.004
Total number of courses of tobramycin	43	0.487	1.058
Cumulative dose of tobramycin (mg/kg)[#]	43	0.469	1.000
Total number of days of ceftazidime	43	0.604	1.002
Total number of courses of ceftazidime	43	0.584	1.035
Cumulative dose of ceftazidime (mg/kg)[#]	43	0.549	1.000
Total number of days of colistin	43	0.717	1.005
Total number of courses of colistin	43	0.718	1.077
Cumulative dose of colistin (Units/kg)[#]	43	0.717	1.000

[#] Patients where the maximum daily dose (instead of mg/kg dose) was administered, the weight was assumed as 50kg.

2.5 Discussion

This study provides measurements of ^{51}Cr-EDTA GFR in a large group of people with CF. This is the first study to measure GFR, using a ^{51}Cr-EDTA method, in children with CF. The whole study cohort had a 9% prevalence of low GFR (<90 ml/min/1.73m^2). Excluding the patients who were post lung transplant, this amounts to an overall 6% prevalence of low GFR. Although there were only 2 children with a GFR value of <90 ml/min/1.73m^2, interestingly, almost one in three children had a GFR value higher than 90th centile of the reference values. This study demonstrates that when compared with the gold standard of mGFR, i.e. ^{51}Cr-EDTA GFR, eGFR is an inaccurate marker of true GFR in people with CF. This was particularly so for the paediatric patients, where the Schwartz formula overestimated the GFR and the 95% CI of the level of agreement ranged widely on either side of the mean difference between two values. Thus estimated GFR based on Schwartz formula should not be relied upon to predict true GFR in children with CF. Hearing deficit was identified in 25% of the population which did not correlate with cumulative aminoglycoside exposure. Though people with CF require administration of higher doses of aminoglycosides compared to general population due to altered aminoglycoside pharmacokinetics and pharmacodynamics related to the lung pathology, this study found no association between cumulative antibiotic exposure and GFR. This lack of association may be related to change in management practices to less nephrotoxic regimens (e.g. once-daily aminoglycosides) and recovery of renal insult between subsequent IV aminoglycoside courses.

The largest study of a general population of children assessing ^{51}Cr-EDTA GFR (*421*), by Piepsz et al., showed the mean GFR between 2 to 15 years age to be 104.4 (SD 19.9, 10th and 90th centile values of 81 and 135 respectively) ml/min/1.73m^2. The mean GFR of our paediatric group (124.8 (SD 22.3) ml/min/1.73m^2) is clearly much higher than that found in the Piepsz et al. study (*421*). Although they used the same tracer

substance, the GFR measurement was based on a single sample collected at 2 hours post-tracer injection. On adjusting their results to the method recommended by BNMS (*419*) i.e. slope-intercept method and Bröchner-Mortensen's correction, their mean GFR was even lower i.e. 93 (SD 12) ml/min/1.73m^2 (*421*), thus making the difference from our paediatric population even higher. Another study using ^{51}Cr-EDTA tracer and BNMS method (*466*), showed a mean GFR of 109 (SD 16.8) ml/min/1.73m^2 in 2 to 17 years old children. It was a small study with only 24 subjects, who were being tested following urinary tract infections, which might have led to hyperfiltration and contributed to higher GFR values (*467,468*). Granerus et al. compiled various epidemiological studies to establish the reference range for ^{51}Cr-EDTA in adults (*422*); mean GFR at different ages were shown as 105 ± 26ml/min at 30 years, 98 ± 23 ml/min at 50 years and 78 ± 24 ml/min at 70 years. Although the mean GFR of our adult patients (99.6 (SD 27.5) ml/min/1.73m^2) falls within the normal range, the majority of our adult cohort was younger. The mean GFR in our group of adults with CF, however, was closer to that of GFR in the higher age group from the general population, i.e. 50 years in the Granerus study. This might have been related to the confounding effect of people with lung transplant and when transplant recipients were excluded, the mean GFR of the adults between 18-50 years age was 109.2 (SD 18.1) ml/min/1.73m^2, which is similar to the results in the Granerus study (*422*).

A limited number of studies have tried to assess the prevalence of CKD in CF population. I found a low GFR in 3.8% children and 14% adults (excluding lung transplant recipients). Previous studies have shown variable results, which appear to be mainly related to the method of GFR measurement or estimation. Using the recent CKD guidelines (*189*), only 3 patients from this study cohort fulfilled the criteria of CKD (stages G2A2, G2A3 and G3b, please refer to Table 1-4 and Table 1-5) two of whom had received a lung transplant. Many studies from the previous

century measured renal function in CF population using the gold standard inulin clearance or isotope clearance methods but with small patient groups and with an aim to assess the renal electrolyte handling rather than to identify CKD (*214*). Studies that are more recent have derived prevalence of CKD based on creatinine clearance or GFR estimated by creatinine dependent equations. The largest study assessing the prevalence of stage 3 or higher CKD in (about 11,000) adult people with CF was done by Quon et al. (*4*). They found a 2.3% prevalence of CKD, which increased from 0.6% in the age group of 18-25 years to 19% in people >55 years old. This was a US CF Foundation Registry-based study, which defined CKD by low eGFR for 2 consecutive years. Although the usefulness of eGFR in CF has been questioned (*429*), such large studies may only be possible with these limitations. Pederson et al. (*213*) assessed creatinine clearance in people with CF (age range 8 to 35 years) and demonstrated a low clearance of <96 ml/min/1.73m^2 in about 40% study population. Similarly, Al-Aloul et al. (*5*) showed a 42% prevalence of creatinine clearance less than 80ml/min/1.73m^2 in a group of 80 adult CF patients. This study had a significant role in increasing awareness of the possibility of CKD in CF. However, all these results are based on creatinine clearance, which may be unreliable, firstly due to unpredictable creatinine secretion in renal tubules (*397,403,404*), and more so, in people with a nutritional deficiency or reduced muscle mass affecting the creatinine production. Furthermore, the need for collection of 24-hour urine samples increases the chances of error. The reliability of creatinine-based GFR estimations in the setting of CF has been questioned by Edwards et al. (*469*), and the results of our study reinforce these concerns. A study in a paediatric CF population measured creatinine clearance and used eGFR, based on the Schwartz equation, to assess the prevalence of CKD in CF (*216*). They showed a creatinine clearance of <90 ml/min/1.73m^2 in 14/24 (58%) subjects, but the eGFR (Schwartz) did not show any cases of CKD (median values of the eGFR ranging between 123 to 161 and ages ranging from 1-15 years). Of note in the study by

Andrieux et al. (*216*), there was mildly elevated albumin-creatinine ratio in 58% children and urolithiasis in 2 infants. There was a high prevalence of urolithiasic risk factors (hyperoxaluria and hypocitraturia) in their population (*216*). This aspect was not assessed in our study, but this finding highlights the presence of different risk factors for renal disease in the CF population.

A limited number of studies have measured GFR by plasma clearance of radioactive tracers, and these show a low prevalence of CKD in paediatric age group. Soulsby et al. measured GFR (^{99m}Tc-DTPA injection followed by 3 blood samples) in a cohort of 20 adults and 20 children with CF, mean age 26.8 (SD 9.2) years and 11.4 (SD 4.7) years respectively (*215*). No paediatric patients but 4/20 (20%) adult patients had GFR below 90 ml/min/1.73m^2. A larger study (*214*) using ^{99m}Tc-DTPA in paediatric age group (N=63, mean age 9.6, SD 5 years) found one patient with GFR <90 ml/min/1.73m^2, 3 patients with recurrent microscopic haematuria and an overall prevalence of renal dysfunction of 6%. All these studies agree with a low prevalence of CKD in children with CF. The prevalence of low (<90 ml/min/1.73m2) GFR, when measured by radioactive tracers, in adults with CF was shown to be 20% by Soulsby et al.; however, this result was not adjusted for confounders and was limited by a small number of subjects (*215*). One recent study on adults with CF showed a mean GFR, measured by Iothalamate clearance method, of 104 (SD 32.2) ml/min/1.73m^2, which was similar to the healthy control group (*430*).

The level of agreement between different tracer clearances is still not fully agreed upon and appears complicated. There is strong evidence regarding equivalence of ^{51}Cr-EDTA GFR to inulin clearance (*404,470*). There is insufficient evidence for the accuracy of plasma clearance of ^{99m}Tc-DTPA against inulin clearance (*404*). Although there are studies to show its good correlation with ^{51}Cr-EDTA GFR (*471,472*), some authors suggest an overestimation of about 7.5% (*473*), whereas others showed an underestimation by about 10% (*474*). Correlation, however, does not

describe the level of agreement between the two methods and is simply a measure of association (*475*). Bland-Altman plot allows measurement of bias and limits of agreement and is recommended for method comparison studies (*475*). Many recent studies that have used Bland-Altman analysis have shown that ^{51}Cr-EDTA GFR has a good level of agreement with inulin clearance with a mean bias of 3 ± 6 ml/min (*470*). Many studies have shown protein binding of ^{99m}Tc-DTPA whereas others have shown this to be related to the type of preparation used (*474*). Protein binding of ^{51}Cr-EDTA has not been shown by some authors (*476,477*), whereas another group (*478*) described it to be equivalent to ^{99m}Tc-DTPA (*479*). To add to the confusion is a study by Spino et al. where they assessed plasma clearance of DTPA along with its renal clearance (*480*). Comparing 8 CF patients with 10 non-CF control subjects, they showed a non-renal clearance of 26.6 ml/min/1.73m^2 and 11.8 ml/min/1.73m^2 respectively. Extra-renal clearance of ^{51}Cr-EDTA GFR has been shown to be around 4 ml/min across all ranges of GFR by some (*478,481*) and even higher values by other authors (*472*). I did not find any studies that have assessed the extra-renal clearance of ^{51}Cr-EDTA in CF population. With all these caveats in mind, if there is a 10% extra-renal clearance of ^{99m}Tc-DTPA (*214*), then the mean GFR of our paediatric patients is in agreement with the paediatric CF patients in the studies by Soulsby et al. (*215*) and Prestidge et al. (*214*), who suggested a mean GFR of 142 ± 27.2 and 140 ± 22ml/min/1.73m^2 respectively.

Various guidelines for the management of renal disease define low GFR, but there is no cut-off point suggested for high GFR (*482*). Our study did not have a control group; hence I chose to use reference values from Piepsz study, which used plasma clearance of ^{51}Cr-EDTA in children (*421*). Our paediatric population showed a 29% prevalence of high GFR, i.e. >90th centile (glomerular hyperfiltration). Using the same definition as we did, Prestidge et al. showed hyperfiltration in 56% subjects (*214*); after correction for 10% extra-renal clearance of ^{99m}TC-DTPA in CF, they found

hyperfiltration in 21% patients. Two studies on adult CF patients, including about 20 patients each and using radioisotope GFR measurements (Iothalamate (*430*) and ^{99m}Tc-DTPA (*215*)), showed mean GFR values of 104 ± 32.2 and 113 ± 25.3 ml/min/1.73m^2 respectively, which fit within normal ranges by Piepsz et al. for age 30years. However, the study by Soulsby et al. shows a large range of GFR in adult patients (68-149ml/min/1.73m2). Many (*191,329,483-485*) but not all (*193,331,486,487*) studies published in 80's and 90's describe high mGFR values in people with CF compared to control groups. However, the primary objectives of these studies were to evaluate fluid, and electrolyte and sodium handling by the kidneys or to assess drug clearance in CF. The patients were in varying states including fasting, fluid deprivation, some having concurrent IV fluids or antibiotic administration. GFR in our study was measured while the patients were in a steady state (i.e. not during an exacerbation and at a median of 127 (IQR 49 to 638) days from the end of last IV antibiotic course), and most of them were in generally good health (mean FEV_1 80%, 95% CI 73.5 to 86.3). Similarly, GFR measurements in the Soulsbly study (*215*) were also at least 8 weeks after the recent course of antibiotics. Our study showed no correlation between cumulative IV antibiotic exposure and GFR. Thus, the high GFR in our paediatric cohort does not appear to be driven by increased disease severity or higher number of antibiotic courses received. Strandvik et al. proposed that renal changes in CF might be related to essential fatty acid deficiency (*483*). After IV supplementation of essential fatty acids for a year, the GFR decreased from the high value before the start of the study (133 ± 18ml/min/1.73m^2) to a value equivalent to controls (111 ± 14 ml/min/1.73m^2). This study has not been replicated so far. Abnormal fatty acid levels have been shown in the blood and tissues of people with CF; most common abnormality is decreased linoleic acid levels (*488,489*). It has been related to increased expression and activity of fatty acid desaturases, which help to metabolise linoleate to arachidonic acid (*489,490*). Abnormal desaturase expression has been linked to CF

mutations. There is evidence to suggest that abnormal fatty acid levels in CF are not related to malnutrition, and the changes persist even in people who are adequately managed with enzyme replacement and nutritional support (*489,491*). There is speculation that abnormal fatty acids affect the biophysical properties of epithelial cell membrane altering the function of membrane proteins (*489*) and possibly make them more permeable to sodium and potentiate proximal tubular sodium reabsorption (*483*). This should result in decreased fluid delivery to distal tubules with subsequent activation of tubuloglomerular feedback (*492*). However, there are some studies on mouse model of CF that suggest that proximal tubular function is preserved in CF with normal sodium excretion in both, salt-depleted and salt-replete conditions (*493,494*). Furthermore, a study on mice fed on essential fatty acid deficient diet showed a reduction in GFR to about half and increased prominence of proximal tubular brush border (*495*). Thus, more research is needed before the above hypothesis to explain hyperfiltration in CF could be accepted.

Glomerular hyperfiltration and the underlying mechanism leading to it have been studied in the context of diabetes mellitus, but this phenomenon has also been described in other diseases like sickle cell disease, obesity/metabolic syndrome, hypertension, high protein diet, autosomal dominant polycystic kidney disease, etc. (*496,497*). Hyperfiltration is a result of afferent arteriolar vasodilation or efferent arteriolar vasoconstriction due to activation of the renin-angiotensin system (*496*). This, in turn, can be related to a number of factors like tubuloglomerular feedback suppression associated with high glucose load; increased proximal tubular sodium reabsorption; increased vasodilatory prostaglandin levels; and the role of hormones such as insulin-like growth Factor (*498,499*) and atrial natriuretic peptide (*492,500*). The consequences of hyperfiltration are again mainly described in the diabetic population. A systematic review concluded that

the risk of progression of diabetic patients to diabetic nephropathy is more than doubled in people with glomerular hyperfiltration compared to diabetic people with normofiltration (*501*). However, this has been disputed by others (*502*). Further research is needed, using standardised conditions, reliable GFR estimation and long-term follow-up to evaluate the association and progression of high GFR levels in some people with CF.

The effect of age on GFR observed in our study agrees with the previous observations that GFR is constant during the childhood; it remain stable or declines at a very slow rate in early adulthood followed by a faster decline after about 40-50 years of age. Piepsz et al. (*421*), retrospectively assessed ^{51}Cr-EDTA GFR in a group of 623 children with unilateral renal pathology (e.g. hydronephrosis, reflux, megaureter, etc.) and equal radioisotope uptake in both kidneys (i.e. no renal functional impairment). They established that GFR increases consistently from birth up to 2 years age and then it remains constant until age 15 years. On the other hand, reference values for adult patients show that ^{51}Cr-EDTA GFR declines with age at the rate of 4 ml/min/decade below the age of 50 followed by a faster decline, at the rate of 10 ml/min/decade, at higher age (*422*). Similarly, Grewal et al. (*423*) showed that GFR remains constant at 103 (SD 15.5) ml/min/1.73m^2 until about 40 years age and then declines at a rate of 9.1 ml/min/1.73m^2 every decade. Although our adult cohort comprised mainly of people below 40, the rate of decline detected in our analysis was much higher (i.e. 1.6 ml/min/1.73m^2 decline per year); a sensitivity analysis excluding the lung transplant recipients and restricting the age limit to the highest of 50 years, there was no significant association of GFR with age (P=0.620). A possibility of bias cannot be excluded in this relationship, as our adult group was small, comprising of only 16 patients, which did not allow adjustment for confounders.

To my knowledge, this is the first study assessing the CKD-Epi eGFR in people with CF. Compared to adjusted MDRD eGFR, CKD-Epi eGFR

showed less (minimal) bias, more precision and higher accuracy with almost 60% of the eGFR values being within 10% of the corresponding mGFR. However, our adult patient group was small, and these results may not be generalisable. Agreement of creatinine-based eGFR with ^{51}Cr-EDTA GFR in people with CF has not been evaluated so far. There have been some previous studies assessing the agreement of various creatinine-based equations with mGFR (measured with other tracers or creatinine clearance) in individuals with CF. Many studies have instead compared the eGFR results to creatinine clearance. A large study on 74 adults with CF by Al-Aloul et al. assessed agreement of eGFR calculated by 10 different equations to creatinine clearance; they compared the results to a matched control group of healthy adults (*429*). All the equations performed similarly with a large bias and a wide range of 95% levels of agreement. Moreover, the accuracy was less in people with CF compared to controls. Touw et al. showed poor precision and a wide range of deviation (-25 to + 62%) of eGFR from the measured values of creatinine clearance (*326*). In a paediatric study, Andrieux et al. showed that eGFR overestimates creatinine clearance; however, they did not report bias and precision (*216*). There is evidence now that creatinine clearance measurement may be no more (or even less) reliable than the GFR estimated using creatinine-based equations to predict the true GFR in both, adults and children. Hence, it is difficult to rely on creatinine clearance as a reference method for comparisons of eGFR. There are a few studies in literature within the context of CF population, which have assessed agreement of eGFR (both creatinine and cystatin C equations) with mGFR using tracer related methods. Beringer et al. (*430*) compared eGFR equations (MDRD, Cockcroft and Gault and a cystatin C-based equation by Tidman et al. (*503*)) against a group of healthy controls using Iothalamate clearance as a reference method. Similar to the results by Al-Aloul et al., the creatinine-based equations were less accurate in people with CF compared to the control group. The bias was similar for all the equations; however, they showed better precision with cystatin C

equation. This effect was not observed in a study by Soulsby et al. (*215*), where they assessed a group of adults and children with CF for the level of agreement between ^{99m}Tc-DTPA GFR and the GFR estimated using creatinine and cystatin C-based equations. They showed a large bias and a wide range of (95%) level of agreement for different equations, i.e. 6.6 ml/min/1.73m^2 and 92 ml/min/1.73m^2 respectively for the Schwartz equation and -64 ml/min/1.73m^2 and 294 ml/min/1.73m^2 respectively for the MDRD equation. There was no significant improvement in accuracy with cystatin C-based equation (Macisaac equation (*504*)). The results by Soulsby et al. (*215*) for the Schwartz equation are comparable to our study results.

Various studies on non-CF children showed that bias for Schwartz equation ranged from -0.4 to 10 ml/min/1.73m^2 and SD between 2 to 20 ml/min/1.73m^2, with 75% of eGFR estimates being within 30% of inulin clearance (*188*). The^{51}Cr-EDTA method is shown to give results close to inulin clearance (*404*). NKF KDOQI clinical practice guidelines (2002) recommend that an eGFR within 30% of mGFR is a satisfactory marker for clinical performance and accuracy of estimation equation. They recommended a P30 of >90% in the validated population (*188*). Gretz et al. highlight that precision of ± 20% in adults and ± 30-40% in children is generally found while estimating GFR from creatinine-based equations (not specific to CF) (*505*). Based on these figures, though we did not have a control group for comparison, our results are more or less what can be expected from these equations in a non-CF population. Taking our 95% limits of agreement for Schwartz equation, any child with a GFR of 100 can have an eGFR value anywhere from 45 to 165 ml/min/1.73m^2. Certainly, in any population where there is recurrent exposure to potential nephrotoxic treatment including the possibility of a lung transplant in future, this equation cannot be recommended. We need more studies in future assessing newer eGFR estimation equations, particularly those combining creatinine and cystatin C. At the same time,

we should also be looking out for new simplified methods for GFR measurements to allow better monitoring of our patients.

Our results did not show any correlation of GFR with a cumulative dose of IV antibiotics including aminoglycosides i.e. did not show an evidence of cumulative nephrotoxicity of antibiotics in both adult and paediatric patients. This included comparing levels of exposure in different forms including the number of courses of ≥7 days duration, number of days of antibiotics received and the total dose received in mg/kg. Previous reports suggested an association of AKI with aminoglycosides, in particular, gentamicin (*200*). The effect of cumulative exposure on renal function has been studied in a limited number of studies. The largest study was done by Al-Aloul et al. in a group of 80 adults with CF, which showed an inverse relationship of creatinine clearance with increasing aminoglycoside exposure (*5*). The effect was potentiated by coadministration of colistin, but colistin on its own did not seem to affect the creatinine clearance. Similarly, Etherington et al. showed a positive association of NAG levels with previous antibiotic exposure over a period of 6 years; however, when analysed individually (*274*), there was a significant association of baseline NAG levels with previous colistin exposure but not with previous tobramycin exposure. We did not replicate the association found in these two studies. However, our results are in agreement with many other studies. Pederson et al. (*213*) and Andrieux et al. (*216*) did not find a correlation between cumulative tobramycin exposure and creatinine clearance. Both these studies used tobramycin, in thrice-daily and once-daily doses respectively. An important difference from our study is the use of a different method of GFR measurement. Creatinine clearance may be subject to various inaccuracies related to non-GFR determinants of creatinine (*404,420*) and compliance with collection of a 24 hr urinary sample, particularly in paediatric patients. One study that measured GFR with ^{99m}Tc-DTPA method did not find any correlation with cumulative aminoglycoside

exposure (*214*). The effect of a course of IV aminoglycoside (including a once-daily dose of tobramycin) has been shown to cause increased excretion of certain enzymes in urine that specify renal injury. This suggests that there is a renal insult during the treatment course (*205,206,274,305*). The aminoglycoside-induced renal damage is, however, thought to be reversible. It is possible that the renal injury following one treatment course with IV antibiotics is repaired during the time interval before another course of IV antibiotics is commenced. This may explain the lack of cumulative toxicity of repeated antibiotic courses observed in our study. Studies in people with CF that showed an elevation of NAG levels during a course of IV aminoglycosides have also shown that the levels return to baseline or near baseline in a large proportion of patients followed up for a median of 50 days post-treatment (*274,305*). There is a possibility that this process of healing continues beyond 50 days, which may explain why Glass et al. showed an elevated NAG level at 4 weeks after treatment, whereas the level at the start of therapy was normal even in the children who had received tobramycin previously (*305*). In people with CFRD, NAG showed the same 3.5 times increase over baseline seen in other CF patients. However, a higher proportion of people with CFRD were shown to have elevated NAG levels at baseline and continued to have elevation in urinary NAG levels compared to baseline at follow-up (*274*). It is possible that the process of healing is slower in people with other comorbidities (such as CFRD) or other nephrotoxic influences. We did not find any association of GFR with the presence of CFRD; however, the numbers with CFRD were small (6/68). Another aspect to consider is the effect of antibiotic prescribing practices, which have changed over time. There is a move away from the use of gentamicin to tobramycin (*301,303,353*). In our study, only a small number of patients, i.e. only 4 children and one adult patient had ever received a course (≥7 days) of IV gentamicin. Most other studies have used tobramycin, though Al-Aloul et al. report using a mix of aminoglycosides including gentamicin (*5*). Another practice that has

changed is the use of once-daily tobramycin, which was shown to be less nephrotoxic than thrice-daily tobramycin (*217*). It is possible that with better antibiotic prescribing practices and with improved management (including better nutrition), we are able to prevent or reduce the renal insult caused by individual courses of antibiotic treatment that allows time for recovery prior to administration of subsequent antibiotic courses.

This study found an increased hearing threshold in 25% of all subjects, which combined prevalence of 20% in paediatric population, and 40% in adults. Within the non-CF population, a variable prevalence of aminoglycoside-induced hearing loss has been described, which ranges from 2-25% (*435*). The majority of the studies in literature from people without CF are based on single aminoglycoside courses (*435,436,449*). The population of CF is unique with different pharmacokinetics from the general population, the requirement of higher doses and repeated courses in many cases. Again, there is a considerable variation in the prevalence of aminoglycoside-induced ototoxicity in people with CF (*320*). Most of the evidence is based on retrospective studies and case reports or case series (*167*). Mulheran et al. (*449*) showed a 17% prevalence of hearing loss in their cohort of 70 patients (including both adult and children); however, 11/12 patients with hearing deficit were adults. Our study shows a higher prevalence of hearing loss, which is similar to some other studies, published more (*450,506,507*) recently.

As the literature evidence of correlation of antibiotic exposure with hearing loss in CF is divided, our results of lack of correlation between increased hearing threshold and cumulative aminoglycoside exposure in children are in agreement with some studies but not with others. One early study did not show significant ototoxicity associated with cumulative exposure to tobramycin in a group (age range 10-35 years) that had received median 20 courses of aminoglycosides (*213*). Mulheran et al. showed a significant difference between the number of

aminoglycoside courses received by the groups (including both adult and paediatric patients) with and without hearing loss (*449*). However, the relationship was non-linear in nature. A small study on adults with CF did not show any difference in hearing threshold levels after a median of 3 (range 1-8) courses of tobramycin (*508*). On the other hand, Al-Malky et al. demonstrated ototoxicity in 21% children with CF who had received >5 courses of aminoglycosides (*506*) and in 44% children with CF who had received >10 courses (*450*). Geyer et al. showed a much higher proportion of hearing loss (high frequency, 83%) in people with CF (age 7-20 years) who had received >10 courses of aminoglycosides. There was a significant association between increased hearing threshold in high-frequency audiometry and the number of courses of aminoglycosides received (*451*). Another important aspect in the context of results of our study is that a large proportion of people with CF do not develop ototoxicity despite having had similar amount of aminoglycoside exposure. Similar observation has been made in the studies that have shown an association of hearing loss with cumulative aminoglycoside exposure (*506*). Thus, there are other factors in play, which may be idiosyncratic (*449*) or genetic in nature. Mulheran et al. calculated a 'per aminoglycoside course cochleotoxic risk' in their cohort of people with CF to be about 1.7%. The authors speculated that patients with CF might have a shorter half-life of aminoglycosides in the cochlea or have an increased tolerance to aminoglycosides related to higher renal clearance. However, we did not find any association of GFR with hearing loss in our study (analysed in paediatric population). This is the first time ^{51}Cr-EDTA GFR has been assessed along with hearing in people with CF. A limitation of our study was that we performed pure tone audiometry at conventional frequencies only whereas all the above studies also used high-frequency audiograms. The latter has been shown to be more sensitive to detect the high-frequency hearing loss (*450*). Indeed, Al-Malky et al. found that the hearing loss was in the high-frequency range in their cohort (*506*). On the contrary, there are other studies, which have

used only conventional pure tone audiometry and have shown an increased prevalence of ototoxicity with increasing cumulative dose of aminoglycoside (*507*). If we had measured high-frequency hearing thresholds (i.e. >8000Hz) in our cohort, the overall prevalence of hearing loss might have been higher. One weakness of our study is that we used a set of screening questions and history to rule out middle ear disease but did not undertake tympanometry. If there were any cases with unidentified middle ear problems, our prevalence is likely to have been an inflated one. Results may have been influenced in a similar way towards higher prevalence of hearing loss by any ambient noise present in the quiet rooms where the audiograms were undertaken. We did not have genetic screening for mitochondrial mutation in our cohort; however, considering the low prevalence of these mutations, testing of whole study cohort would have been prohibitive due to additional cost. We were unable to make any comparisons between ototoxicity of different aminoglycosides as only a small number of patients received aminoglycosides other than tobramycin. However, on individual assessment, we did not find any association of hearing deficit (in children) with the level of exposure to tobramycin or gentamicin (the number of courses and days of therapy or cumulative dose/kg). No studies have assessed this aspect so far in CF population (*167*).

The prevalence of hearing deficit in CF in our study and other studies in literature is significantly higher than the prevalence described in the UK CF Registry report, i.e. 3.8% combined prevalence in adults and children (*446*). In our study, none of the patients complained of any hearing difficulty apart from the ones who had already been diagnosed. Many cases of hearing loss in CF may be asymptomatic and may remain undiagnosed until significant damage has been done (*449,509*). The presentation of hearing loss may not be a characteristic complaint of difficulty in hearing, particularly in children (*510*). This signifies the

importance of regular audiological assessment in this group of patients, which still lacks a standard protocol.

One major strength of our study was that we measured GFR by an accurate, gold standard measure in a large group of people with CF. This is the first time that a radioactive tracer based GFR has been measured in a large group of CF patients in a research setting during a stable state. One other study using ^{99m}TC-DTPA tracer measured GFR in children (*214*), however, on a retrospective basis, in a clinical setting, and during the time when patients were receiving IV antibiotics, i.e. were likely to have been unwell. A limitation of our study was a small adult group. Having a larger group of adult patients could have provided a more generalisable data. Due to the nature of the study procedures (the measurements took most of the working day), the study recruitment, particularly for adult patients, was a challenge. Considering the already high burden of disease, it is important to develop accurate and simple ways to predict or measure GFR. We included 2 patients in the adult group who had received lung transplant; their GFR was significantly lower than the rest of the adult patients, which could have confounded the results. I calculated the mean GFR values including and excluding the values from these patients. This highlights the fact that GFR falls down significantly after lung transplant (Section 1.3.5.4), and it is critical to preserve the renal health in this group of patients in anticipation of a transplant.

The selection of patients for the study was not random in nature, which might have introduced a selection bias. One would generally expect the cumulative antibiotic exposure to increase with increasing age. However, our data showed that though the range of the number of antibiotic courses received by adults was larger than children, the median cumulative antibiotic exposure was smaller in adults than that in children. This is an unexpected finding and may indicate an unintentional bias in patient selection. It is possible that due to higher disease burden and increased demand on their time, (adult) patients who were sicker or

having frequent antibiotic courses, did not consent to participate whereas more adult patients with less disease burden (i.e. healthier CF patients) chose to participate; approximately 40% of our adult cohort had an FEV_1 of >85 percent predicted. Similarly, those patients who received their care at more than one centre were more likely to be excluded from the analysis due to difficulties in organising medical notes review from multiple centres. At the same time, this unexpected observation of higher median antibiotic exposure in paediatric group compared to the adult group may also be related to missing some part of the information and lack of completeness of data, particularly for adult patients as the records go back to several years.

Another limitation of this study is that GFR was measured at two laboratories due to the distance between the research sites. The research protocol for ^{51}Cr-EDTA measurement was prepared according to the protocol followed in the nuclear medicine department at Nottingham University Hospitals; the same protocol was shared with and used by the nuclear medicine department at Birmingham Children's Hospital. Though both the laboratories used the same protocol, no specific external quality standard was used, so a small variation in results may be possible. A national audit of GFR measurement (across the UK), compared the results from various centres (*511*) with national median and a reference value calculated by the method recommended in the BNMS guidelines (*419*). The audit found widespread standardisation of GFR measurement across participating centres, and the results from Nottingham University Hospital laboratory followed the reference results (R^2=>0.997); the other research site did not participate in the audit.

The cross-sectional nature of our study inherently limits any inference regarding causation and disease progression. It will be interesting to see the progression of GFR in people with high GFR to be able to assess the cause and natural history of hyperfiltration in future studies. We did not evaluate the impact of inhaled aminoglycoside therapy and oral

macrolide therapy on renal function and hearing. Inhaled antibiotics (tobramycin) have been implicated in case reports of AKI (*199,512*), however, in studies evaluating evidence of acute renal damage assessed by enzymuria, no association was observed (*206*). Other antibiotics like macrolides have been suggested to cause ototoxicity (*507*) whereas drugs such as PPI have been associated with acute and chronic renal damage in general population (*392-394*). Future studies will be needed to assess the impact of these drugs that are so commonly used in CF population. In addition, there is still a need for validation of GFR estimation equations, possibly based on a combination of creatinine and cystatin C for accurate prediction of GFR in this group while comparing them to the gold standard measures.

2.6 Conclusion

This study showed that renal function measured by ^{51}Cr-EDTA GFR was normal in most CF patients with a low prevalence of CKD. One in three children showed high GFR suggestive of glomerular hyperfiltration, which will need further investigation to establish its cause and progression. We could demonstrate no relationship between cumulative antibiotic exposure and renal function. This could be attributed to better management practices or reversible nature of any acute insults caused by IV antibiotic courses. However, we need to be cautious, as there are other nephrotoxic risk factors, e.g. CFRD and likelihood of receiving a lung transplant. One in four people had increased hearing threshold, which could not be explained by previous aminoglycoside exposure and needs further exploration to identify other factors involved. We established that GFR estimated by creatinine-based formulas is a poor predictor of mGFR, and there is a need to develop and validate more accurate prediction equations in this population. At the same time, there is a requirement for simplification of methods to measure GFR accurately, in a convenient and less invasive way.

Chapter-3 Animal Models of CF

3.1 Introduction

The outlook for the patients with CF has improved significantly due to comprehensive management strategies. Understanding of the disease process has substantially increased over the last several decades; however, the spectrum of CF disease is varied and complex and is yet far from being fully comprehended. With obvious limitations of clinical studies on humans, development of the animal model was an essential step towards resolving the pathophysiological enigmas of CF.

3.1.1 Mouse model

The CF gene was identified and cloned in 1989 (*55*). This accelerated research to explain the pathophysiology of CF and identify a genetic cure.

Three groups initially developed mouse models of CF: the North Carolina (*513*), Edinburgh (*514*) and Cambridge (*515*). Since then, fourteen mouse models of CF have been characterised and reported (*516*). Many of these involved use of embryonic stem cells and homologous recombination technique, a specific genetic method to target a mutation to a specific site in the mouse genome (*517*). Initial mouse models were associated with mutations that led to complete loss of function of CFTR or with some normal residual activity. These were followed by models with specific clinically relevant mutations, e.g. Phe508del mutation, G480C and G551D mutation models. Transgenic mouse models (*518*) express human CFTR with a specific mutation (G542X) in the knockout (CFTR -/-) mice (*517,519*).

Mouse models of CF have been used extensively not only to understand the CF pathophysiology but also have been indispensable in novel therapy including gene therapy trials as well as the study of CF and non-CF lung responses to bacterial infection and inflammatory challenges (*520,521*). Despite several similarities between mice and humans regarding clinical

phenotype of CF, there are numerous differences also, the most striking one being the lack of development of significant lower airway disease that is so characteristic of CF in human beings. Besides, mice also fail to develop pancreatic, vas deferens, liver and gallbladder disease that are present in humans (*522*). Furthermore, although intestinal abnormalities do occur consistently in all mouse models of CF, these are phenotypically different from meconium ileus in newborn babies with CF. Despite these differences in phenotypes, mouse models have made an extremely valuable contribution to the research in CF.

3.1.2 Pig model

Considering the limitation of interspecies CF phenotype differences observed with the mouse models of CF, research was focussed on the search for other animal models in CF, which included sheep, pig and ferret. To date, pig and ferret models of CF have been developed (*523,524*). These larger animal models have shown phenotypical similarities to human CF disease seen in newborn babies (*10,524*).

3.1.2.1 Development of pig model of CF

Somatic cell nuclear transfer (SCNT) is a technique used for cloning; nucleus is removed from a donor egg cell, which is then fused with the nucleus from another cell to generate an embryo. This technique was used in cloning of Dolly the sheep (*525*). Homologous recombination is a process of exchange of genetic material between two strands of DNA containing a stretch of similar base sequences. This occurs naturally as a DNA repair process and during meiosis. This technique is utilised in genetic engineering for gene targeting, i.e. for altering a specific gene. With this technique, an engineered mutation (e.g. a premature stop codon, a drug resistance marker) can be introduced at a specific locus of a gene.

Researchers from the University of Iowa developed the first pig model of CF in 2008 (*11*) by using homologous recombination in fetal fibroblasts to

disrupt the exon 10 of the CFTR gene of domestic pigs. Fetal fibroblasts from domestic pigs (Sus scrofa) were used as these cells had previously been used successfully for SCNT. A "null" target construct including neomycin resistance cassette (Neo®) was designed to disrupt CFTR exon 10 (required to produce a functional CFTR). Neo® contains a neomycin resistance complementary DNA (cDNA) driven by phosphoglycerate kinase and is flanked by loxP sites. Adenovirus-associated vector was used to introduce the target construct into the nucleus of fetal fibroblasts using the principle of homologous recombination. The infected fibroblasts were then used as nuclear donors for enucleated oocytes. The embryos were transferred to surrogate female pigs (*523*), which gave birth to CFTR +/- heterozygote pigs; the offspring of heterozygote pigs were then used to generate CFTR -/- pigs.

The mutation Phe508del (deletion of a three base-pair at position 508 in exon 10 of the CFTR gene leading to loss of amino acid phenylalanine) is the commonest CF-associated mutation. A Phe508del target construct was also designed similar to the "null" target construct described in the previous paragraph. Phe508del heterozygote pigs were developed using a similar methodology as used for the production of CFTR +/- pigs. The offspring of heterozygote Phe508del pigs were used to generate homozygous Phe508del mutation pigs. For the current study, only CFTR -/- piglet kidneys were available.

3.1.2.2 Studies on the porcine model of CF

Potential benefits of the pig model of CF include their similarity with humans in various organs regarding anatomical, histological, physiological, biochemical, immune and inflammatory responses. Due to their larger size and longer lifespan, this offers an opportunity to investigate pathogenesis and therapies over time (*523*). The phenotype of CFTR -/- piglets was similar to that of a human newborn with CF (*523*). Abdominal lesions dominated the initial clinical picture and were more severe than those in humans. CFTR -/- piglets showed meconium ileus,

pancreatic destruction, early focal biliary cirrhosis and abnormalities of the gallbladder and bile ducts (*526*). Newborn pig models of CF (CFTR -/- and CFTR Phe508del/Phe508del) revealed partial or total vas deferens and epididymis atresia at birth that were normal in wild-type littermates (*527*). The pig model of CF spontaneously developed hallmark features of CF lung disease including airway inflammation, remodelling, mucus accumulation and infection. The lungs of newborn pig models of CF did not have evidence of inflammation, but there was impaired bacterial elimination within hours of birth, suggesting a defect in the bacterial host defence (*10*). Additionally, tracheal changes were noted in CFTR -/- piglets including a reduced tracheal lumen, which has also been shown in newborn babies with CF (*528*).

So far, mouse models of CF have been used to help understand the pathophysiology of kidney disease in CF including the ion transport and renal handling of fluid and electrolytes (*493,529,530*). CFTR is located in the mouse kidneys in the apical area of proximal tubular cells with maximum intensity in the straight part of proximal tubular cells. Mouse model of CF has been shown to have defective receptor-mediated proximal tubular endocytosis associated with a defective cubilin and significant low molecular proteinuria (*232*).

Kidneys of the pig model of CF have not been tested so far. A study aimed to establish CFTR expression in pigs showed it to resemble largely that of human CFTR expression except for the kidney, brain, and cutaneous glands which lacked CFTR expression (*531*). This is in contrast to both humans and mice where CFTR is abundantly expressed in kidneys. The question of whether this is a true species-specific difference in the expression of CFTR or an issue of assay sensitivity, as suggested by the authors, needs to be addressed. Even if there is not a primary, CF-related defect, considering the lifespan of pigs, the pig model of CF will provide an opportunity to explore complications related to other environmental factors. As renal manifestations in CF are highly influenced by other

factors like use of nephrotoxic drugs, the presence of diabetes, etc., an opportunity to study kidneys before and after nephrotoxic exposure will be valuable to explore the renal pathophysiology in CF.

3.1.3 Role of CFTR in kidneys

The CFTR mRNA is expressed in the kidneys, abundantly in the cortex and outer medulla, but much less in inner medulla (*233*). CFTR was found to be present in the proximal tubule, thin limbs of the loop of Henle, the luminal membrane of distal tubule, cortical collecting duct and the inner medullary collecting duct by immunocytochemistry. It was suggested that the protein was restricted to the apical rather than the basolateral regions of epithelial cells within the apical membrane and in the membrane of the vesicles occupying the apical regions of the cell (*234*).

A splice variant of CFTR, TNR-CFTR, was also shown to be present. This was associated with small endosomal populations and highly expressed in the renal medulla. In vitro functional studies in Xenopus oocytes and mammalian cells showed that TNR-CFTR possessed cAMP-dependent single chloride channel properties like the wild-type CFTR though with lower efficiency (*233*). Its specific function was not very clear but was thought to be involved in vesicular trafficking.

In the apical membrane of epithelial cells, CFTR is known to function as a chloride channel and plays a key role in transepithelial ion transport and fluid movement in various organs including intestine, pancreas, sweat glands and airway epithelia. CFTR also interacts with other channels including ORCC, ENaC, ROMK-2. Furthermore, a variety of intracellular functions have been attributed to CFTR including acting as an intracellular chloride channel, regulating vacuolar acidification and regulating vesicular trafficking of CFTR-containing vesicles and transport of small anions (*532*).

However, the role of CFTR in the renal epithelia is not clear. People with CF have been shown to have impaired salt reabsorption and a reduced

ability to dilute and concentrate urine (*484,533,534*). They are also known to have increased clearance of penicillin and aminoglycoside antibiotics (*327,535*). This has led some groups to question whether there is a primary, CF-specific, renal defect related to CFTR (*195,533*).

Renal sodium clearance of the mouse model of CF (*Cftr*^{tm2cam}) was similar to the wild-type mice, both in control conditions as well as after extracellular volume expansion. There was no difference in GFR and fractional sodium excretion. However, in the event of volume expansion, the mouse model of CF did not significantly increase their GFR in the same way as the wild-type mice and instead, used a different mechanism, i.e. increased fractional excretion of sodium to achieve diuresis. Furthermore, the mouse model of CF displayed an increased amiloride sensitivity compared to the controls thus suggesting that the former rely more on the distal tubule and collecting ducts to increase sodium reabsorption instead of more proximal sites (*494*).

CFTR has also been linked to the regulation of ROMK channel, which is an ATP-sensitive channel, critical for secretion of potassium by the cells of thick ascending limb of the loop of Henle and distal nephron segment (*530*). This implies that potassium secretion would be maintained during water diuresis leading to increased urinary potassium loss, which may contribute to the tendency of CF patients to develop hypokalemic metabolic alkalosis (*536*).

CFTR was suggested to play a role in receptor-mediated endocytosis. CFTR was shown to be located abundantly in the apical area of the terminal part of proximal tubular cells codistributing with Cl^-/H^+ exchanger CLC-5 and Rab5a in endosomes. CFTR deficient mice showed defective receptor-mediated endocytosis evidenced by impaired uptake of ^{125}I-β2- microglobulin, decreased expression of cubilin receptors in the kidneys and significant excretion of cubilin and its low molecular weight ligands into the urine. CF patients also showed low molecular weight

proteinuria (*232*). How CFTR deficiency leads to defective endocytosis and the proposed cubilin instability is not clear, but the resulting proteinuria could be a risk factor for progression of renal disease (*537-539*).

3.1.4 Endocytic receptor proteins

A large amount of proteins including albumin and low molecular weight proteins are present in the glomerular ultrafiltrate. It is reabsorbed by the proximal tubular cells to return the proteins to the blood supply (*540-542*). Absorption of filtered substrates in the renal tubules is a very efficient process in normally functioning kidneys that leaves almost no proteins to excrete in the urine.

As depicted in Figure 3-1, receptor-mediated endocytosis involves two interacting receptors namely cubilin and megalin, which form a complex with a transmembrane protein, AMN. These 3 receptors are located very closely (*543*). Megalin is a 4600 amino acid transmembrane protein with molecular weight of ~517 kDa. It has an extracellular domain (with ligand-binding region), transmembrane domain and a cytoplasmic tail (Figure 3-1). Cubilin is a protein with 3600 amino acids, which has no transmembrane domain. It has a molecular weight ~ 400 kDa. The extracellular domain contains ligand-binding sites. It depends on other receptors for its endocytosis. AMN is a 434 amino acid protein with single transmembrane protein and has a molecular weight of ~46 kDa (*542*).

3.1.4.1 Receptor-mediated endocytosis

AMN binds with cubilin with high affinity and is required for the presence of cubilin at the plasma membrane (*544*). Megalin plays a role in the internalisation of the cubilin-AMN complex (*543,545,546*). Interaction with both megalin and AMN are essential for the intracellular stability of cubilin (*547*). This system is involved in reabsorption of several important substances, e.g. albumin, various low molecular weight proteins and

carrier bound vitamins (vitamins A & D) and trace elements, drugs including aminoglycosides.

The mouse model of CF showed increased excretion of cubilin and its ligands in the urine (*232*). Similarly, in patients with CF, increased urinary loss of low molecular weight protein was documented compared to non-CF controls (*232*). The cause of increased excretion of cubilin is not clear. Lack of AMN expression can be hypothesised to be a potential mechanism to explain the increased loss of cubilin as AMN plays a role in the stability of cubilin.

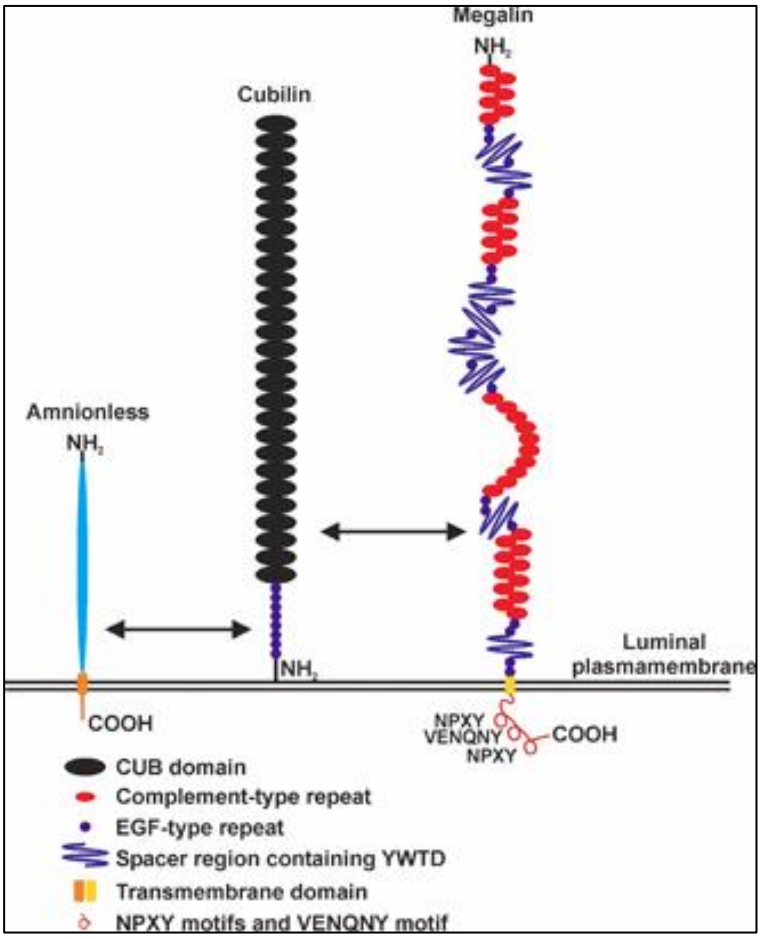


Figure 3-1 Structure of Megalin, Cubilin, and AMN (Reference: Birn 2006, (542)); CUB: cubilin, AMN: amnionless; EGF: epidermal growth factor; YWTD: tyrosine-tryptophan-threonine-aspartic acid; NPXY and VENQNY motifs: short linear amino acid sequences located at the carboxy terminals of endocytic receptors that play a role in internalisation of receptors and sub-cellular sorting and trafficking.

3.2 Aims

I undertook this study with the following objectives:

1. To test the hypothesis that there are no renal histological differences between newborn pigs with genotypes CFTR -/- (CFTR knockout) and pigs with genotypes CFTR +/- (heterozygotes) or CFTR +/+ (wild-type)

2. To compare gene expression of CFTR and endocytic receptors: cubilin, megalin and AMN, between CFTR knockout and CFTR heterozygotes or wild-type piglets by using quantitative polymerase chain reaction (qPCR) technique

3. To determine colocalization of CFTR, cubilin, megalin and AMN by immunohistochemistry

4. To test the hypothesis that there is no increase in urinary loss of low molecular weight proteins (transferrin) and endocytic receptors (cubilin, megalin and AMN) in CFTR knockout piglets compared to CFTR heterozygotes or wild-type piglets

This chapter will explain the basic science theory and experimental procedures used in the study.

3.3 Methods

3.3.1 Study Design

CF pig kidney specimens were used for gene expression experiments, and pig urine samples were used for ELISA (enzyme-linked immunosorbent assay) experiments. The study was designed jointly by Professor Alan Smyth, Professor Michael Symonds and me. I carried out the gene expression and ELISA experiments in the Division of Child Health, Obstetrics & Gynaecology at the University of Nottingham. The experimental methods used were either as recommended by the manufacturers or suppliers or as per the protocols optimised within the Division of Child Health, Obstetrics & Gynaecology at the University of Nottingham. I received training from Dr Mark Pope, Victoria Wilson, Dr Shalini Ojha and Dr Vivek Saroha in conducting the experimental techniques. The histology department at Nottingham University Hospital NHS Trust sectioned the liquid paraffin formalin-fixed tissue. Dr Tom McCulloch (renal histopathologist) and Dr Claire Hawkes (scientist) undertook the staining, histological examination and immunohistochemistry experiments following their local protocols.

3.3.2 Collection and handling of tissue samples

CFTR knockout pigs were genetically engineered and born in the University of Iowa, Iowa, USA. The animal model was developed using principles of SCNT where fetal fibroblasts from wild-type pigs were used. Adenovirus-associated vectors helped transfer an engineered “null” target construct (with a stop codon at exon 10 location of CFTR gene) into the nucleus of fibroblasts by homologous recombination. The resulting cell was then fused with enucleated oocytes to create heterozygote embryo, which was implanted in surrogate female pig to generate heterozygote (CFTR +/-) pig. The offspring of CFTR heterozygote pig were used to produce CFTR -/- piglets.

The piglets were euthanased in the newborn period, and various organs including lungs and intestines were used for experiments in Iowa. Kidneys and urine from these pigs were provided to the Division of Child Health, Obstetrics & Gynaecology at the University of Nottingham for research purposes. Approval for import of animal tissue from overseas was taken from the Department for Environment, Food and Rural Affairs, under the terms of Regulation 4 of the Products of Animal Origin (Third Country Imports) (England) Regulations 2006 (as amended) (*548*), authorisation number:POAO/2011/181. The frozen kidney tissues and urine samples were transferred from Iowa to Nottingham in dry ice containers complying with the International Air Transport Association's (IATA) Dangerous Goods Regulation, 52nd Edition, 2011 (*549,550*), meeting UN1845 requirements. These specimens were stored at -80°C in freezers and kept on dry ice during transit for sampling or processing, to protect the integrity of genetic material. The desired amount of kidney tissue (100-150 mg) was chopped for experiments, and the remaining tissue was returned to -80°C storage. A random code was generated for specimens from each pig using block randomisation method. Each pig was allocated randomly to one of five blocks and assigned a code number (by Dr Andrew Prayle). The scientists (Dr Tom McCulloch, Dr Claire Hawkes and I) were blinded to the identity and genotype of the specimens to avoid any bias. The samples were kept in duly labelled, double plastic bags to prevent any contact with each other.

3.3.3 Experimental Methods –Gene expression analysis

All experiments were performed while wearing gloves and using autoclaved or sterilised equipment including filter pipette tips. The work surfaces, lab shelves and equipment were cleaned with RNaseZap®(RNase decontamination solution, Ambion, California, USA) or 1% Virkon disinfectant (Antec International, Suffolk, UK), and 70% denatured ethanol (Ecolab, Surrey, UK). Sterile nuclease-free water (Ambion Inc, UK) was used for gene expression experiments. While

dealing with hazardous biological agents, experiments were conducted under a laminar flow hood.

On naked eye examination of frozen kidney tissue, it was not considered possible to isolate renal cortex, medulla or tubules; hence, the kidney tissue was homogenised as a whole. Expression of any gene in this experiment, therefore, may be indicative of the presence of the said gene in any cells from porcine kidneys.

For each sample, mRNA was extracted, purified, quantified and stored. Messenger RNA was reverse transcribed into cDNA. Primers were designed for genes of interest and were used for generating standard curves. A classical PCR reaction was conducted to amplify the cDNA using the primers in a reaction mediated by thermostat Taq polymerase enzyme. Gel electrophoresis and DNA extraction were carried out to test the purity of the PCR product and optimisation of the primers. These primers were then used to perform qPCR, using SYBR™ Green I dye as a fluorescence marker. Fluorescence was quantified to indicate the amount of mRNA in relation to the gene of interest in the original sample. The expression levels of various genes in kidney tissues from CFTR -/- pigs were compared to the expression in CFTR +/+ and CFTR +/- pig kidneys.

3.3.3.1 Ribonucleic acid (RNA) extraction

3.3.3.1.1 RNA extraction Principle

Total RNA in the tissue samples was extracted by an adapted version of "the single step method" (*551*) followed by purification and elution using RNeasy plus mini extraction kit (Qiagen, West Sussex, UK) according to manufacturer's protocol. The single step method is a quick and efficient method for isolating total RNA in undegraded form and with little or no contamination with DNA or proteins. Its basic principle involves homogenisation of tissue samples in a monophasic solution of guanidine thiocyanate and phenol, which leads to the dissolution of protein, DNA and RNA in the solution and inhibition of RNase activity. The next step is

the addition of an organic solvent (chloroform), which leads to the formation of three layers; top aqueous layer contains RNA, interphase and the bottom organic layers contain DNA and protein respectively. The aqueous phase is separated and passed through gDNA Eliminator spin column (RNeasy plus mini extraction kit, Qiagen, West Sussex, UK) to ensure efficient elimination of genomic DNA. The aqueous RNA solution is then treated with 70% ethanol and then run through an RNeasy mini spin column (RNeasy plus mini extraction kit, Qiagen, West Sussex, UK). Ethanol provides appropriate condition for RNA to bind the silica membrane in the mini spin columns. These columns have a maximum binding capacity of 100 μg RNA composed of >200 nucleotides in size. A series of washing steps including ethanol and guanidine salt-based buffers are undertaken before the elution of RNA in nuclease-free water.

A spectrophotometer was used to measure the RNA concentration and purity. The principle of spectrophotometry is that the concentration of a solute is proportional to the amount of light absorbed at a particular wavelength. Every substance can absorb or transmit only a certain wavelength of radiant energy. Nucleic acids absorb ultraviolet (UV) light at 260 nm. RNA concentration is proportional to the amount of light absorbed at the 260 nm wavelength (one optical density (OD) unit absorbance at 260 nm=40 μg RNA/ml). Measurement is done at two wavelengths (260 and 280 nm), and relative absorbance value is calculated to determine the purity of the RNA. Proteins absorb UV light at 280 nm, which should be less than the absorbance at 260 nm. A 260/280 ratio ≈2.0 suggests pure RNA. A 260/280 ratio of ≈1.8 is indicative of DNA, and a lower absorption ratio (i.e. stronger absorbance at or near 280 nm) indicates protein contamination (*552,553*). Absorbance ratio at wavelengths 260/230 is expected to be in the range 2-2.2, and lower values may indicate the presence of contaminants that absorb UV light at 230 nm, e.g. EDTA, carbohydrates, phenol, etc. For the purpose of this

study, 260/280 ratio of 1.9 to 2.1 was considered suitable for downstream applications.

3.3.3.1.2 RNA extraction procedure

Porcine kidney tissues (100-150 mg) were chopped for each sample and added to 1 ml TRI Reagent® (Sigma Chemical Co. Poole, UK) individually. The samples were homogenised using a Dispomix homogeniser (Medic Tools, Zurich, Switzerland) at 3000 rpm and then centrifuged at 4000 rpm for 1 min at 4°C. The homogenate was transferred to a sterile 1.5 ml Eppendorf tube, and 200 µl of analytical grade chloroform (Fisher Scientific, Leicestershire, UK) was added and mixed by inversion. This was incubated for 10 min at room temperature followed by centrifugation at 13,000 rpm for 15 min at 4°C temperature. The homogenate separated into 3 phases; the top layer containing RNA was carefully aspirated without disturbing the interphase and the lower phase that were discarded. The aspirate was pipetted on a gDNA eliminator column, which was then centrifuged at 13,000 rpm for 30 sec at room temperature, and the column was then discarded. An equal volume of 70% ethanol was added to the remaining solution and mixed with the pipette.

The solution was transferred to an RNeasy mini spin column (maximum 700 µl at each step) and centrifuged at 13,000 rpm for 15 sec at room temperature, and the flow-through was discarded; the process was repeated if there was any residual sample volume. Then, 700 µl of RW1 buffer was added to the column and centrifuged at 13,000 rpm for 15 sec, and, the flow-through was discarded. After that, 500 µl of RPE buffer was added to the column, centrifuged at 13,000 rpm for 15 sec, and, the flow-through was discarded; this process was repeated once. The Mini spin column was then transferred to a new tube and spun again at 13,000 rpm for 1 min to eliminate any RPE buffer that could affect the results. The column was transferred to another 1.5 ml Eppendorf tube and 50 µl of nuclease-free water was added to the column, centrifuged at 13,000

rpm for 1 min, and the solution was aspirated. This step was repeated once to increase the RNA concentration.

3.3.3.2 Measurement of RNA concentration and purity

RNA concentration and purity were measured using Nanodrop®ND-1000 (Nanodrop Technologies, Wilmington, USA) spectrophotometer, selecting RNA 40 constant (1 OD=40), using nuclease-free water for blank measurement (0.0), and using 1 µl of RNA elute. The final RNA concentration was determined by taking an average of three readings. A 20 µl solution of 1 µg/µl RNA concentration was prepared with nuclease-free water to be used for RT-PCR (reverse transcriptase polymerase chain reaction) analysis with an aim to reduce the freeze-thawing of the stock RNA solution. Both the solutions were stored at -80°C in an RNA freezer until used for further analysis.

3.3.3.3 Reverse Transcription

3.3.3.3.1 Reverse Transcription Principle

This is a reverse transcriptase (RT) enzyme-mediated process of transcription of single-stranded RNA (mRNA) into cDNA. This allows detection of a small number of mRNA molecules following amplification of the DNA in a PCR. Reverse transcription is a crucial step for the accuracy of PCR as any amount of cDNA generated reflects the amount of input mRNA. Several different types of enzymes are available with most being the engineered forms of enzymes derived from Moloney Murine Leukemia Virus (MMLV) and Avian Myeloblastosis Virus (AMV) (*554*) which have different efficiencies. It is recommended to use same experimental conditions and protocols for comparable results.

The reaction requires the addition of primers (*555*). There are three types of primers including oligo (dT) primers, random sequence primers and gene-specific primers. An oligo (dT) primer anneals to the poly (A) tail of mRNA and initiates reverse transcription from (3') end of RNA. Poly (A) tails are found on most RNA molecules but absent from certain RNAs e.g. prokaryotic genes, and may be lost with RNA degradation. Random sequence primers are short oligomers of all possible sequences generated randomly and can anneal throughout the target RNA sequence, thus producing higher cDNA yield. In this study, I used High Capacity RNA to cDNA kit (Applied Biosystems, CA, USA), which incorporates all the components of the reactions into two tubes, thus giving an advantage of fewer pipetting steps (fewer chances of error and improved performance), and has short reaction time. One of the tubes has RT Buffer mix, which contains oligo dT primers, random octamer polymers and deoxyribonucleoside triphosphate (dNTPs) mix (a premixed solution containing sodium salts of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate) to provide building blocks for DNA production. The other tube has an enzyme mix containing MuLV and RNase inhibitor protein

(inhibition of RNase activity in the enzyme is crucial to obtain higher DNA yield).

3.3.3.3.2 Reverse Transcription Procedure

A high Capacity RNA to cDNA kit (Applied Biosystems, CA, USA) was used. Eppendorf tubes (0.2 ml) were labelled for each specimen and the controls. The tubes were placed on Eppendorf PCR cooler (Sigma-Aldrich) before adding the reagents. A total reaction volume of 20 µl was made up using 10 µl of RT buffer mix, 1 µl of RT enzyme mix, 1 µg mRNA (1 µg/µl), and 8 µl nuclease-free water. A 'no reverse transcriptase enzyme control (NRTC, contains every reagent and RNA sample excluding the enzyme) and a no template negative control (NTC, contains all the reagents but no RNA sample) were run concurrently to ensure that RNA was free of genomic DNA and that the reagents were not contaminated respectively. For NRTC and NTC reactions, total volume was made up to 20 µl by adding 1 µl of nuclease-free water instead of RT enzyme mix and RNA sample respectively.

After centrifugation at 13,000 rpm for 1 min to spin down the contents, the plates were transferred to Techne Touchgene Gradient thermal cycler (Techne Incorporated, NJ, USA). The reverse transcription included incubation of the reaction mix at 37°C for 60 minutes for transcription of RNA to cDNA, and then heating to 95°c for 5 min to stop the reaction (for denaturation of double-stranded DNA to single-stranded DNA) and then held at 4°c. The cDNA product was stored in a freezer at -20°c).

3.3.3.4 Primer Design

A primer is a short sequence of nucleotide bases; forward and reverse primers are runs of nucleotide bases complementary to the two opposite strands of DNA and are the starting and finishing points for the imprinting of the new DNA. Designing a primer is a crucial step for PCR experiments. The success of a PCR is defined by its specificity and efficiency, and it is important to achieve a balance between the two to attain the best results from the experiments. In an ideal scenario, only the correct product should be synthesised, and after each PCR cycle, the full length of target molecules will be doubled (efficiency 100%).

A primer determines the length of the PCR product, its melting temperature (Tm) and the final yield of the reaction. A poorly designed primer can affect the specificity of the reaction by formation of a wrong product or no product at all, the formation of a secondary structure or primer dimers. This can contribute to the reduced PCR efficiency. Various criteria help select a primer like its length, annealing temperature, guanine (G) –cytosine (C) content (the number of Gs and Cs in the primer as a percentage of the total number of bases), its location on the target sequence and the length of the amplicon (final product of PCR).

In general, oligonucleotides between 18-24 bp usually work well and tend to be very sequence specific (*556*). A reasonable guanosine cytosine (GC) content, between 45-60% is required to ensure optimal melting temperature, Tm (Tm=2(A+T) + 4(G+C), A=adenosine, T=thiamine) between 52-58°c. Melting temperature is the temperature at which the two strands of the DNA separate (by breaking of the hydrogen bonds) yielding single-stranded DNA. A Tm of more than 65°c should be avoided due to a potential of formation of secondary structures at higher Tm (*557*). While optimising primers, utilisation of shortest primers that require an annealing temperature of >54°c provide the best chances of specificity and efficiency.

The length of the sequence amplified during PCR (PCR targets or amplicons) is kept short, generally between 60-200 bp as shorter amplicons are more likely to result in the complete synthesis of a product within a given cycle in comparison to a much longer amplicon. The amplicons should span an exon-exon boundary to avoid amplification of genomic DNA taking advantage of the fact that introns are present in genomic DNA but are absent in mRNA.

Forward and reverse primers should not be complementary to each other particularly at their 3'ends. Secondary structures or primer dimers can be a result of an interaction between forward and reverse primers, a primer folding on it, forward-forward or reverse-reverse primer annealing, etc. Primer-dimers are more likely to form in reactions with a lower amount of template (higher dilutions) or negative template controls. This can lead to false positive results and can affect the efficiency of the reaction by creating competition among reaction components.

A traditional method for detection of primer-dimers is gel electrophoresis, where they appear as diffuse smudgy bands near the bottom of the gel. Contamination with genomic DNA will show a separate band, which migrates less than the cDNA. Primer-dimers can also be identified in the melt curve analysis at the end of a qPCR, as they have a different melting temperature to the target amplicon. If any primer-dimer peak is present only in the non-template control but absent in the template curves, these may not affect the PCR efficiency.

Various bioinformatics programs are available to help with primer selection from a transcript, generally conforming to certain basic rules. In this study, I designed primers (Table 3-1) for genes of interest using Primer3 plus website (www.bioinformatics.nl/primer3plus/) (*558*), using the pig genome sequence from Ensembl online database (*559*) version 66-67. Primers for the reference genes were either designed in the same way (beta-actin (ACTB)) or sequences that were previously published in

the literature (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)) or were previously optimised in the Division of Child Health, Obstetrics & Gynaecology at the University of Nottingham (Importin 8 (IPO8); ribosomal phosphoprotein (RPO) and 18S ribosomal RNA (18S)) were used (please refer to Table 3-2). These oligonucleotide sequences were then checked for their specificity to ensure that they bind to the correct region of the pig genome (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (*560*).

The oligonucleotides were purchased from Sigma-Aldrich, Dorset, UK. They were suspended with an appropriate volume of nuclease-free water to prepare a stock solution of 100 µmol/L concentration and stored at -20°c.

Table 3-1 Primers used in the study for genes of interest					
Target gene	**Target sequence (Forward)**	**Target sequence2 (Reverse)**	**Annealing temperature (°C)**	**Amplicon length (Base pair)**	**Source sequence accession no.**
Amnionless (AMN)	CCGATAACTGGAGCCAGAAC	ATCCGAGATGCTGTGACCTT	60	107	ENSSSCT00000002803
CFTR	ACAGTTGTTGGCAGTTGCTG	ACCCTCTGAAGGCTCCAGTT	60	88	ENSSSCT00000018101
Megalin	CAGAGATGGTTGCTTCACCA	GCGAATCGTTTCAGAGTTCC	60	128	ENST00000263816
Cubilin like mRNA (Cubilin)	GGGCTTTAACGCATCCTTTC	GTGCCAAGAGCAGTTGAGGT	61	118	ENSSSCT00000012072

CFTR: cystic fibrosis transmembrane conductance regulator; mRNA: messenger ribonucleic acid

Table 3-2 Primers used in the study for reference genes					
Target genes	**Target sequence (Forward)**	**Target sequence (Reverse)**	**Annealing temperature (°C)**	**Amplicon length (Base pair)**	**Source sequence accession number**
Importin 8 (IPO8)	GCCCTTGCTCTTCAGTCATT	GTGCAACAGCTCCTGCATAA	52	93	NM_001190995.1 Pope, et al (2014) (*561*)
Beta-Actin (ACTB)	TCCCTGGAGAAGAGCTACGA	CATGATCGAGTTGAAGGTGGT	60	147	ENSSSCT00000025260
YWHAZ*	TGTAGGAGCCCGTAGGTCATCT	TTCTCTCTGTATTCTCGAGCCATCT	59	102	Garcia-Crespo D, et al (2006), (*562*)
Ribosomal Phosphoprotein (RPO)	CAGCAAGTGGGAAGGTGTAATC	CCCATTCTATCATCAACGGGTA	60	74	NM_001098598 Almond, et al (2015) (*563*)
18S Ribosomal RNA	GATGCGGCGGCGTTATTC	CTCCTGGTGGTGCCCTCC	59	125	AK2372246, Almond, et al (2014) (*563*)

YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

3.3.3.5 Principle of PCR

PCR is a method of amplifying particular segments of DNA/RNA in vitro. This process uses the enzyme DNA polymerase that directs the synthesis of a DNA using deoxynucleotide substrates on a single-stranded DNA template.

Steps of a basic PCR reaction (Figure 3-2):

1. Denaturation: Splitting of the original double-stranded DNA (dsDNA) to single strands takes place by heating the template to 94°C.
2. Annealing: Binding of primers to their complementary sequences on the template strands takes place after cooling the reaction to a certain temperature (usually between 50-70°C).
3. Extension: Heating the reaction to 72°C (optimum temperature for DNA polymerase to act) allows the reaction of the enzyme leading to the addition of nucleotides to single-stranded template DNA and formation of dsDNA. Use of a heat stable DNA polymerase enzyme (Taq polymerase) allows PCR reaction to continue without the need to add enzyme after each round of amplification.

In each cycle of PCR, a single segment of dsDNA template is amplified to two separate pieces of dsDNAs. These are then used as template strands for the next cycle thus generating duplicate copies in an exponential manner. This process is limited by the amount of substrates in the reaction. If PCR is repeated for x number of cycles then, in an ideal situation, the final amount of DNA will be 2^x multiplied by the number of original copies of DNA present at the initiation of the reaction. This final amount is representative of the

amount of cDNA and hence, of the number of intact mRNA molecules of this gene present in the tissue of interest used for mRNA extraction.

3.3.3.5.1 Technique of classical PCR

Conventional PCR was performed for optimisation of primers and for amplification of target cDNA, which was subsequently used to prepare standard dilutions for qPCR reaction. A set of Eppendorf tubes, including a 'no template' negative control for each gene, were labelled. The reaction was carried out on a PCR cooler using cDNA from 3 different specimens. A total reaction volume of 20 µl was used. For each reaction, PCR master mix was made with 10 µl of Thermostart PCR master mix and 4 µl of 1:40 diluted forward and reverse primers each. The thermostart PCR master mix (Thermo Scientific, Abgene, Epsom, UK) contains nucleotides and thermostart Taq DNA polymerase enzyme that is thermosensitive; it is inactive at room temperature and activates on heating up to 96°c for 15 min. To the whole 18 µl master mix, 2 µl of cDNA (1:10 diluted in nuclease-free water) for templates or 2 µl of nuclease-free water (for negative control) was added. The PCR plate was centrifuged up to 13,000 rpm for 1 min and then loaded into Techne thermal cycler and ran on a 60°C hot start PCR program. The steps of PCR program are as shown in Table 3-3.

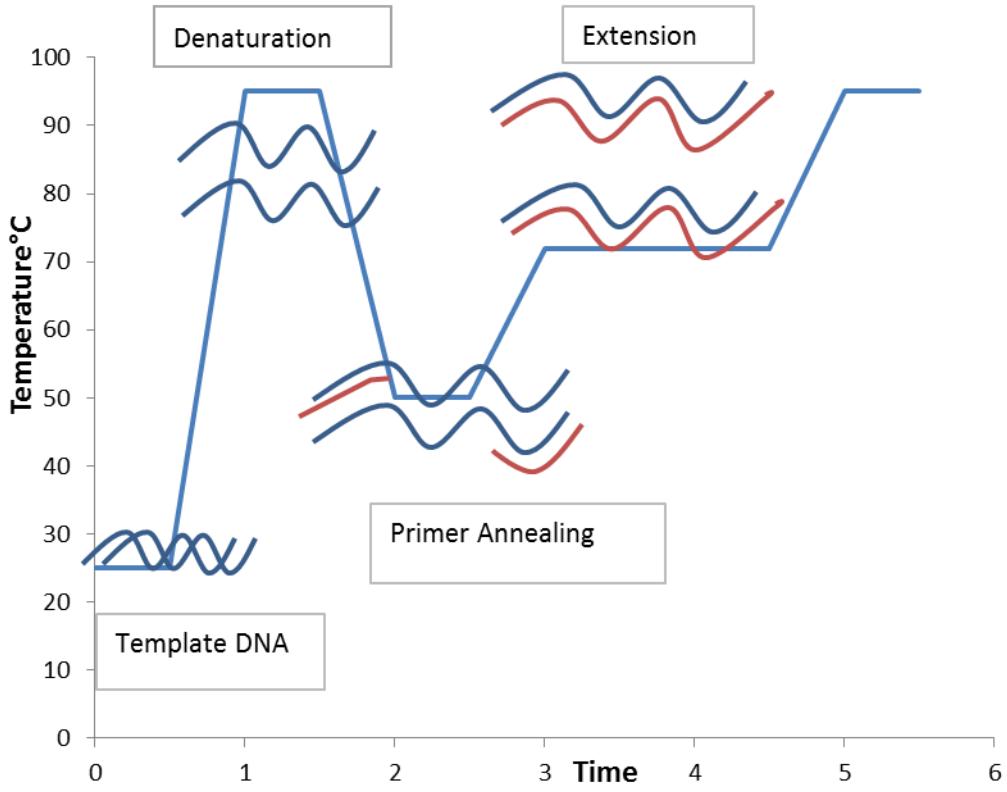


Figure 3-2 PCR (polymerase chain reaction) principle; change of temperature and 3 stages of PCR: denaturation, annealing and extension; DNA: deoxyribonucleic acid

Table 3-3 Hot start PCR program conditions			
Step	**Process**	**Temperature (°C)**	**Duration (mins)**
Step 1	Initiation	105	4
Step 2	Enzyme activation	96	15
Steps 3-5 (45 cycles)			
Step 3	Denaturing cDNA strands	94	0.5
Step 4	Annealing	60	0.5
Step 5	Extension	72	1
Step 6 (single cycle)			
Step 6	Final extension	72	7
Final Hold	Hold	4	Till stored at 20°C

PCR: polymerase chain reaction; cDNA: complementary deoxyribonucleic acid; mins: minutes

3.3.3.6 Gel electrophoresis and DNA extraction

3.3.3.6.1 Principle

Agarose gel electrophoresis is an efficient way of separating DNA fragments of varying sizes. When set in a gel form, agarose polymers form a network of bundles and act like a sieve for nucleic acids. For gel electrophoresis, DNA is loaded into preformed wells in the gel. On application of electric current, DNA molecules (negative charged) migrate towards the positively charged anode. The distance travelled is inversely proportional to the log of molecular weight of DNA. Thus, molecules of different weights are separated. This allows separation of DNA molecules between sizes 100 bp and 25 kb. Separated DNA bands can be stained with a dye (commonly ethidium bromide). The dye fluoresces under UV light allowing visualisation of the DNA bands. DNA standards (DNA ladder) are used to compare and determine the size of DNA fragments. Gel bands with DNA are cut and then commercial kits are used for DNA extraction. QIAquick ® Spin Extraction kit uses high DNA affinity silica membrane column to separate DNA. This is followed by several steps of washing with buffers to eliminate contamination by the gel. Purified DNA can be eluted in nuclease-free water or elution buffer, and concentration can be determined using a spectrophotometer.

3.3.3.6.2 Technique

A 2% (w/v) agarose gel was prepared by dissolving 2 g of agarose (electrophoresis grade (Invitrogen Life Technologies) in 100 ml Tris-acetate-EDTA (TAE) buffer. This buffer was taken from a 50X stock solution of 2 M tris (hydroxymethyl) aminomethane base (TRIS), 1M glacial acetic acid (Fisher Scientific) and 50 mM Na_2EDTA buffer. The solution was heated in a microwave for about 3 minutes to mix, and then 0.5 µl/ml of Ethidium Bromide (10 mg/ml) dye was added. The solution was cooled and allowed to set as a gel for about 20-30 min

with a comb inside to form the wells. The gel was transferred to the electrophoresis chamber (filled with TAE buffer) that can be connected to electrodes to provide electrical fields.

PCR products (20 µl) were individually mixed with 5 µl gel loading buffer (0.0016% v/v saturated bromophenol blue, 0.15%(w/v) EDTA and 20% (w/v) glycerol. This mixture was loaded into the wells of the gel. In addition, 5 µl DNA ladder (100 bp; Blue eXtended DNA Ladder, BIORON GmbH, Ludwigshafen, Germany) was loaded on the side wells for comparison.

Electrophoresis was run at 100 volts for about 35 minutes (BIORAD Powerpac 300 (Bio-Rad Laboratories, Hemel Hempstead, UK). The gel was then visualised under a UV transilluminator camera (Fujifilm Luminescent image analyser LAS-1000 v 1.01) using a UV lens for imaging. Fluorescent gel bands were identified, photographed and their size was estimated by comparison with DNA ladder. Gel bands specific to the size of PCR product were cut and transferred to sterile 1.5 ml Eppendorf tubes. These gels were used for DNA extraction using a QIAquick® gel extraction kit (Qiagen).

The gel bands were weighed, dissolved in 300 µl GQ buffer per 100 µg of gel and heated at 50°c for 10 min while being vortexed every 2-3 min to mix properly. Next, 100 µl isopropanol (Fisher Scientific) was added per 100 µg weight of original gel band, vortexed and then transferred to QIAquick spin column. The columns were centrifuged at 13,000 rpm for 1 min to precipitate the DNA onto it and the flow through with the impurities was discarded. For further purification, 500 µl GQ buffer was added to the column and centrifuged at 13,000 rpm for 1 min. To remove any impurities, 750 µl PE buffer containing ethanol was added to the column and allowed to stand for 4 min and then, centrifuged at 13,000 rpm for 1 min. The column was then

placed in new 1.5 ml Eppendorf tubes, and 30 µl elution buffer was added to elute the DNA. The tubes were centrifuged at 13,000 rpm for 1 min, and the column was discarded. The DNA concentration of elutes was measured by spectrophotometry with a Nanodrop (as described in Section 3.3.3.2). Elution buffer was used for blank measurement. An average of three readings was taken as the final DNA concentration that was used for the preparation of standards of the qPCR reactions. The DNA samples were stored at -20°c until used in downstream applications. Sample from cDNA (for CFTR) was sent for DNA sequencing to the Centre for Genetics and Genomics, the University of Nottingham (based at Queen's Medical Centre, Nottingham) for cross-reference against the online Ensembl database (*559*).

3.3.3.7 Quantitative (Real Time) PCR

3.3.3.7.1 QPCR Principle

In qPCR, detection and quantification of nucleic acid are done in real time i.e. as the reaction progresses thus eliminating the need for additional steps. This technique uses a fluorescent reporter that binds to the PCR product (dsDNA) and reports its presence by fluorescence. There is a baseline fluorescence, which cannot be differentiated from fluorescence generated by initial cycles. The level of fluorescence increases as the reaction progresses leading to the doubling of reaction products after each cycle. The cycle when the fluorescence exceeds a "chosen" threshold level above the background fluorescence is called "cycle threshold (Ct)" or "crossing point (Cp)". Cycle threshold is used for quantification of DNA, and it correlates with the initial amount of target template.

The PCR reaction is depicted graphically as a sigmoidal curve. The exponential amplification of fluorescence, when there is doubling of

PCR products after each cycle, is followed by linear phase, when the reaction starts to slow down, and plateau phase that depicts saturation of the reaction due to running out of reaction components (Figure 3-3). The exponential curves are supposed to be parallel in the growth phase and are separated according to the initial amount of template (higher the initial concentration, earlier the curve). The difference can be quantified by the number of cycles required to reach the Ct value (compare with classical PCR, where measurement is taken after the end of the reaction, and no quantification is possible as all the reaction curves saturate at the same level).

Fluorescence reporting is done by using sequence-specific probes or nonspecific labels such as dyes. Asymmetric cyanine dye, SYBR™ Green I is a commonly used dye for reporting fluorescence. There is no fluorescence when the dye is free in the solution, but it becomes brightly fluorescent when it binds to DNA. However, the dye gives rise to fluorescence in the presence of any dsDNA including primer dimers, which may lead to erroneous results. It can be checked by undertaking melt curve analysis. After the PCR cycle is complete, the temperature of the reaction is gradually increased, and fluorescence is measured against temperature. When the reaction reaches Tm, dsDNA separates to single-stranded DNA, and the dye comes off suddenly leading to an abrupt drop in fluorescence.

Tm is influenced by length and sequence base; hence, the melt curve of one product produces a single peak. Primer dimer products are shorter than PCR products and melt at a lower temperature generating a separate peak in melt curve analysis. DNA sequencing of the product after gel electrophoresis allows further qualitative analysis of PCR products.

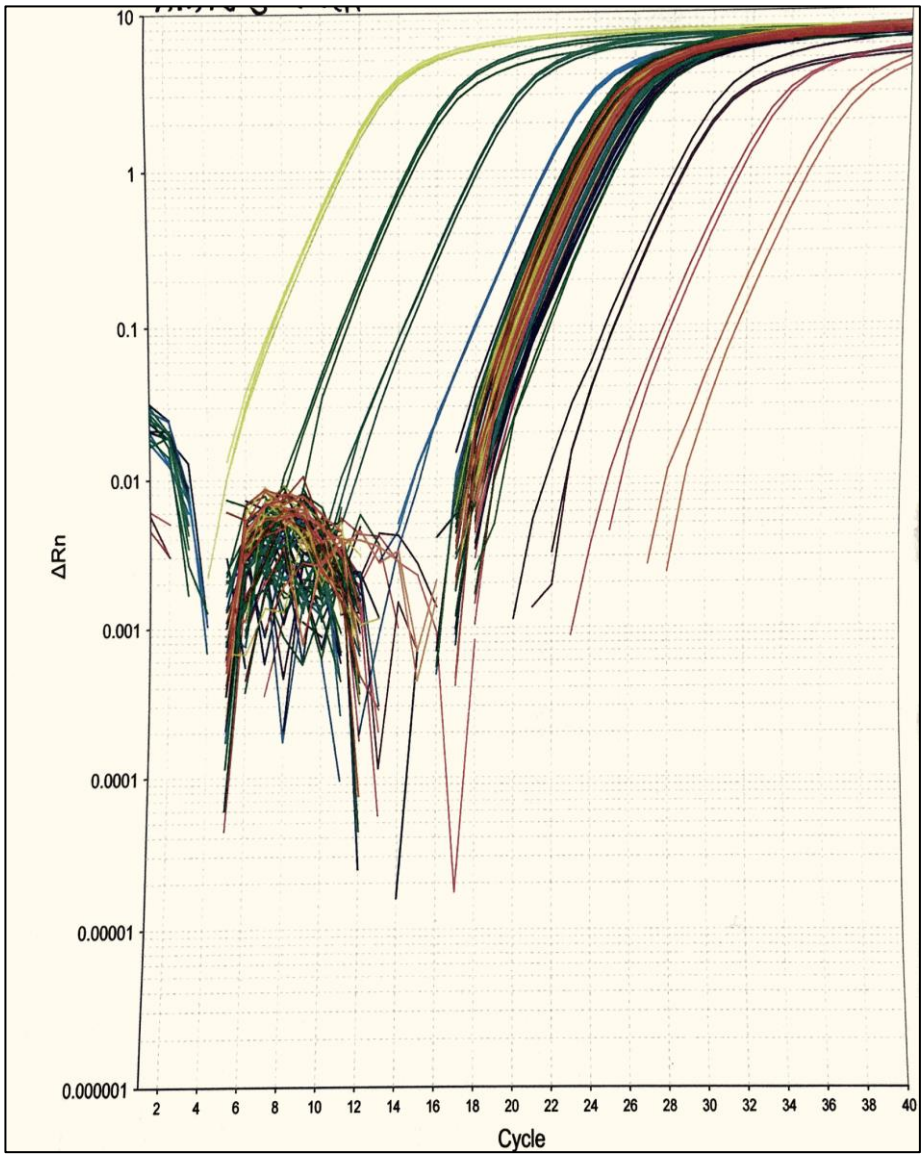


Figure 3-3 Log view of qPCR amplification plot showing fluorescence (ΔRn) on y- axis against increasing cycles of PCR on x-axis; with a higher concentration of cDNA (complementary deoxyribonucleic acid), less number of cycles are required to cross a threshold value; towards the end, amplification reaches a plateau phase which depicts saturation of the reaction; Rn is fluorescence of the reporter dye normalised to the fluorescence of a passive reference dye which is used to correct well to well non-PCR fluctuation; ΔRn depicts Rn (fluorescence at a particular point) minus the baseline fluorescence; qPCR: quantitative polymerase chain reaction

3.3.3.7.2 QPCR method

QPCR was performed using Step One Plus Real Time PCR System (Applied Biosystems). For every gene analysed, standard serial dilutions were prepared to start from 10^{-1} to 10^{-8} concentration using a cDNA template of 1 ng/µL concentration. As a first step to the reaction, to establish the transcription efficiency, standards were run in PCR instrument along with two samples and two negative controls (cDNA replaced by nuclease-free water). In the next stage, PCR was run on a 96 well plate (Applied Biosystems, ThermoFisher Scientific, Waltham, USA) including all standards (10^{-1} to 10^{-8}), unknown samples and NTC. All the samples and negative controls were run in duplicate, and the coefficient of variation (CoV) was calculated within each experiment. All the standards were run in duplicate for cubilin and in triplicate for AMN, CFTR and megalin. Samples with CoV >5% were excluded from analysis. Fast SYBR™ Green Mastermix (Applied Biosystems, ThermoFisher Scientific, USA) was used that contained the following:

SYBR™ Green I Dye, AmpliTaq® Fast DNA Polymerase UP (ultrapure), Uracil DNA Glycosylase (UDG), dNTPs, ROX™ dye passive reference, optimised buffer components.

Taq polymerase enzyme allows the instant hot start of the PCR reaction. UDG treatment prevents reamplification of the carryover PCR products by removing any Uracil in the amplicons. The ROX™ dye provides a passive internal reference against which the reporter dye (SYBR™ Green I) signals are normalised during data analysis. This is necessary to correct for fluorescence fluctuations due to changes in concentration or volume.

Each well of PCR plate reaction was prepared as per Table 3-4. The PCR plates were sealed by MicroAmp Optical Adhesive Films (Applied Biosystems). The reaction was run on StepOnePlus Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, Waltham, USA). The qPCR steps are shown in Table 3-5.

Table 3-4 The components of qPCR reaction mix for each PCR reaction

Component	Volume for one reaction (10 µL)
Fast SYBR™ Green Master Mix*	5 µL
Forward and Reverse primers (1:40)	1 µL each
cDNA template / Nuclease-Free water (NTC)	3 µL
Total volume	10 µL

: Power SYBR™ Green Master Mix was used for CFTR (cystic fibrosis transmembrane conductance regulator) detection; other reaction conditions were the same; qPCR: quantitative polymerase chain reaction, cDNA: complementary deoxyribonucleic acid; NTC: no template negative control

Table 3-5 The qPCR steps including melt curve analysis			
Process	**Temperature (°C)**	**Duration (Seconds)**	**No. of Cycles**
DNA polymerase activation	95°C	20 Sec.	Hold
Denaturation	95°C	3 Sec	40 cycles
Annealing	60°C	30 Sec	
Melt curve	65° C to 90°C in 1°C increments	15 min	Incremental stages, holding for 1 min at each step

QPCR: quantitative polymerase chain reaction; DNA: deoxyribonucleic acid; No.: number; min: minutes; sec: second

During the qPCR cycles, there is no additional step for primer extension; it occurs while the reaction temperature increases from 60°C (annealing temperature) to 72°C before the start of next cycle. Fluorescence is measured at the end of each cycle. After the set number of cycles are completed, the temperature is increased in steps by 1°C at each step followed by a hold for 10 seconds until the temperature increases from 65° to 95°C. Fluorescence is measured at each step allowing for the formation of melt curves.

QPCR analysis was done using the Step One™ Version 2.2 Software Package (Applied Biosystems). Experiments with R^2 value >0.990 were included. The measured Ct values were used to calculate relative gene expression and normalisation was undertaken by the geNorm software package (*564*).

3.3.3.7.3 Relative quantitation of mRNA (Standard curve method)

Relative standard curve method is the most commonly used technique for relative quantitation of DNA. It involves preparing serial dilutions of a known concentration of target DNA. The known standards are run on the same 96 well plate as other unknown or control samples. Plotting the log concentration of standards (x-axis) against their Ct values (y-axis) creates a linear graph (Figure 3-4)**,** which can be used to calculate relative quantities of genes of interest. The standard curve also helps establish the efficiency of PCR amplification (E) by calculating the slope of the graph and calculates the correlation coefficient.

PCR efficiency (E) can be calculated by the following formula:

PCR efficiency:

$$PCR\ Efficiency\ (E) = 10^{[-\frac{1}{slope}]}$$

Equation 3-1

For an ideal situation, with doubling of the PCR product in each cycle, PCR efficiency is 100% (amplification factor=2), and the slope of the linear graph is 3.322. The correlation coefficient is calculated as another quality control measure suggesting linearity of the standard curve.

Relative expression of Cp values was calculated by Delta-Cp method where Cp value of genes is compared with the control sample with highest expression (or lowest Cp value) (*565*):

$$Delta\ Cp = E\text{^}(control\ Cp - Sample\ Cp)$$

Equation 3-2

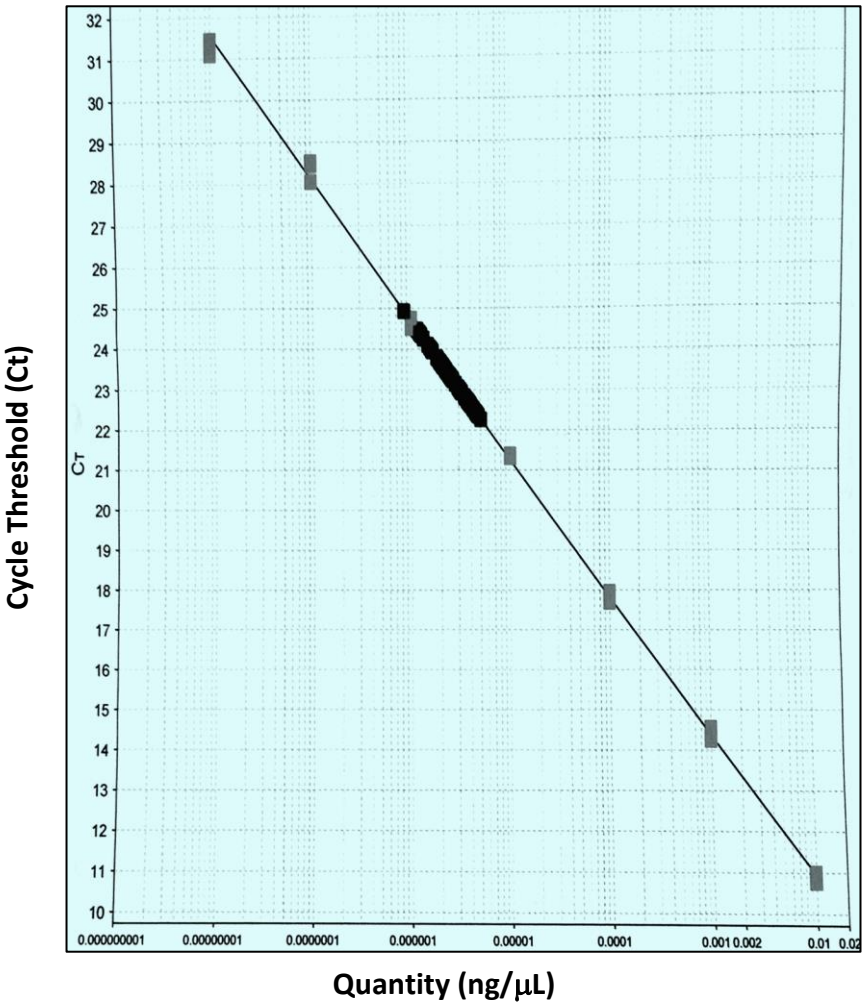


Figure 3-4 Standard curve of a PCR reaction; cycle threshold (Ct) values on y-axis and standard cDNA quantity on x-axis (ng/µL) ranges from 10^{-2} to 10^{-9}; R^2=0.999, Slope=-3.436, PCR Efficiency=95.5%; PCR: polymerase chain reaction; cDNA: complementary deoxyribonucleic acid

3.3.3.8 Selection of reference genes and normalisation of gene expression

For any gene expression study, it is vital to normalise the samples to correct for differences in the initial RNA abundance. Reference genes (housekeeping genes) are genes that are expressed at constantly high levels in all the cells and are not affected by the treatment in question. Several reference genes are tried, and the most stable ones are selected for normalisation. For this study, five reference genes were trialled, and 3 of these were used for normalisation. Visual basic application (VBA) in Microsoft Excel (geNorm version 3.5 (*564*)) was used to select the most stable reference genes based on gene stability measure (M value) (please refer to Figure 3-5). The gene stability measure is based on a principle that the expression ratio of two ideal internal control genes is identical in all the samples. Increasing variation in the expression ratio of the samples suggests decreasing expression stability, i.e. either or both of the genes are not consistently expressed. For each reference gene, pairwise variation is calculated with all other reference genes as the SD of log-transformed expression ratio. Average pairwise variation of any particular gene with all other control genes is defined as gene stability measure (M value). VBA geNorm software eliminates the worst scoring reference gene in a stepwise manner and ranks the reference genes according to their expression stability. It also calculates the systematic variation, i.e. machine, enzyme, pipetting variation, etc. as V value for repeated PCR on the same gene.

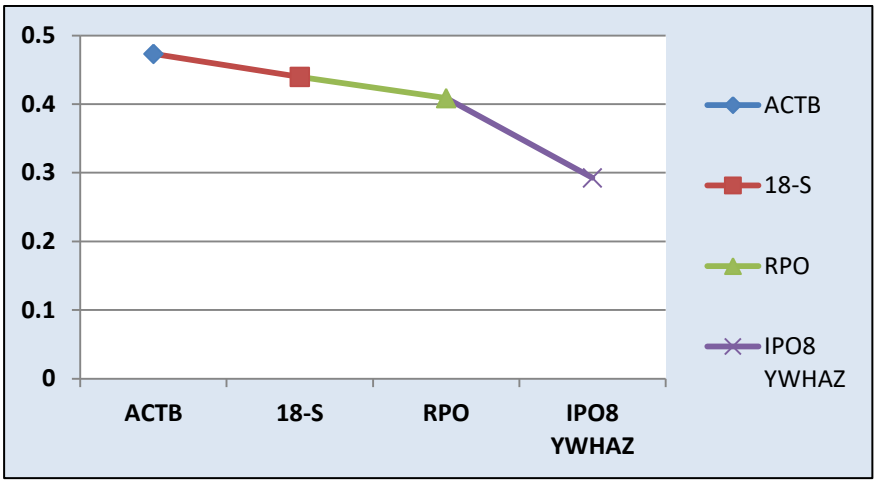
A normalisation factor based on the geometric mean of the best-performing reference genes is provided by geNorm software (*564*). Geometric mean provides better control for outlying values and abundance differences between different reference genes compared to the arithmetic mean. It is recommended to use multiple reference

genes for normalisation, starting with minimal three reference genes followed by stepwise inclusion of more control genes until the addition of more genes does not significantly alter the newly calculated normalisation factor. A pairwise variation of 0.15 is suggested as a cut-off value below which addition of any more reference genes is not required (Figure 3-6).

The normalisation of relative expression of genes is achieved by the following formula:

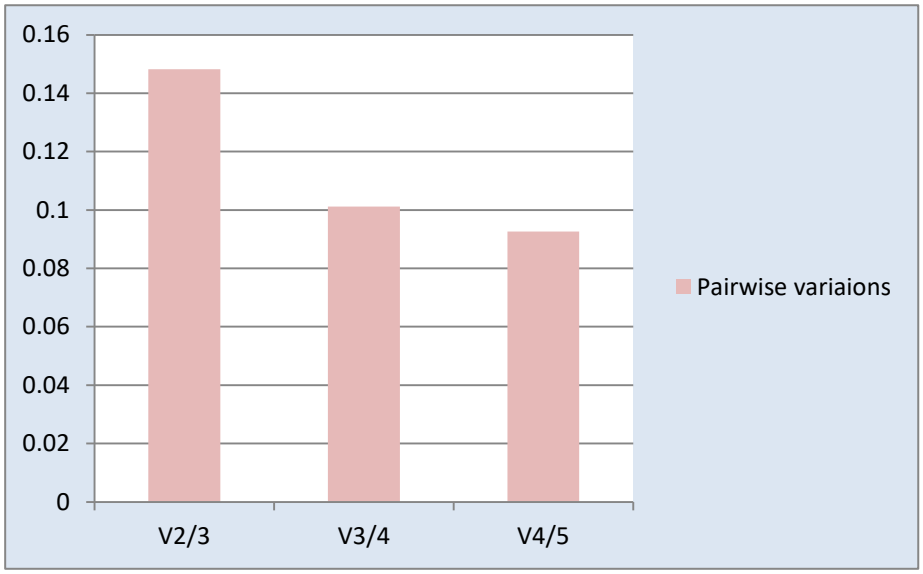
$$Normalised\ relative\ gene\ expression = \frac{Delta\ \ Cp}{Normalisation\ factor\ for\ reference\ genes}$$

Equation 3-3



ACTB	18S	RPO	IPO8 YWHAZ
0.473163	0.439733	0.408748	0.292499

Figure 3-5 Average expression stability (M value) for reference genes; genes with lower M values are more stable; ACTB: beta-actin; 18S: 18S ribosomal RNA; RPO: ribosomal phosphoprotein; IPO8: importin 8; YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide



V2/3	V3/4	V4/5
0.148219	0.101162	0.092608

V1/2=YWHAZ/IPO8
V2/3=YWHAZ-IPO8/RPO
V3/4=YWHAZ-IPO8-RPO/18S
V4/5=YWHAZ-IPO8-RPO-18S/ACBT

Figure 3-6 Pairwise variations to select reference genes ; ACTB: beta-actin; 18S: 18S ribosomal RNA; RPO: ribosomal phosphoprotein; IPO8: importin 8; YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

3.3.4 Experimental Methods: Histology

Histology is the study of microscopic anatomy and architecture of sections of tissues by use of staining. Formalin-fixed and paraffin-embedded tissues are sectioned with a microtome, and the morphology is enhanced by the use of various stains to visualise the tissues. Formalin-fixed pig kidney tissue was provided to the histology department at Nottingham University Hospital NHS trust. Sectioning and Haematoxylin & Eosin (H&E) staining were undertaken in the histology department. Dr. Tom McCulloch (Consultant Renal Histopathologist) performed histological examination as collaboration. The specimen identities were blinded from the investigator.

3.3.4.1 Sectioning Protocol

1. Excess paraffin wax was scraped from the sides of blocks.
2. Block was carefully trimmed until tissue surface was revealed.
3. The block was placed on ice to cool down.
4. Sections of 2 µm thickness were cut from each block onto slides.
5. Sections were allowed to dry for 30 min before staining.

3.3.4.2 H&E Staining Principle

H&E staining is one of the most commonly used methods for tissue staining. Haematoxylin itself is not a dye and does not directly stain the tissue. It is used in combination with a mordant (a compound that helps it bind to the tissues), generally a metal cation, such as Aluminium. When oxidised haematoxylin (haematein) binds to the mordant, it becomes positively charged and binds to the negatively charged, basophilic elements of tissues (including nucleic acids in the nuclei), which gives a blue colour. Eosin is a negatively charged dye that stains the positively charged eosinophilic components of tissues, e.g. cytoplasm. It provides pink colour to the tissue.

The staining protocol involves initially over staining the tissue with haematoxylin and then by process of differentiation using acidic solutions, removing excess background staining. Bluing by use of alkaline solution converts the colouration of nuclei from reddish purple to crisp blue purple and enhances the contrast. The next step is eosin staining, which is differentiated by washing with tap water.

3.3.4.3 H&E Staining Protocol

The protocol had following steps:

1. Water→ 2 min
2. Haematoxylin→ 2 min, Tap water→ 30 Sec.
3. Acid alcohol→ Few seconds until background is clear (differentiation)
4. Tap water→ 30 sec.
5. Borax→ 30 sec. (Bluing)
6. Eosin solution→ 10 sec.
7. Tap water wash
8. Dehydration and mounting

3.3.5 Experimental Methods Immunohistochemistry

3.3.5.1 Immunohistochemistry Principle

Immunohistochemistry is a technique that uses a combination of immunological, histological and biochemical methods to visualise and localise specific cell components within a tissue. The reaction is based on a specific antigen-antibody reaction that is tagged with a visual label (fluorescent indicator). Immunohistochemistry involves three steps:

1. Processing and epitope/antigen retrieval

   During the process of formalin-fixation, morphological features are preserved, but the antigenicity is compromised to some

extent. There is a semi-reversible alteration in the tertiary and quaternary structure of proteins. However, there is not a complete loss of antigenic determinants.

The first step for immunohistochemistry is unmasking the antigens altered by formalin fixation. Commonest antigen retrieval technique to reinstate the tertiary structure is by heating the tissue sections in water or buffered solutions (e.g. citrate/ EDTA buffer).

2. Antigen-Antibody Interaction

   Direct immunohistochemistry requires the use of a primary antibody (conjugated with the marker) to bind the target antigen to produce a colour signal. Indirect immunohistochemistry involves two layers of antibodies (primary antibody and a secondary antibody conjugated with the marker). The latter greatly increases the specificity and versatility of immunohistochemistry as this allows the use of a wide range of unlabelled primary antibodies.

3. Visualisation through different detection systems

   The results are visualised microscopically to view or locate the fluorescent markers in the background of (stained) surroundings of the tissue.

Antibodies are conjugated to enzymes, (e.g. Horseradish Peroxidase, HRP) which form coloured, insoluble precipitates upon addition of chromogenic substrates (such as 3,3'-diaminobenzidine (DAB), gives a brown to black colour). The intensity of colour correlates with the concentration of antibody and the respective antigen. Positive and negative controls should be used for each staining run to ensure quality control. A positive control is a sample that is known to contain the antigen of interest and is stained with the same method as the tissue of interest. A negative control is the same sample as the positive

control where the staining method remains the same, but the primary antibody is replaced by a non-binding antibody from the same species.

3.3.5.2 Immunohistochemistry Procedure

Immunohistochemistry was performed by Dr Tom McCulloch and Dr Claire Hawkes from histopathology department, Nottingham University Hospital NHS Trust. A slide from each specimen, a positive and a negative control slides were used for staining. A Bond Max Automated Immunohistochemistry System (Leica Microsystems GmbH, Wetzlar, Germany) was used. The staining procedure was followed as per the protocol from Leica Bond Polymer Refine Detection Kit as follows:

1) Dewax
2) Antigen retrieval step (HIER, heat induced epitope retrieval, using citrate buffer at pH 6.0 or EDTA buffer at pH 9.0) 20 min (microwave at full power for 10 min and half power for 10 min)
3) Wash
4) Peroxide block 5 mins
5) Wash
6) Primary antibody (optimally diluted) 15 mins
7) Wash
8) Post-primary antibody 8 mins
9) Wash
10) Polymer 8 mins
11) Wash

12) DAB 10 mins

13) Wash

14) DAB enhancer 5 mins

15) Wash

16) Haematoxylin counterstain 5 mins

Antibodies used are described in Table 3-6.

Table 3-6 Antibodies used for Immunohistochemistry				
Antigen	**Name of antibody**	**Supplier**	**Antibody type, HIER buffer & dilutions used**	**Species specificity**
Amnion-less (AMN)	HPA000817 Rabbit Polyclonal antibody	Atlas Antibody Stockholm Sweden	Polyclonal, IgG Citrate & EDTA 1:200, 1:300, 1:400	Humans, sequence 82% similar* to porcine Amnionless-like mRNA
Megalin	bs-3909R Rabbit Anti Megalin Polyclonal antibody	Bioss Antibodies Bioss Inc. MA, USA	Polyclonal, IgG Citrate 1:100, 1:200	Humans, mouse & rat, sequence 82% similar* to porcine Megalin mRNA
	ab76969 Rabbit polyclonal antibody	Abcam plc. Cambridge, MA USA	Polyclonal, IgG Citrate 1:50, 1:100, 1:200	Humans, monkeys
Cubilin	SC23644,Goat Polyclonal IgG (Cubilin antibodyT-16)	SantaCruz Biotechnology Inc., Texas, USA	Polyclonal IgG Citrate & EDTA 1:50, 1:100, 1:200	Humans, pigs nblast sequence 83% similar* to porcine Cubilin mRNA
CFTR	Bs-1277R Rabbit Anti-human CFTR Polyclonal antibody	Bioss Antibodies Bioss Inc., MA, USA	Polyclonal, IgG Citrate & EDTA 1:100, 1:200	Humans, mouse & rat, sequence 90% similar* to pig CFTR mRNA
	LS-C87635, Mouse Anti-human CFTR Monoclonal antibody	Life Span Biosciences Inc., Seattle, WA, USA	Monoclonal, IgG Citrate & EDTA 1:25, 1:100	Humans, mouse & rat

HIER: Heat Induced Epitope Retrieval; *sequence similarity assessed using nBLAST search engine (560); CFTR: cystic fibrosis transmembrane conductance regulator; IgG: immunoglobulin G; EDTA: ethylenediamine tetraacetic acid; mRNA: messenger ribonucleic acid

3.3.6 Experimental methods: ELISA Methods

3.3.6.1 ELISA Principle

Enzyme-linked immunosorbent assay (ELISA) is a commonly used technique for identification and quantitation of substrates, particularly antigens or antibodies in samples. The principle of ELISA is similar to that of immunohistochemistry. A labelled antigen-antibody complex is used to produce a detectable signal (colour or fluoroscopy) that can be measured to inform the concentration of substrate. For a sandwich ELISA, a microtiter plate is prepared by coating of wells with a capture antibody, i.e. an antibody that will bind the antigen in question. On addition of sample, the antigen binds the antibodies. This step is followed by use of a detection antibody that will bind the antigen (hence called sandwich ELISA). The detection antibody can itself be linked to an enzyme or, in a next step, is attached to a secondary antibody linked to an enzyme. In between each step, the plate is washed with buffer solution to remove excess reagents/unbound substrates. After the final wash, an enzyme substrate is added that produces a colour signal. The intensity of the colour is in proportion to the quantity of the antigen in the sample.

3.3.6.2 ELISA Technique

3.3.6.2.1 Sample Handling

Pig urine samples were provided by the University of Iowa. The urine samples were frozen at -80°C after collection and transported in dry ice (Section 3.3.2) shipment. The samples were kept frozen at -80°C until processed. Before starting the ELISA experiments, all the samples were thawed and centrifuged to remove debris. The supernatant was collected and stored in aliquots at -80°C until used. All the samples were blinded to the investigator (myself) as described in Section 3.3.2. Details of ELISA kits used are given in Table 3-7. As cubilin, megalin and

AMN ELISA kits were targeted for human biological fluids, all ELISA experiments for these antigens were run with an additional control specimen of human urine. Transferrin ELISA was run with human plasma (as this was a porcine ELISA kit for plasma). Prior to experiments, the urinary pH was normalised by dilution to ½ or ¼ with Phosphate Buffer solution (PBS).

3.3.6.2.2 Cubilin ELISA technique

Details of Cubilin ELISA kit are as per Table 3-7.

The assay was performed as per manufacturer's instructions provided in the insert. All the reactions were performed in duplicate. The manufacturer provided a microplate precoated with biotin-conjugated antibodies specific for cubilin. Standards were prepared as with concentrations ranging from 0.78 to 50 ng/ml and standard diluent was used for blank well. Standards or samples were added to microtiter plate wells and incubated at 37°C for 2 hours after covering with plate sealer.

A secondary antibody was added and incubated. After washing the plate several times, avidin conjugated to HRP was added to each well and incubated. In the next step, TMB (Tetramethylbenzidine) substrate was added that exhibits change in colour only in the wells containing cubilin conjugated to antibodies. The enzyme reaction was terminated by addition of sulphuric acid, and the colour change was measured spectrophotometrically at a wavelength of 450 nm. The concentration was analysed by a standard curve made by plotting concentration on y-axis and OD on the x-axis (log-log graph) and using regression analysis to draw the best-fit straight line.

3.3.6.2.3 Megalin ELISA technique

Please refer to Table 3-7 for details of Megalin ELISA kit. The protocol provided by the manufacturer was followed. The protocol was similar

as described with cubilin ELISA. Standards were prepared ranging from 0.625 to 200 ng/ml.

3.3.6.2.4 Amnionless ELISA technique

Please refer to Table 3-7 for details of Amnionless ELISA kit. The protocol provided by the manufacturer was followed. The protocol was similar as described with cubilin ELISA. Standards were prepared ranging from 0.312 to 40 ng/ml.

3.3.6.2.5 Transferrin ELISA technique

Please refer to Table 3-7 for details of Transferrin ELISA kit. Instructions provided by the manufacturers were followed. The principles of sandwich ELISA were same as described above. Balance solution was added to the wells if the samples were not serum or plasma. Standard solutions ranged from a concentration of 25 ng/ml to 500 ng/ml.

3.3.6.2.6 Creatinine ELISA technique

Creatinine levels were measured to help normalise concentrations of various proteins. The details of the ELISA kits are as in Table 3-7. Parameter creatinine assay is based on Jaffe reaction where creatinine is treated with picrate solution to yield a bright orange-red colour (in the intensity proportionate to the concentration of creatinine). Assay protocol instructions provided by the manufacturers were followed. Urine samples were diluted to 20 fold; however, for the samples where the reaction signals were poor, the test was repeated as 10 fold dilution of urine. The standards ranged from 0.31 mg/dl to 20 mg/dl, and the OD was read at 490nm.

Table 3-7 Details of ELISA kits used					
Protein/ Substrate	**Kit Details**	**Provider**	**Samples & Species**	**Intra-Assay CoV**	**Inter-Assay CoV**
Megalin	ELISA for Low-Density Lipoprotein Receptor-Related Protein (LRP2), E93101Hu, 0.625 to 40 ng/ml	USCNK Life Sciences Inc. Wuhan, China	Serum, plasma and other biological fluid Human	<10%	<12%
Cubilin	ELISA for Cubilin, E92411Hu, 0.78 to 50 ng/ml	USCNK Life Sciences Inc. Wuhan, China	Tissue homogenates and biological fluids Human	<10%	<12%
Amnionless (AMN)	ELISA for Amnionless, E92295Hu, 0.312 to 20 ng/ml	USCNK Life Sciences Inc. Wuhan, China	Tissue homogenates and biological fluids Human	<10%	<12%
Transferrin	Porcine Transferrin ELISA Kit, E07T0130, 25 to 500ng/ml	Bluegene Biotech, Shanghai, China	Serum, plasma, cell culture supernatants, body fluids and tissue homogenates Pig	<9%	<10%
Creatinine	Parameter Creatinine Assay, KGE005	R&D Systems, Abingdon, UK	Urine	3.2 to 3.5%	4-5.5%

CoV: coefficient of variation, ELISA: enzyme-linked immunosorbent assay

3.3.6.3 ELISA Troubleshooting Methods

3.3.6.3.1 Spike and Recovery analysis

Spike and recovery assays are used to validate and assess the accuracy of ELISA experiments. In some cases, certain components of the sample matrix (specimen or diluent) itself can interfere with ELISA results. For spike and recovery assay, a known amount of ELISA standard is spiked into a sample, and the resulting concentration (recovery) of the spiked sample is compared to the expected return. Serial dilutions can be used to test for linearity of the experiment. The concentration of the sample should fall in the middle to high end of the standard curve range. The recovery is calculated by the following equation:

$$\% \, Recovery = \frac{Observed - Neat}{Expected}$$

Equation 3-4

Observed=Spiked sample return; Expected=Amount spiked;
Neat=Unspiked sample concentration;

An acceptable recovery range is described as 80-120% of the amount spiked. A low recovery for spiked samples compared to the spiked standard is suggestive of interference by sample matrix.

3.3.6.3.2 Dialysis and Lyophilisation

This procedure was undertaken with two aims: removal of any potential inhibitors to the reaction and concentration of the samples to allow detection of substrates present in low quantity. Ready to use Spectra/Por® Float-A-Lyzer® G2 dialysis devices (Spectrum Laboratories Inc., CA, USA) were used (Figure 3-7). The protocol was followed as described by Norden et al.(*566*) and manufacturer's instructions were followed for the handling of dialysis tubes. The dialysis tubes were cylindrical in shape, made of cellulose ester that is

a low protein binding synthetic membrane. The molecular weight cut-off (MWCO) for the tubes used was 20 kD (i.e., it contains substrates larger than and up to 20 kD within the tube). The screw cap is described as leak-proof, and the device is supposed to have 95-98% sample recovery while maintaining 99% sample purity and <1% sample dilution.

3.3.6.3.2.1 *Dialysis Principle*

Dialysis is a technique for separation of substrates based on the principle of diffusion. It facilitates removal of unwanted compounds in the solution through a semipermeable membrane. The sample and a buffer solution (called dialysate) are kept on the opposite sides of the membrane. The process is facilitated by movement of the molecules (by stirring on a stirrer and using a magnetic flea) and by changing the buffer at intervals to maintain the pressure gradient. Molecules larger than the MWCO will stay in the sample; hence, I selected an MWCO of 20 kD for this study. The smallest molecular weight of the substrates of interest was 35 kD for AMN.

3.3.6.3.2.2 *Dialysis Technique*

Urine samples in Eppendorf tubes were centrifuged at 1000 G at 4°C for 20 min, and the supernatant was transferred to a new tube. Dialysis devices (Spectra/Por® Float-A Lyzer® G2 ready to use dialysis devices, 5ml) were submerged in distilled water for 30 min and then rinsed with ammonium bicarbonate. The urine samples were transferred to dialysis tubes, which were then transferred to buffer solution with float applied to the tube (Figure 3-7).

Four litres of 50 mM ammonium bicarbonate (molecular weight 79.06 g/M) was used as a buffer solution, which was prepared as follows:

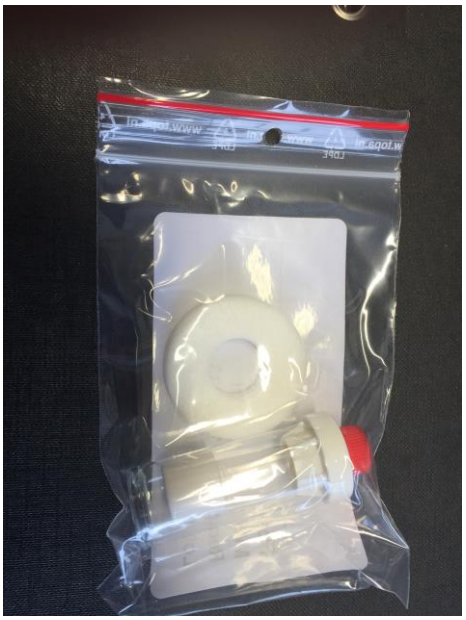
$$4 * 0.05M * 79.06\frac{g}{M} = 15.8g$$

Equation 3-5

The volume of 4 litres was selected as roughly 100 times the total volume of samples. The buffer solution with the dialysing device was kept on a stirrer along with a magnetic flea in the bottom of the beaker at 4°C for 4 hours. The buffer solution was changed after 1 hour. The dialysed samples were then transferred to Eppendorf tubes that were then freeze-dried in the fungal laboratory in the University of Nottingham. The dried specimens were then resuspended in half the original volume (same volume for the samples with small initial volumes-total 8/33) of 1% PBS and then used in downstream ELISA experiments.

3.3.7 Statistical Methods

IBM SPSS (22) was used for statistical analysis. Gene expression analysis values of different groups were assessed for normality by reviewing the histograms. Data with a normal distribution (gene expression values for cubilin and AMN) were compared using independent samples t-test (CFTR and non-CFTR groups). Expression values with non-normal distribution were used after log transformation to normalise (CFTR and megalin). GraphPad Prism version 7 was used to generate standard curves and interpolate unknown concentrations in ELISA experiments.



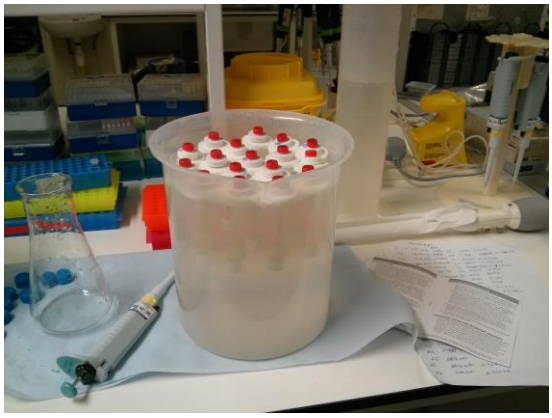


Figure 3-7 Dialysis of urine samples**; **the picture on top shows the Float-A-Lyzer® G2 device; the bottom picture shows the devices loaded with the specimens, submerged in the dialysate

3.4 Results

There were 39 pig kidney samples including 17 CFTR -/-, 6 CFTR +/- and 16 CFTR +/+ genotypes (so phenotypically kidney tissue from two groups of animals, 17 from pig model of CF and 22 from control pigs). During the initial mRNA expression data analysis, 3 kidney tissue samples K025, K033 and K039 consistently showed extreme outlying values compared to the rest of the group. These samples were tracked back to tissues from one box that was delayed for one day in transit (while being shipped from Iowa to Nottingham) compared to the other containers. There was a small amount of dry ice left in this container when collected from the airport; hence, considering a possibility of exposure to higher temperature and associated protein degradation, I excluded these samples from gene expression analysis. This left 36 pig samples for gene expression. Details of genotypes and demographics are given in Table 3-8***.***

Urine samples were available for 33 of 39 pigs including 14 CFTR -/- pigs. The urinary pH was normalised by dilution to ½ or ¼ with PBS. Urine dipstick results are presented in Table 3-9**.** No statistical comparison was made between the two groups for the number of urine samples from each group showing proteinuria; 4 of the total 6 pig urine samples showing grade 3 or more proteinuria also had grade 2 or higher haematuria, which could have contributed to higher protein levels and inaccurate results.

Table 3-8 Demographic parameters for pigs		
Parameter	**CFTR +/- or +/+**	**CFTR -/-**
Number (N)	21	15
(Genotype)=N	(+/-)=6, (+/+)=15	(-/-)=15
Males : Females	15:6	5:10
Mean BW (Kg), (SEM)	1.34 (0.05)	1.24 (0.07)

CFTR: cystic fibrosis transmembrane conductance regulator gene; BW: birth weight; SEM: standard error of mean

Table 3-9 Urine dipstick results for pig urine samples			
Parameter	**CFTR +- or +/+**	**CFTR -/-**	**Total**
Number (N)	19	14	33
pH (range)	5-8.5	5-8	32
Specific gravity (range)	<1.005 to 1.015	<1.005 to 1.030	32
Glucose	(Nil=19)	(Nil=12), (5.5 mmol/L=2)	33
Ketones	(Nil=19)	(Nil=10), (Trace=4)	33
Nitrites	(Nil=19)	(Nil=14)	33
Leucocytes	(Nil=19)	(Nil=14)	32
Proteins trace/Nil	6	5	
Proteins 30-100 mg/dl (+/++)	11	4	
Proteins >100 mg/dl) (+++/++++)	2	4	
Blood trace/nil	16	4	
Blood 25-80 Ery/µL (+/++)	2	3	
Blood >200 Ery/µL (+++/++++)	1	6	

CFTR: cystic fibrosis transmembrane conductance regulator gene; Ery: erythrocytes

3.4.1 PCR Results

Images for gel electrophoresis are presented in Figure 3-8**.** Gel electrophoresis performed on cDNA developed from PCR on CFTR primer sequence provided by Iowa, Figure 3-8 (A), did not show any bands in CFTR -/- well; however due to lack of optimum PCR efficiency, these primers were not used in downstream applications, and new primers were designed in-house. Gel electrophoresis on the cDNA from the new CFTR primers showed CFTR cDNA to be present for all genes including CFTR cDNA for the knockout and wild-type CF groups (Figure 3-8 (B)). The same was found to be true in gene expression analysis (please refer to Section 3.5.1 for possible explanation). The cDNA was sent for DNA sequencing, and the results were blasted against porcine CFTR DNA (nBLAST, www.ncbi.nlm.nih.gov/**blast**/) *(560)*; this showed 100% sequence resemblance for pig CFTR gene for forward and reverse primer sequences respectively, Figure 3-9**,** (nblast results in Appendix Chapter III Section 8.3.1)**.** Figure 3-10 shows a single peak of melt curve for the PCR reaction with primers designed in-house for CFTR gene.

3.4.1.1 Relative gene expression results

Relative gene expression ratios for different genes are presented in Table 3-10. CFTR and megalin expression ratios followed non-normal distribution hence median values and IQR are presented. These data were transformed to $\log_{10}$ CFTR and $\log_{10}$ megalin respectively for independent samples t-test (results in Table 3-11). There was no difference between the two groups for relative gene expression of any of the four genes studied.

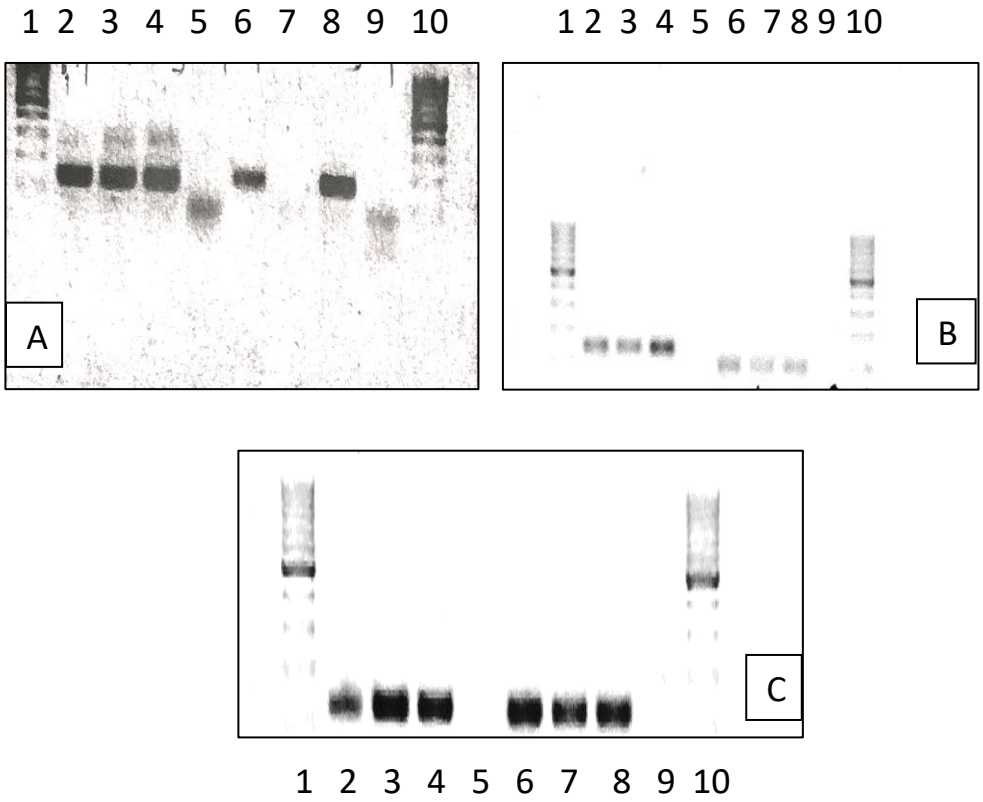


Figure 3-8 Photographs of amplified cDNA, in agarose gel stained with ethidium bromide and visualised under ultraviolet illumination; in all the images, the columns are numbered from left to right, and there is a DNA ladder at the end on either side (i.e. columns 1 and 10).

Image A: cDNA bands amplified from mRNA extracted from CFTR +/+, CFTR -/-, CFTR +/- pig kidney tissues and negative control for amnionless gene (columns 2-5) and CFTR gene (Columns 6-9) from left to right; the CFTR cDNA was amplified using primers shared by Iowa; Image B: cDNA bands amplified from mRNA extracted from CFTR +/+, CFTR -/-, CFTR +/- kidney tissues and negative control for amnionless gene (columns 2-5) and CFTR gene (columns 6-9-using primers designed in-house) from left to right-); Image C: cDNA bands amplified from mRNA extracted from CFTR +/+, CFTR -/-, CFTR +/- kidney tissues and negative control for megalin (columns 2-5) and cubilin genes (columns 6-9) from left to right; CFTR: cystic fibrosis transmembrane conductance regulator gene; cDNA: complementary deoxyribonucleic acid; mRNA: messenger ribonucleic acid

CFTR-2 Forward sequencing result:

TCTAACCCCCGGCGCAGAATCACTTCTGATGATGATTATGGG

AGAACTGGAGCCTTCAGAGGGTA

CFTR-2 Reverse Sequencing result:

GAACCAGCGCCGTGATGTCTTGCCTGCTCCAGTAGATCCAGCAACTGCCA

Figure 3-9 Gene Sequencing results for CFTR cDNA (primers designed in-house); nBlast of forward and reverse primer sequencing results against pig genome showed 100% identity to Sus scrofa CFTR mRNA (NM_001104950.1 (560)); CFTR: cystic fibrosis transmembrane conductance regulator gene; mRNA: messenger ribonucleic acid

Table 3-10 Relative gene expression levels for CFTR knockout and control pigs				
Gene	**Gene Expression CFTR knockout (N=15)**	**95% CI/ range**	**Gene Expression control (N=21)**	**95% CI/ range**
CFTR (Median) (IQR)	0.449 (0.33)	0.24 to 0.81	0.45 (0.28)	0.28 to 1.23
Cubilin (Mean) (SE)	0.408 (0.04)	0.32 to 0.49	0.498 (0.03)	0.44 to 0.56
Megalin (Median) (IQR)	0.334 (0.164)	0.22 to 0.51	0.334 (0.22)	0.21 to 0.73
AMN (Mean) (SE)	0.538 (0.42)	0.45 to 0.63	0.536 (0.33)	0.47 to 0.61

CF: cystic fibrosis; CI: confidence interval; CFTR: cystic fibrosis transmembrane conductance regulator gene; AMN: amnionless; SE: standard error; IQR: interquartile range; CFTR knockout: CFTR-/- genotype; control: CFTR +/- and +/+ genotype

Table 3-11 Independent samples t-Test for Relative expression of genes				
Gene	**P value**	**Mean difference**	**SE difference**	**95% CI difference lower to upper**
CFTR (Log_{10})	0.687	0.0235	0.05777	-0.0939 to 0.1409
Cubilin	0.062	0.0902	0.0467	-0.0047 to 0.1851
Megalin (Log_{10})	0.165	0.0667	0.04701	-0.02884 to 0.16225
AMN	0.983	-0.001148	0.052838	-0.108529 to 0.106232

CFTR: cystic fibrosis transmembrane conductance regulator; SE: standard error, CI: confidence interval; AMN: amnionless

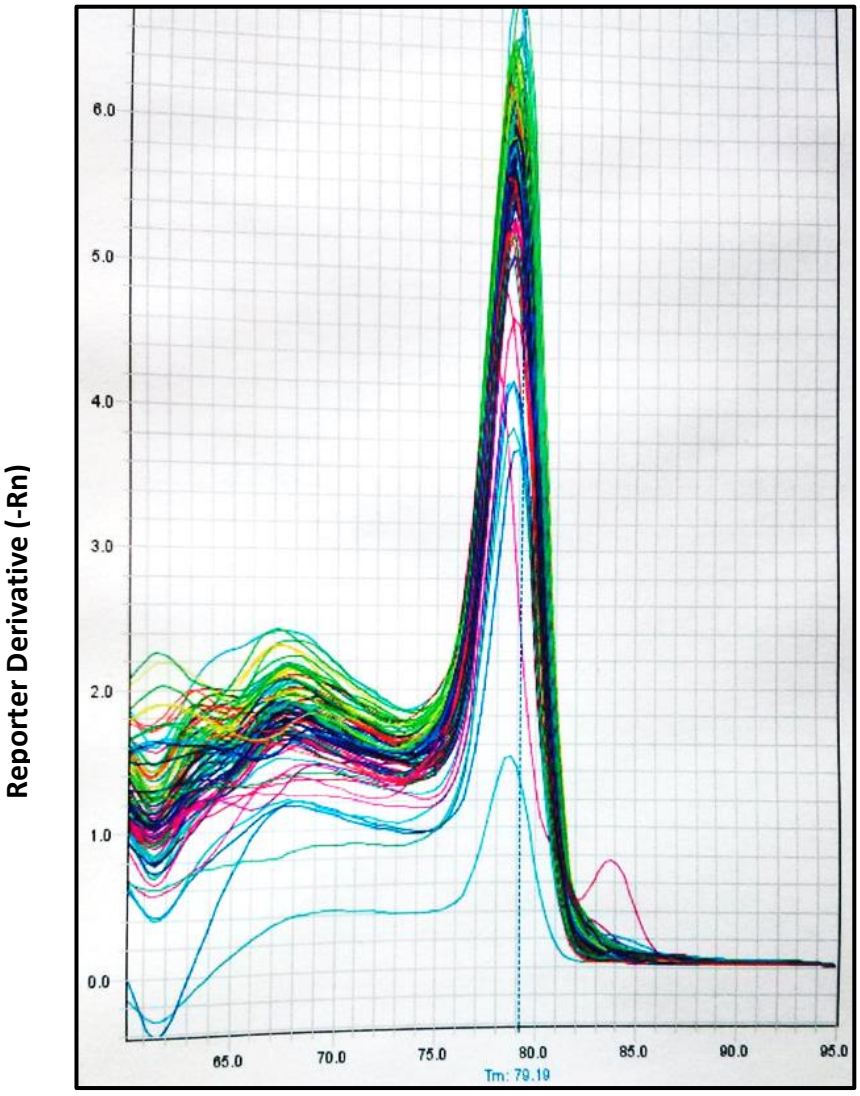


Figure 3-10 Melt curve analysis for CFTR Quantitative PCR shows a single peak; y-axis represents the negative of the first derivative of fluorescence intensity (-rate of change in fluorescence, -ΔRn/ΔT(time) with respect to temperature on x-axis; Tm: melting temperature; (PCR efficiency for the reaction=95.5%, R^2 for slope of standard curve=0.999); CFTR: cystic fibrosis transmembrane conductance regulator gene; PCR: polymerase chain reaction

3.4.2 Results of histological examination

Histological examination did not show any difference in morphology between CFTR -/- genotype and CFTR +/+, +/- genotype piglet kidney sections. Example histological images from CFTR -/- genotype and CFTR +/+, +/- genotype pig kidney sections are presented in Figure 3-11, Figure 3-12, Figure 3-13, Figure 3-14, Figure 3-15, Figure 3-16, and Figure 3-17. These images were randomly chosen as representative of that group.

3.4.3 Results ELISA

The ELISA reactions were performed as a preliminary experiment on a small number of samples (including all genotypes: CFTR +/+, CFTR +/- and CFTR -/-) in different dilutions. Standard curves were optimum with good efficiency for all proteins; however, there was no colour in any of the sample wells in any dilution (made with PBS). Because of low absorbance, the concentration of unknown samples could not be interpolated by the standard curve. Spike and recovery analysis undertaken showed poor recovery of 16%/47% with pig urine samples/spiked standard diluent. Further experiments conducted to explore various possibilities leading to these results are described in Section 3.3.6.3 under the heading 'ELISA troubleshooting'. Despite dialysis of the samples to remove any potential impurities or inhibitors and concentrating the samples, no colour was noted in the specimen wells. The absorbance for unspiked pig urine samples was still close to or less than the blank value, and concentration could not be interpolated with the standard curve. Additional control assays run parallel to the experimental assays using human urine (cubilin and AMN) did not show any colour signals suggestive of low concentrations in urine or abundance of inhibitors/competitors. Transferrin and Megalin ELISA experiments were run in parallel to human plasma as control specimens, which showed positive results

and about 70% spike recovery. The results of the spike and recovery experiments are summarised in Table 3-12 suggesting improvement in spike recovery post dialysis and concentration of specimen. For further explanation, please refer to the discussion in Section 3.5.3.

Table 3-12 Summary of spike and recovery ELISA tests

Protein name	Control Specimen (Human)		Spike recovery (%)			Interpret.
	Nature Dilution	Concentr. (ng/ml)	Control	Pig urine$ (average)	Stand.	
AMN	Urine£ 1:1	No results	*No results	69	68	Reaction condition influence on plasma/pig urine
	Plasma 1:100	No results	72%			
Megalin	Plasma 1:1000	37.2	**No results	85	130	Sample matrix influence
Cubilin	Urine 1:1	0.902	17%	53	120	Sample matrix influence
TNF	Plasma 1:1	66.3	66%	131	105	Good return for pig urine
	Plasma 1:100	No results	134%			Better recovery with plasma dilution

£: There was poor or no substrate detection with undiluted human urine specimen; $: Pig urine specimen post-dialysis and concentration; *absorbance level below blank (no antigen /no reaction); **: absorbance higher than highest standard (above the standard curve range); ELISA: enzyme-linked immunosorbent assay; concentr.: concentration; stand.: standard; interpret.: interpretation; AMN: amnionless; TNF: transferrin

3.4.4 Results Immunohistochemistry

Staining for AMN was successful at 1/400 dilution. AMN stained the brush border of proximal tubules and, apart from a weak blush in the cytoplasm of the proximal tubular cells, did not stain any other structure (please refer to Figure 3-18, Figure 3-19 and Figure 3-20). The staining for megalin and CFTR was unsatisfactory despite various attempts at repeat staining, use of different protocols and different antibodies (please refer to Table 3-6). The AMN staining was graded according to its intensity in three groups: grade-1 (low intensity), grade-2 (medium intensity) and grade-3 (high intensity). The tissue from piglets with genotype CFTR +/- and +/+ had a higher proportion with medium or higher intensity staining compared to tissues from CFTR -/- genotype piglets (17/22 versus 9/17 respectively, Table 3-13; however, the difference was not statistically significant with a P value of 0.110. For the Chi-square test, I grouped the tissues as low intensity (grade-1) and high intensity (grade-2 and grade-3) as the number of piglets with grade-3 staining were low affecting the validity of the test.

Table 3-13 Amnionless IHC staining grades as per genotypes

Staining intensity	CFTR -/-	CFTR +/-	CFTR +/+	Total
Low (Grade-1)	8	1	4	13
Medium (Grade-2)	8	3	11	22
High (Grade-3)	1	2	1	4
Total	17	6	16	39

IHC: immunohistochemistry, CFTR: cystic fibrosis transmembrane conductance regulator

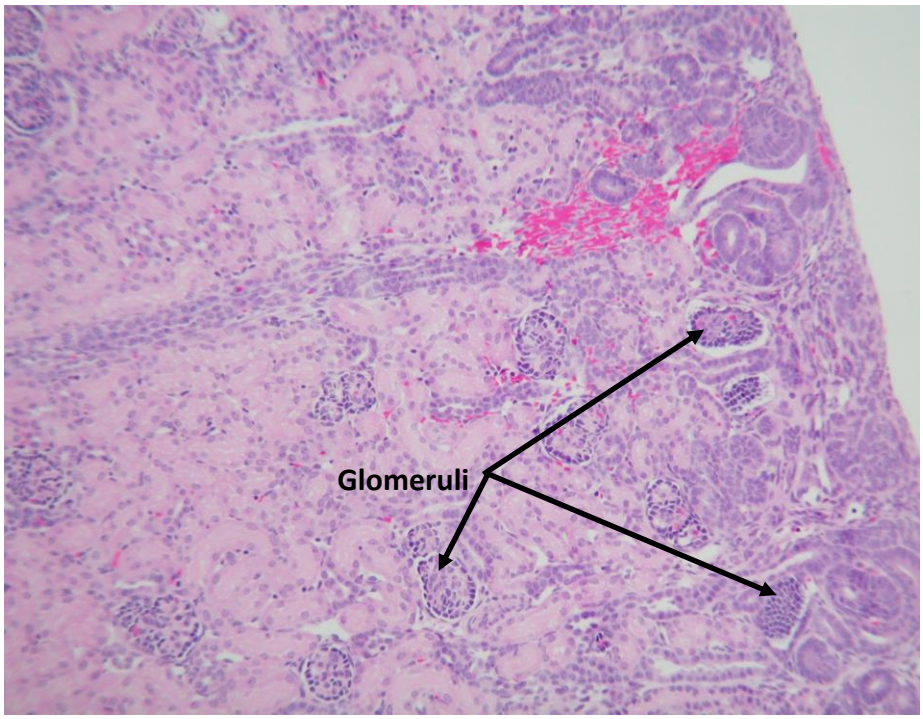


Figure 3-11 Randomly selected histological image of CFTR -/- pig kidney, magnification X 100 H&E staining, viewed under Leica DM LB microscope; H&E: haematoxylin & eosin staining; CFTR: cystic fibrosis transmembrane conductance regulator

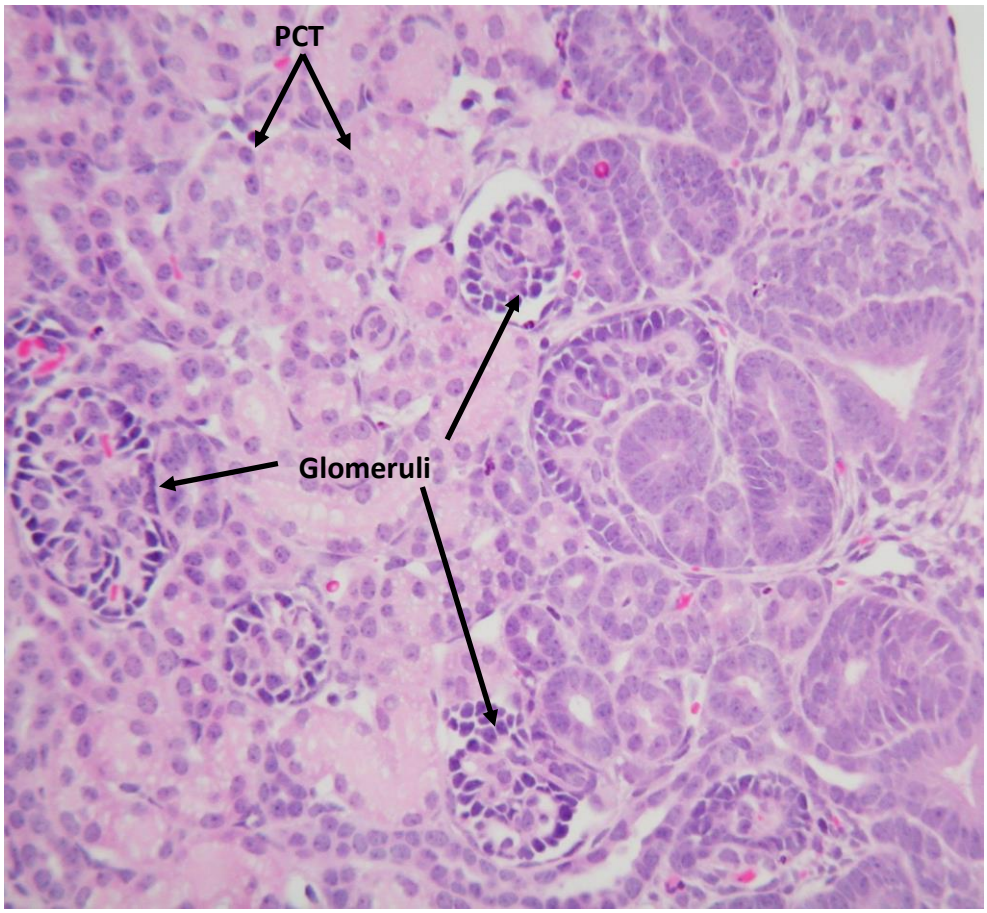


Figure 3-12 Randomly selected histological image of CFTR -/- pig kidney, magnification X 200; H&E staining, viewed under Leica DM LB microscope; PCT: proximal convoluted tubules; H&E: haematoxylin & eosin staining; CFTR: cystic fibrosis transmembrane conductance regulator

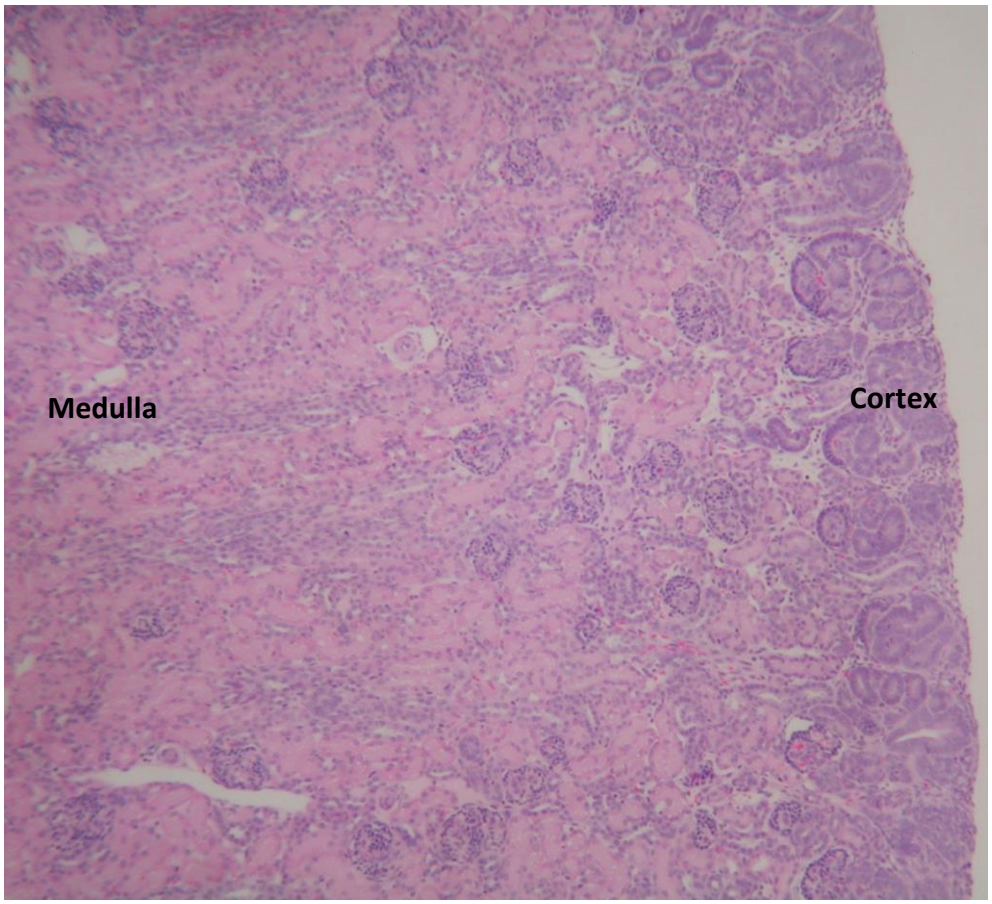


Figure 3-13 Randomly selected histological image of CFTR +/- pig kidney, magnification X 100; H&E staining; viewed under Leica DM LB microscope; H&E: haematoxylin & eosin staining; CFTR: cystic fibrosis transmembrane conductance regulator

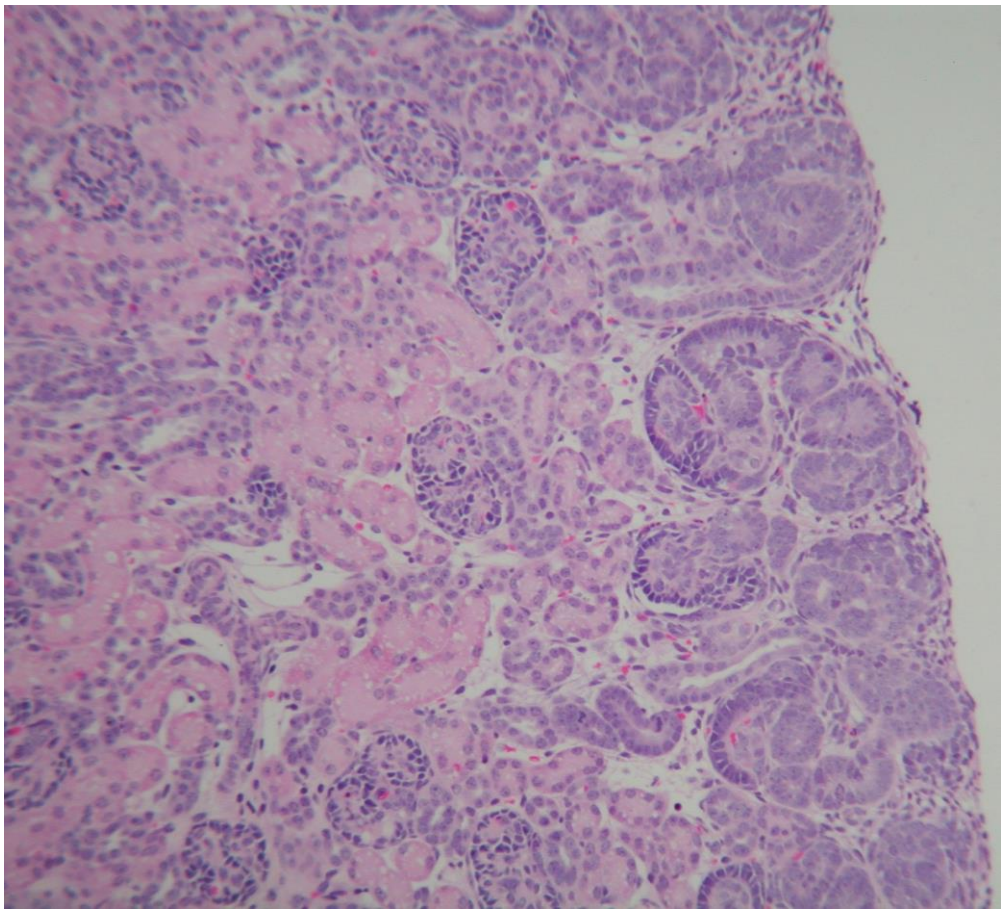


Figure 3-14 Randomly selected histological image of CFTR +/- pig kidney, magnification X 200; H&E staining, viewed under Leica DM LB microscope; H&E: haematoxylin & eosin staining; CFTR: cystic fibrosis transmembrane conductance regulator

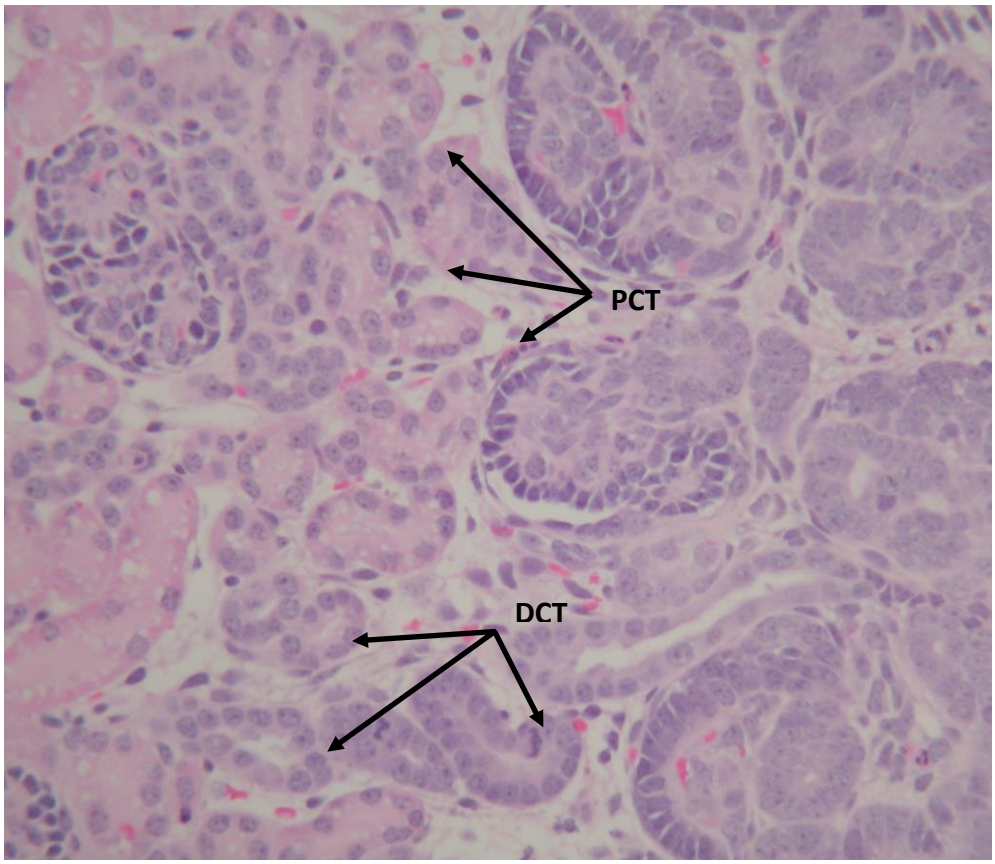


Figure 3-15 Randomly selected histological image of CFTR +/- pig kidney, magnification X 400; H&E staining, viewed under Leica DMLB microscope; PCT: proximal convoluted tubule; DCT: distal convoluted tubule; H&E: haematoxylin & eosin staining; CFTR: cystic fibrosis transmembrane conductance regulator

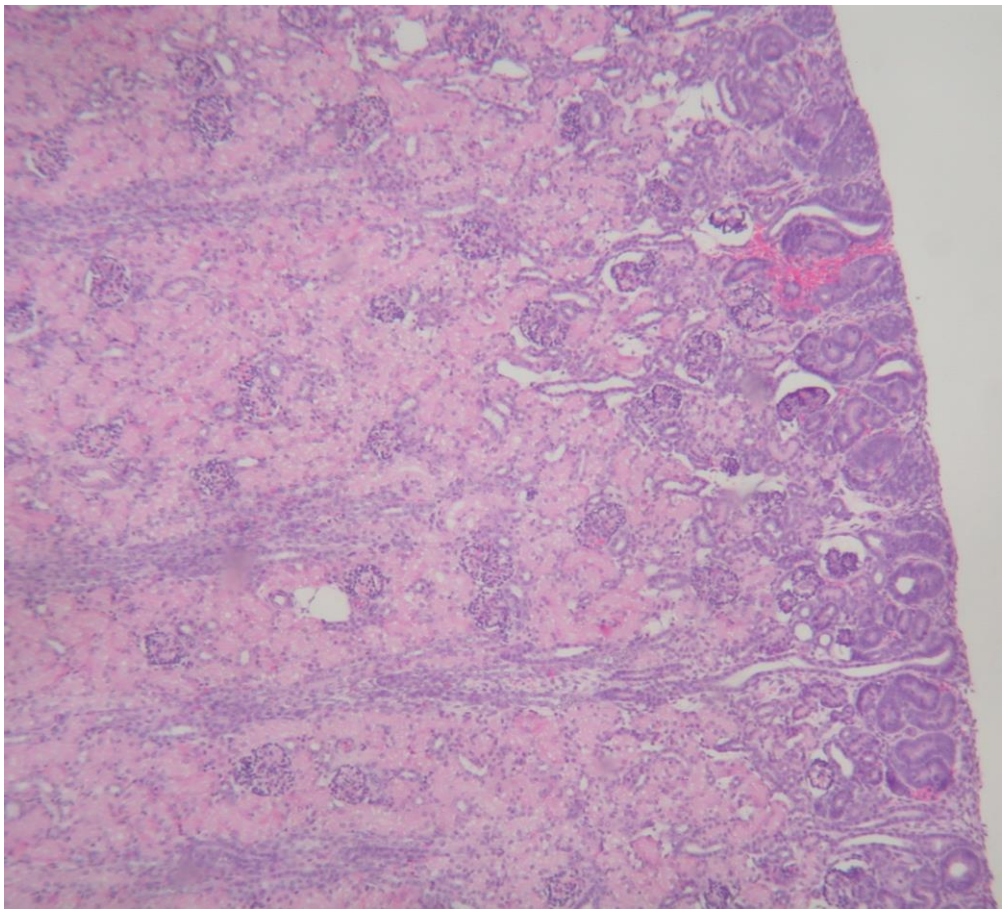


Figure 3-16 Randomly selected histological image of CFTR +/+ pig kidney, magnification X 100; H&E staining, viewed under Leica DM LB microscope; H&E: haematoxylin & eosin staining; CFTR: cystic fibrosis transmembrane conductance regulator

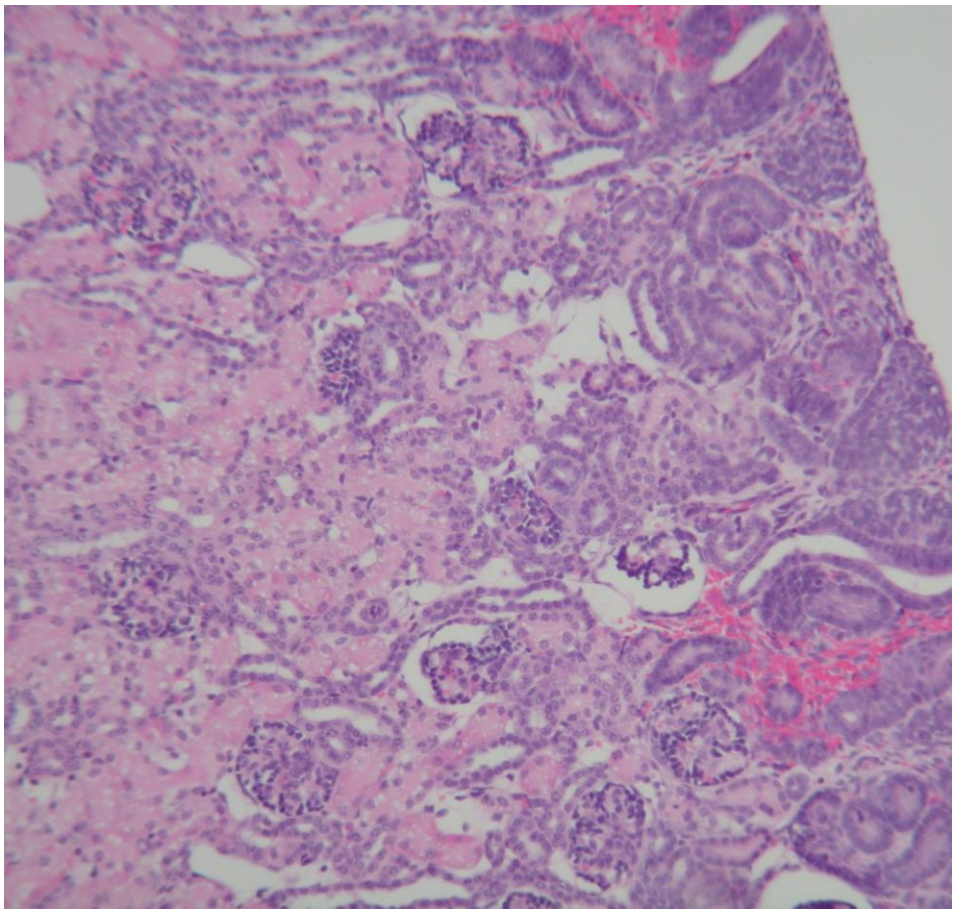


Figure 3-17 Randomly selected histological image of CFTR +/+ pig kidney, magnification X 200; H&E staining, viewed under Leica DM LB microscope; H&E: haematoxylin & eosin staining; CFTR: cystic fibrosis transmembrane conductance regulator

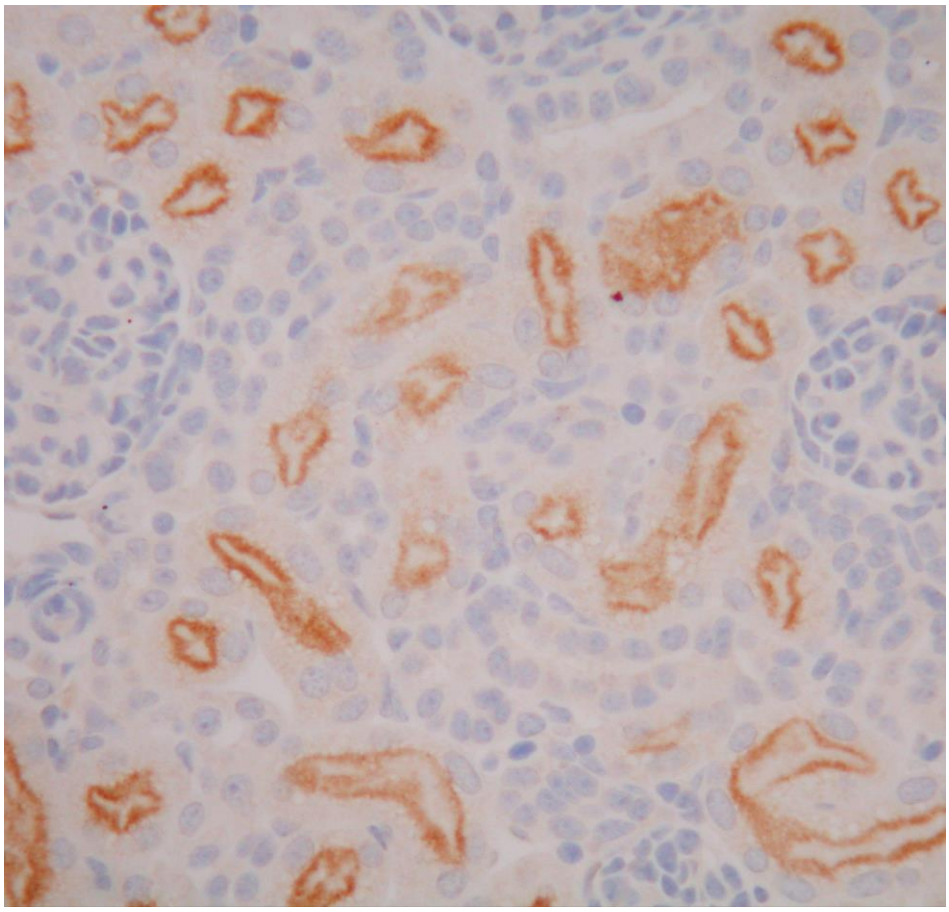


Figure 3-18 Immunohistochemistry staining results for amnionless, grade-1 staining, viewed under Leica DM LB microscope, magnification X 400, using a rabbit polyclonal antibody (Table 3-6)

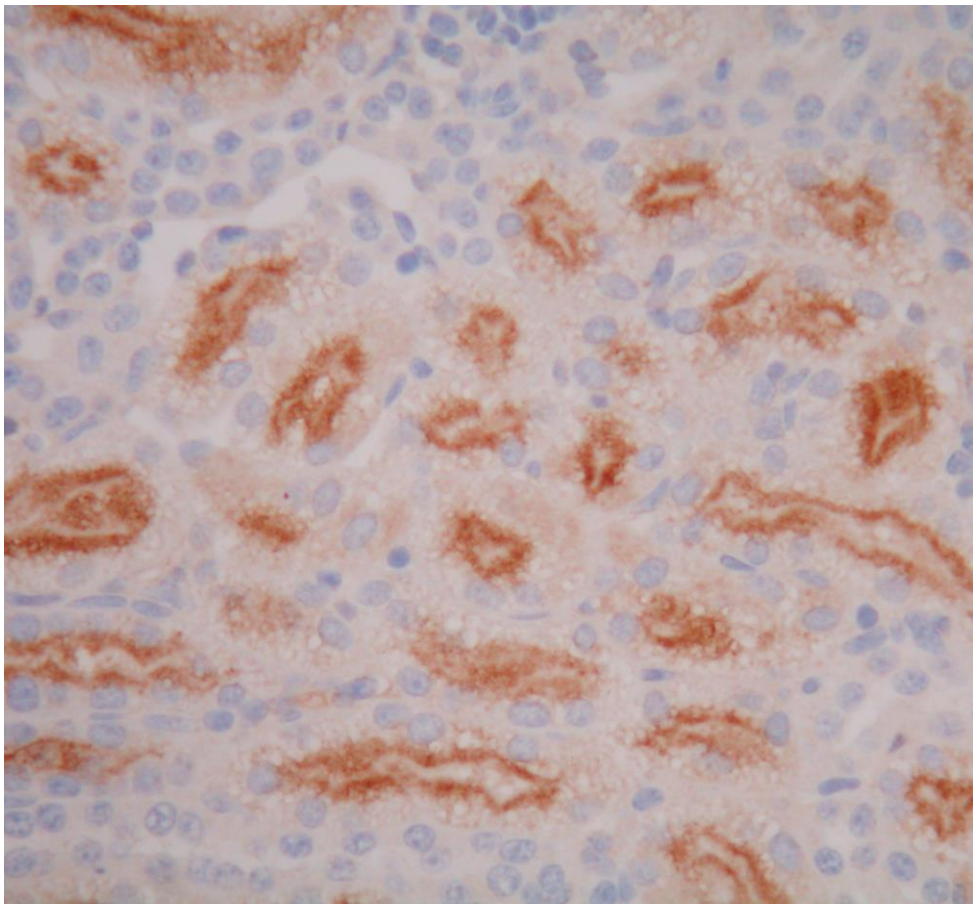


Figure 3-19 Immunohistochemistry staining results for amnionless, grade-2 staining viewed under Leica DM LB microscope, magnification X 400, using a rabbit polyclonal antibody (Table 3-6)

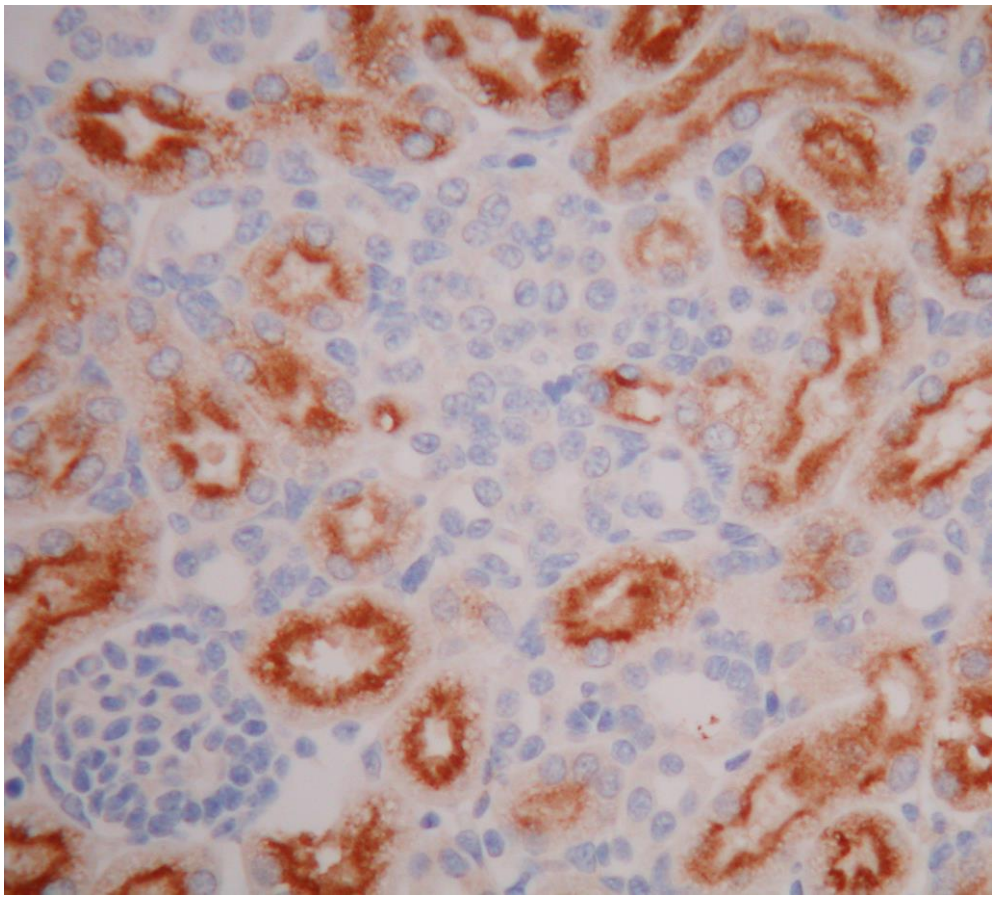


Figure 3-20 Immunohistochemistry staining results for Amnionless, Grade-3 staining;(brown colour staining of proximal convoluted tubules); viewed under Leica DM LB microscope, magnification X 400, using a rabbit polyclonal antibody (Table 3-6)

3.5 Discussion

This is the first study on kidneys from pig model of CF. Results of our study show no difference in kidney morphology between newborn CFTR knockout piglets and non-CF (heterozygous and wild-type) piglets. This finding supports the notion that kidneys are normal at birth in people with CF, though species differences may of course exist. We have established that CFTR mRNA is expressed in pig kidneys; however, similar expression levels of the CFTR gene in the kidneys from piglets with genotype CFTR -/- and piglets with genotypes CFTR +/+ and +/- is intriguing and requires further study and explanation. We have also established that the expression of endocytic receptor proteins is similar at the molecular genetic level, and any difference in the amount or stability of proteins is likely to have been driven by changes at the protein or epithelial levels. Our results for detecting differences in protein levels including ELISA and immunohistochemistry were unsuccessful, limited in most likelihood, by the lack of pig specific antibodies.

Several studies (*3-5,200*) have suggested an increased risk of acute and chronic kidney disease in patients with CF, which has been associated with various environmental influences including repeated use of antibiotics. At the same time, association with loss of CFTR function has also been implicated. Although CFTR is widely present in the kidneys, its exact role in renal physiology is not entirely defined (*567*). CFTR has been shown to act as a chloride secretory channel in distal tubules and drive abnormal fluid secretion into the renal cysts of autosomal dominant polycystic kidney disease. CFTR inhibitors are considered as potential therapeutic options for this condition. Study on kidney cell lines showed increased cell surface expression of CFTR in hypertonic shock thus suggesting its role in the regulation of renal

medullary hypertonicity (*241*). CFTR knockout mice were shown to have low molecular weight proteinuria (*232*) and increased cubilin loss in urine. This study provided an opportunity to assess renal morphology at birth. Previous (autopsy) studies showed several abnormalities in renal histology, both at glomerular and interstitial levels, in patients with CF from a wide age group that spanned from infancy to adulthood (*218-220*). These changes included glomerular enlargement, mesangiopathic changes, amyloidosis, acute and chronic tubular damage including abundant tubular lysosomal proliferation and tubular atrophy suggestive of chronic aminoglycoside injury. Review of renal biopsy of adult patients with CF showed multiple histopathological changes of varying nature within the glomeruli and within the renal interstitium (*223*). The commonest lesions were Kimmelstiel-Wilson nodular glomerular sclerosis (3/13) and amyloidosis (3/13); the rest of the lesions were of varying nature. Interstitial fibrosis was present in 7/13 patients. Our study established that kidneys are histologically normal at birth in pig model of CF; this finding is different from the results shown in various studies on (non-newborn) human kidneys. It is possible that the kidneys of newborn CFTR knockout pigs were normal, as they had not been exposed to any nephrotoxic influences. It may be speculated that various morphological changes (as shown in the renal histological studies of people with CF) develop with time after enduring a series of nephrotoxic insults. The pig model of CF may provide an opportunity to explore the impact of nephrotoxic factors, e.g. exposure to repeated courses of antibiotics, over a life course of the pig model of CF.

3.5.1 Relative gene expression experiments

Expression of CFTR mRNA in pig kidneys does not come as a surprise considering the widespread expression of CFTR mRNA in human kidneys. However, a previous study evaluating cellular expression patterns of porcine CFTR found an absence of CFTR expression in kidneys at both mRNA and protein levels (*531*). The difference in results from our study could be due to different assay sensitivities. Expression of CFTR mRNA in CFTR knockout pigs is an interesting and intriguing finding. There could be one possible explanation. The stop codon for generation of CFTR knockout pigs was introduced in exon 10 (*11,568*). However, a primer sequence (AGAATTTCATTCTGCTCTCAGT), described to be from exon 10 in the original paper (*568*), anneals to exon 11 of the current genome (the sequence is underlined in Figure 3-21). The 'Pig whole genome sequencing project' was launched in 2006 followed by the release of various draft sequences and the publication of the whole genome sequence for domestic and wild pigs in 2012. The difference in the location of the primer found in this study may be a reflection of a change in pig CFTR sequence with the addition of a new exon. As the primers used were in exon 10 (Figure 3-21), there may be a possibility that the translation occurred until the point of stop codon allowing amplification and detection of our amplicons. Generally, mRNA containing premature stop codons are recognised and degraded, by a process called nonsense-mediated decay, to prevent possible toxic effects of truncated peptides. Though it happens mostly at nuclear level in mammalian cells, it may follow a cytoplasmic pathway of degradation (*569*), and this is when a nonsense codon can be recognised. There is a possibility that due to lack of CFTR function, overall translation rate is higher in CFTR -/- genotype pigs (as a feedback loop); however, due to being 'balanced by' nonsense-mediated decay, CFTR mRNA relative expression level

was found to be equal to CFTR +/- and CFTR +/+ genotype pigs. This possibility might become evident with inhibition of translation as shown in a study using chlorhexidine (*569*). Further studies will be required to explain and explore this issue. Similarly, more studies should be conducted to assess any difference beyond molecular level, e.g. the effect of loss of CFTR function on the amount of protein loss in urine. In the current study, urine dipstick tests showed grade-3 or higher proteinuria in 6 samples; however, due to concomitant haematuria, the significance of these results could not be evaluated and no statistical tests could be undertaken, which should be addressed in future studies.

cDNA sequence Ensembl Genome Browser: Transcript ID CFTR-201 ENSSSCT00000018101, accessed on 2015.09.23

TCCAAGTCCACATTGGAGAGCAAATCAATTCTCTCCTCTCTTCATTTTAGCTGGACCAGACCAATTTTGAGAAAAG
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GTTGCTGGATCTACTGGAGCAGGCAAGACATCACTTCTGATGATGATTATGGGAG***AACTGGAGCCTTC***
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TCTTTTTCATTGCCGTTACCTTCATTTCCATTTTAACAACAGGGGAAGGAGAAGGAACAGTTGGGATTATTCTAAC
ATTAGCCATGAATATCATGAGTACATTGCAGTGGGCTGTAAACTCCAGTATAGATGTGGATAGCTTGATGCGATCT
GTGAGCCGAGTCTTTAAGTTTATTGATATGCCAGCAGAAGGAGATCAACCTAACAGGTCGTTCAAACCATCCAAGG
ATGGCCAGCTTTCAAAGGTTATGATTATTGAGAATCAACATGTGAAGAAAGATGACATCTGGCCTTCAGGGGGCCA
AATGACTGTCAAAGACCTCACTGCGAAATATGTAGATGGTGGGAACGCCGTATTAGAGAACATTTCCTTCTCAATA
AGTCCTGGCCAGAGGGTGGGCCTCTTGGGAAGAACTGGATCAGGGAAGAGCACTTTATTATTGGCTTTTTTGAGAC
TGCTGAACACAGAAGGAGAAATACAAGTAGATGGTGTGTCTTGGGATTCAATAACTTTGCAACAGTGGAGGAAAGC
TTTTGGAGTCATACCCCAGAAAGTATTCATCTTTTCTGGAACATTTAGAAAAAACCTGGATCCCTATGGACAGTGG
AATGATCAAGAAATATGGAAAGTTGCAGAGGAGGTTGGACTCAGATCTGTGATAGAGCAGTTTCCTGGGAAGCTTG
ATTTTGTCCTTGTAGATGGGGGTTGTGTGCTAAGCCACGGCCACAAGCAGTTGATGTGCTTGGCCAGATCTGTTCT
TGGTAAAGCAAAGATCTTGCTGCTGGATGAACCCAGTGCTCATTTGGATCCCATAACGTACCAAATCATTCGAAGA
ACCCTGAAACAAGCATTTGCTGATTGCACAGTAATCCTCTCTGAACACAGGATAGAAGCAATGTTGGAATGTCAAC
GATTTTTGGTCATAGAGGAGAACAAAGTGCGGCAGTATGATTCCATCCAGAGGCTGCTGAGCGAGAAGAGCCTCTT
CAGGCAGGCGATCAGCCCCTTGGACCGCCTGAAGCTCCTCCCGCACCGGAACTCTAGCAAGCAGAGGTCTCGGTCC
AAAATCGCAGCTCTGAAGGAGGAAACAGAAGAAGAGGTGCAAGAAACAAGACTTTAG

Figure 3-21 CFTR cDNA sequence and primer locations

CFTR-1 primer location (Primer sequences from Iowa) (highlighted green in exon 11)
CFTR-1 Forward Primer: ctg gag cct tca gag ggt aaa at
CFTR-1 Reverse Primer: agt tgg cac gct ttg atg aca ctc c (complimentary to the sequence shown here)
CFTR-2 Primer location (designed in-house) (bold and red fonts in exon 10 and 11 respectively)
CFTR-2 Forward Primer: ACAGTTGTTGGCAGTTGCTG
CFTR-2 Reverse Primer: ACCCTCTGAAGGCTCCAGTT (complimentary to the sequence shown here)
CFTR: cystic fibrosis transmembrane conductance regulator; cDNA: complimentary deoxyribonucleic acid

3.5.2 The RNA quality before starting the run

It is always preferable to start with a high-quality RNA as qPCR performance, though not the efficiency, are affected by the RNA integrity (*570*). RNA is sensitive to degradation by inadequate sampling and storage processes (*571*). One limitation of my study was that it did not have any formal measurements for assessing RNA quality. However, the tissue was stored throughout at -80°Celsius and all efforts were taken to prevent any contamination with genomic DNA and RNAse nucleases as described earlier (Section 3.3.3). Although some authors have questioned the accuracy of 260/280 method, an OD 260/280 ratio greater than 1.8 is usually considered an acceptable indicator of good RNA (*571*). Moreover, amplification of short PCR products (length 70-250 bp) is said to be independent of RNA quality while amplification of long products of >400 bp is strongly dependent on it. At the same time, the normalised expression differences measured with the qPCR are similar to those obtained from high-quality samples (*571*). Only the non-normalised values show a correlation between RNA integrity and Cp. The reliability of any relative qPCR experiment can be improved by including an invariant endogenous control to correct for sample-to-sample variations and errors in sample quantification (*571*). At times, even intact RNA also does not guarantee good PCR results as the samples may contain inhibitors, which can reduce PCR efficiency, and other factors may play a role such as length of amplicon, secondary structures and primer quality, etc.

3.5.3 ELISA results

The lack of any colour reaction with the samples in ELISA test could have a number of explanation, including low or absent antigen level. Various techniques used to troubleshoot some of these issues are described in the study methods (Section 3.3) i.e. normalising sample pH (aiming 6.5 to 7.5), spike recovery experiment, dialysis and concentration of samples (Section 3.3.6.3).

1. The samples were concentrated contemplating a low concentration of antigens. Dialysis was done with an aim to remove impurities. The dialysed samples were lyophilised and then suspended in a smaller volume. When the antigen concentration is low in the sample, competition by inhibitors increases. I tried to identify if any inhibitors were causing interference by doing spike and recovery experiment. Recovery of spiked samples improved after modification. The ELISA reaction in human urine samples showed poor or no substrate concentration in addition to poor spike recovery. This is suggestive of inhibiting factors in the sample matrix.
2. Another explanation could be that the antigens were undetectable because the urine samples were collected soon after birth and the pigs had not been exposed to any insults so far to allow protein loss in urine. Degradation of antigens or proteins could be another possible reason. However, all the samples had been stored at -80°C in a number of aliquots to minimise freeze-thaw cycles.
3. Lack of sensitivity of antibodies was another possibility considering the antibodies were specific for humans and had not been tested against pigs except transferrin. The “ready to use” ELISA kits were used for convenience sake and keeping in mind that there is a sequence/protein similarity between

humans and pigs. At the same time, porcine specific antibodies, to work in urine samples, were not available for many of these antigens. Due to financial and time constraints, the experiments could not be repeated with other antibodies, which can be planned for a future study.

Due to the difficulties in ELISA assay, a difference in urinary excretion of cubilin and low molecular weight proteins between urines from CFTR -/- genotype and CFTR +/- , +/+ genotype piglets could not be established. Subsequently (if there is a difference as shown in the mouse model of CF), what drives these phenomena remains unexplained. However, this study showed that there is no difference in mRNA expression levels, between CFTR knockout pig kidneys and CFTR heterozygous and wild-type pig kidneys, for any endocytic receptor proteins investigated in this study.

3.6 Conclusion

CF pigs may provide an alternative model to study renal function in CF. In this study, I have established that CFTR is expressed in pig kidneys. No histological changes were identified in the newborn CFTR -/- pigs. The level of expression of endocytic receptor proteins was similar in CFTR -/- genotype and CFTR +/-, +/+ genotype pigs, which rules out any alteration at gene level to explain the increased cubilin expression and the low molecular weight proteinuria noted in the mouse model of CF and in the people with CF respectively. More studies are required to explain the expression of CFTR mRNA in CFTR knockout pigs, firstly, using a primer sequence taken from CFTR exon 10 or less and secondly, using translation inhibitors. As the experiments to assess the protein levels (ELISA and immunohistochemistry) were unsuccessful, further experiments are needed using different (or porcine specific) antibodies to establish if there is any difference between the amount of CFTR or endocytic receptor proteins present in the CFTR knockout pig kidneys and in the wild-type pig kidneys. Environmental influences including repeated antibiotic courses should be explored for effect on renal function in CF.

Chapter-4 Using pharmacokinetics for reducing aminoglycoside-induced renal damage

4.1 Introduction

Aminoglycosides are one of the commonest antibiotics used in CF, and there is a risk of nephrotoxicity. Aminoglycosides have been implicated in both acute (*3,200*) and chronic kidney disease (*5*) in CF.

Various strategies have been employed to reduce the nephrotoxicity associated with aminoglycosides, e.g.use of less nephrotoxic agents like tobramycin (*348,572*) and therapeutic drug monitoring (*573,574*). Recently, an extended-interval dosing of tobramycin was studied in a large, randomised controlled trial (TOPIC study) in both adults and children with CF (*217*). Once daily tobramycin was found to be equally efficacious and less nephrotoxic compared to thrice-daily tobramycin in children with CF. Once daily administration of tobramycin has been adopted as a standard care in CF in many centres (*301*); however, time of day of administration varies.

4.1.1 Circadian variation in pharmacokinetics of aminoglycosides

Circadian variation in aminoglycoside clearance and nephrotoxicity has been demonstrated in various animal experiments (*575,576*); increased nephrotoxicity and reduced clearance were shown when aminoglycosides were administered in the rest period (daytime) compared to the activity period (night).

A circadian rhythm of glomerular filtration has been shown in humans suggesting that GFR is lower (33% variation from the mean) in the night compared to the daytime (*577*), possibly related to fluctuation in

the glomerular capillary hydrostatic pressure, but the exact mechanism is not clear (*578*). Studies in human subjects to investigate the circadian influence on the pharmacokinetics of aminoglycosides have shown conflicting results (*579-587*). Studies assessing chronopharmacokinetics of aminoglycosides that showed reduced drug elimination during the rest period were mainly based on multiple daily doses (*579,581,587*) or a continuous infusion regimen (*582*). Once-daily dosing of aminoglycosides has been shown to be less nephrotoxic than the thrice-daily dosing regimen (*217*). The studies that did not show any circadian variation in drug pharmacokinetics or peak-trough levels were mainly based on the once-daily use of aminoglycosides. These included three large studies, one of which was conducted in intensive care (*583*), and the other two included patients from both general medical wards and intensive care (*580,584*). Although there was no difference in the peak and trough levels of aminoglycosides administered at different times of the day, Prins et al. (*580*) showed increased nephrotoxicity of aminoglycosides when administered during the rest period, measured by a rise in serum creatinine levels. This finding is in contrast to what was found by Maarseveen et al. (*584*). Fauvelle et al. (*583*) did not explicitly compare nephrotoxicity between the groups, but there was no difference in the serum creatinine levels between the morning and evening groups. Some methodological differences could help explain the variation in results. The study by Prins et al. was non-randomised, and there were several confounders related to nephrotoxicity, e.g. patients with severe sepsis, people with hypotension, pre-existing low GFR, and concomitant furosemide therapy. There may be a possibility that the patients who received their first dose of antibiotics during the night (i.e. admitted to intensive care overnight) were sicker than those who received it at other times; hence, they had a greater

nephrotoxicity. On the other hand, Fauvelle et al. randomly assigned about 40 patients to receive netilmicin at different times of the day; those with septic shock were excluded, and no one received diuretics. The difficulty of random assignment of patients in the intensive care setting is understandable. The study by Maarseveen et al. was a retrospective, albeit a much larger study on about 700 adult patients with infections, where they analysed the intensive care and medical ward patients separately. No difference was found in PK parameters or nephrotoxicity in either set of patients.

The mechanisms explaining the temporal variation of renal toxicity of aminoglycosides, if present, are not clear. It has been suggested that interaction between aminoglycosides and proximal tubular luminal cell phospholipids varies as a function of pH and is stronger at lower pH. Since urine pH is higher during the periods of activity and food intake, the cellular uptake is less hence less nephrotoxicity (*588*). There is evidence in the literature to suggest that acute protein loading or chronic increased protein intake lead to an increase in GFR along with diuresis and natriuresis (*589*). Various mechanisms have been implicated to explain the association, which include haemodynamic or renal structural alterations or an effect of tubuloglomerular feedback.

4.1.2 Circadian variation of drug elimination in CF

There is limited literature about the circadian variation of drug elimination in children with CF. Any circadian influence on aminoglycoside elimination and nephrotoxicity has not been explored in this group. There is evidence of reduced clearance of ceftazidime during the resting hours in people with CF receiving the drug as a continuous infusion (*590*). Secondary analysis of data from the TOPIC study (*354*) suggested that the time of day of administration might be a factor affecting tobramycin elimination rate. The thrice-daily

tobramycin group had higher renal elimination rate constant (Kelr) compared to the once daily group (please refer to Figure 4-1). While once-daily dose was mostly administered in the evening (75% patients), the thrice-daily regimen was spread over 24 hours, and the elimination rate constant was an average of three doses. More rapid elimination should lead to reduced nephrotoxicity. If the difference in tobramycin elimination noted in two groups in the TOPIC study is related to a temporal variation in the tobramycin pharmacokinetics, and aminoglycosides are eliminated more rapidly when administered in the morning compared to the evening, then once-daily dosing in the morning could lead to decreased toxicity. This will provide an opportunity for a cost neutral way to reduce nephrotoxicity, which can be implemented readily.

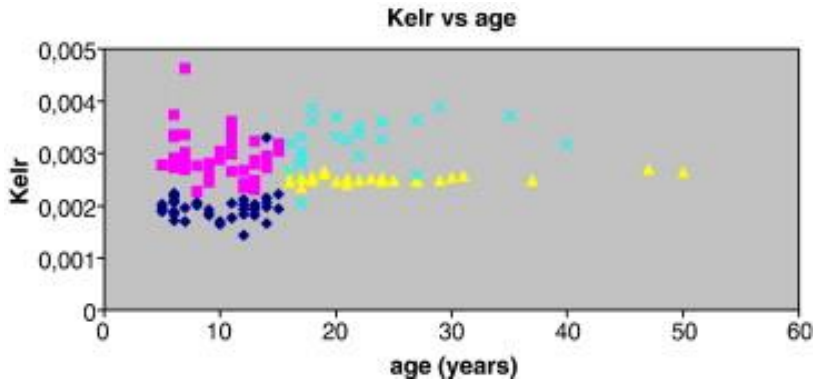


Figure 4-1 Relationship between renal elimination rate and age The paediatric group receiving once-daily tobramycin had lower Kelr (renal elimination rate constant);
Blue diamonds: paediatric patients receiving tobramycin once daily; pink squares: paediatric patients receiving tobramycin thrice daily; yellow triangles: adult patients receiving tobramycin once daily; turquoise crosses: adult patients receiving tobramycin thrice daily. (Reference: Touw et al. (354)).

4.1.3 Circadian rhythm in cystic fibrosis

An altered circadian rhythm could potentially influence any temporal variation in drug elimination. The circadian rhythm in hospitalised children with CF could be altered by illness or hospital admission. However, there are no published studies, which investigate this. In the present study, I aimed to establish whether a circadian rhythm was present in hospitalised children with CF and explore any effects on drug elimination.

4.1.3.1 Dim Light Melatonin Onset (DLMO)

Melatonin is a hormone produced by the pineal gland, which synchronises with the dark phase of the day-light cycle (*591*). It is a robust marker, which can establish the circadian phase in an individual (*592,593*). Rhythmical variation in melatonin levels with the circadian phase has been observed in various body fluids, including serum, saliva and urine (*594*). In normal physiology, melatonin levels are undetectable in the day time, and the rise correlates with the dark phase of the dark-light cycle (*591*). Levels are expected to rise from the evening, around 18:00 to 20:00 hr. Levels reach a peak during the night and decline in the morning, with a nadir in the daytime (*595*). A peak level occurring during daylight hours or a delayed onset of the rise from baseline levels, suggests an abnormal circadian phase (*596*). When only a partial melatonin profile is available, dim light melatonin onset (DLMO) is the most widely used marker of melatonin rhythm (*597*). DLMO is described as a marker of the rise of melatonin level. In individuals with normal circadian rhythm, DLMO is expected about 2-3 hours prior to habitual sleep (*594*). It is calculated as the first interpolated point above a threshold under dim light conditions and is supposed to be observed at 20:07 ± 1:04 (SD) hours in normal children between the ages 6-14 years (*598*). Various methods have been used to describe the threshold value for DLMO:

1. An absolute cut-off threshold of >4 pg/ml (*596*)
2. A threshold calculated at 2 SD above the average baseline (with 3 or more precise samples) (*599*)
3. Visual estimate of the point of change from the baseline to increasing levels (*594*)

The evidence to support either of these methods is limited, and different methods have been used in the literature making comparison difficult. For any method used, it is important to visualise the data for artefacts. In this study, I selected the absolute cut-off method to illustrate the rise in the melatonin levels (DLMO) in the evening to identify people with normal or low melatonin levels. Saliva samples were collected in the middle of the night and during next day to be able to visually inspect the graphs and identify any children with abnormal circadian phase i.e. rise of melatonin levels at a different time of the day (Section 4.3.3.7).

Based on the inferences from the animal studies and results from the TOPIC study, it was hypothesised that renal elimination of tobramycin is lower when administered in the evening (during the rest period) compared to the morning. I undertook the Circadian Rhythm In Tobramycin Elimination in Cystic Fibrosis (CRITIC) study to determine whether there is any difference in the elimination of tobramycin (elimination rate constant, clearance) when administered in the morning versus evening (i.e. 08:00 versus 20:00 hours) in children with CF. In addition, I measured salivary melatonin levels to establish whether a normal circadian rhythm was present in these children.

4.2 Aims

The aim of this study was to determine if the PK profile of IV tobramycin in children with CF varies as a function of the time of day of drug administration.

4.2.1 Primary outcome measure

The main objective of the study was to test the hypothesis that the renal elimination of aminoglycoside is higher in children with CF when IV tobramycin is administered at 08:00 hours compared to 20:00 hours. The elimination was measured as renal clearance of tobramycin.

4.2.2 Secondary outcome measure

The other objectives of the study were as following:

1. to compare the effect of time of day of administration on changes in the following during the course of therapy:
    a. Weight
    b. Pulmonary function- FEV_1, and FVC
2. To establish the circadian rhythm by melatonin assay and any relationship of abnormal rhythm with renal clearance of tobramycin
3. To assess the quality of sleep by a quality of sleep questionnaire

4.3 Methods (CRITIC Study)

4.3.1 Study Design

This study was an open-label, randomised controlled clinical trial. The PK study was designed by Professor Alan Smyth and Professor Dan Touw. The salivary melatonin analysis and sleep study questionnaire were designed by me along with support from Professor Alan Smyth and Dr Birgit Koch. It was conducted in four centres across the Midlands region of the UK including Nottingham University Hospitals, Pilgrim Hospital Boston, Lincoln County Hospital and University Hospitals Leicester.

Regulatory approvals were provided by National Research Ethics Service (Reference number: 10/H0408/98) and Medicines and Health Care Products Regulatory Agency (MHRA). Research and Development departmental approval was obtained for each site prior to undertaking study activities. The study was registered on clinical trials register: ClinicalTrials.gov (NCT01207245) (*600*). Informed consent was obtained by one of the two investigators: Dr A. Prayle or myself. Study samples were obtained by one of the two investigators: Dr A. Prayle or myself. The study was supported by the Medicines for Children Research Network, who provided some nursing and administrative support.

The study was conducted in accordance with the ethical principles that have their origin in the declaration of Helsinki, 1996 (*453*); the principles of Good Clinical Practice (*454*) and Good Laboratory Practice (*455*), the Department of Health Research Governance Framework, 2nd ed., 2005 (*456*), and in accordance with the Human Tissue Act, 2004 (*457*), licence number 12265: granted to the University of Nottingham's Faculty of Medical and Health Sciences and licence number 11035 for Queen's Medical Centre, Nottingham (*457*).

4.3.2 Recruitment methods

4.3.2.1 Inclusion criteria

1. Diagnosis of CF (defined as clinical features of CF with a positive sweat test and / or the presence of 2 genes known to be associated with CF disease)
2. Age between 5-18 years
3. Treating doctor has decided to commence a course of tobramycin on clinical grounds
4. Patient or parent / legal guardian able to give informed consent

4.3.2.2 Exclusion criteria

1. Previous episode of AKI
2. Solid organ transplantation
3. Evidence of renal impairment
4. Once-daily aminoglycoside unsuitable because of hypersensitivity or previous high trough levels on once daily dosing
5. Pregnancy

Participants were recruited from May 2011 until December 2012. Potential participants were introduced to the research team by the treating physician. The research fellow or research nurses gave information about the study, including verbal explanation and information leaflets. Once a decision had been made to commence IV antibiotics for the patients (who had agreed to participate in the study), informed consent was obtained from the patients or, in the case of children <16 years age, from their parents/legal guardians on consent forms approved by ethics committee. Assent was obtained from children <16 years whose parents had given consent. All staff involved in requesting consent (investigators: Dr A Prayle and myself) had received Good Clinical Practice training. Participants had at least

24 hours to consider the information sheets and decide whether to take part in the study.

4.3.2.3 Randomisation

A web-based system provided by Nottingham Clinical Trial Unit randomised participants in a 1:1 ratio to each arm. Patient details including date of birth were entered onto the website. The randomisation code and the time of day of administration (08:00 or 20:00) were then generated on the remote system, and the investigators were informed by an automatic email.

4.3.3 Study Methods

4.3.3.1 Drug Treatment

All children received tobramycin and at least one other IV antibiotic depending on the clinical decision by the treating physicians. The children were randomised to receive tobramycin either at 08:00 hr or at 20:00 hrs. This was the only randomised intervention; all other treatment was similar for both the groups and was according to the CF unit admission protocol. Other concomitant treatment was permitted except non-steroidal anti-inflammatory agents, furosemide and vancomycin.

Tobramycin was administered as a single daily IV infusion over 30 min at the specified time. It was delivered through any IV access device including peripheral cannula, percutaneous long line or a TIVAD. The exact time of initiation and stopping the infusion were recorded. The dose and duration of the therapy were decided by the treating physicians, which was in line with the UK CF trust antibiotic guidelines i.e. 10 mg/kg daily (*296*). The dose was modified, if necessary, according to the trough tobramycin levels prior to administration of the second dose.

4.3.3.2 Other investigations

Serum creatinine level was measured on each participant, prior to administration of the first dose of tobramycin. Weight was measured on day 1, day 8 and the end of therapy in indoor clothes without shoes. Spirometry was performed on the children, more than 6 years old, according to ATS/ERS Task Force recommendations (*601*). All these measures were done as part of routine clinical care of the children by the clinical team.

The work described in this thesis did not measure the difference in nephrotoxicity between morning and evening dosing, but rather the difference in tobramycin elimination between the two dosing times. A parallel study ran concurrently with my work, as another PhD project (Dr Andrew Prayle, University of Nottingham, UK). Dr Prayle's study compared the rise in excretion of various urinary biomarkers during the tobramycin therapy in the morning versus evening groups.

4.3.3.3 Tobramycin pharmacokinetics sampling and analysis

Two blood samples were collected on day 7 (± 2 days) of IV antibiotic treatment. These samples were taken at one hour and 3.5 to 5 hours after the end of the tobramycin infusion. The exact times when the samples were collected were recorded. Blood was taken by venepuncture, from a TIVAD (after taking about 5 ml discard) or from a finger prick. The sampling method was determined by patient choice. The blood samples were collected by one of the investigators (Dr A Prayle or myself) or by trained nurses (clinical) in case of accessing TIVAD. The dose of tobramycin, infusion timings (start and finish) and sample timings were recorded on case report form. The samples were sent directly to the laboratory or stored refrigerated until being sent to the laboratory to be processed. The tobramycin levels were analysed using a homogenous enzyme immunoassay technique (Beckman Coulter Inc, California, USA).

4.3.3.4 Salivary melatonin sampling and analysis

The saliva samples were collected by one of the investigators (Dr A Prayle or myself) or Medicine for Children Research Network nurses. The saliva was collected by passive drooling method into two saliva collection containers (UltraSal-2™, Oasis Diagnostics® Corporation, Vancouver, USA), Figure 4-2. Patients were in dim light (<30 Lx) from one hour before the collection of the first sample and through the night.

Any samples that were visibly contaminated by blood or food were discarded. Various precautions were taken for the saliva collection:

- To avoid contamination with food particles, patients were asked to have any meals at least an hour before the sampling. They were advised to rinse their mouth with water 10 min. before sample collection if they had eaten recently.
- Patients were advised to remain in bed for the most part of the night to minimise the effect of activity and posture changes during the collection period.
- They were asked to avoid ibuprofen and food with high sugar and high caffeine content just before sampling.
- Patients were advised to avoid brushing their teeth 45 min prior to sampling.

All the conditions during collection of salivary samples were recorded on a saliva collection sheet. Six to seven samples were collected on one night (day 7 ± 2 days), at 18:00, 19:00, 20:30, 21:30, and 24:00; and on the next day at 06:00 and 12:00hrs. If the patients were asleep during any of the sampling times, that sample was omitted. The samples were labelled with the study code and a participant code number. The samples were stored at -20° Celsius immediately until next morning after which they were stored at -80° Celcius in the

laboratories based in the Division of Child Health, Obstetrics & Gynaecology at the University of Nottingham. The salivary melatonin analysis was conducted in the Clinical Chemical Haematology Laboratory based at the Gelderland Valley Hospital, The Netherlands. The samples were transported in a dry ice shipment confirming to IATA Dangerous Goods Regulation, 53rd Edition, 2012 (*549,550*). The melatonin salivary assay was undertaken by radioimmunoassay method (*599*) (BÜHLMANN direct saliva melatonin RIA, Schönenbuch, Switzerland).

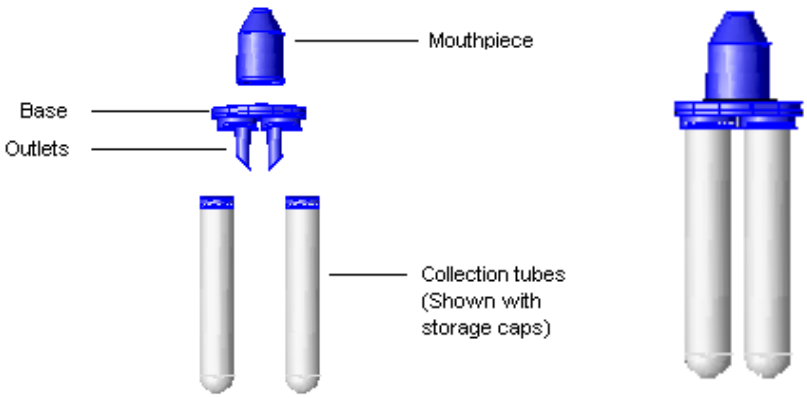


Figure 4-2 UltraSal™-2 Ready to use saliva collection device (Oasis Diagnostics, USA): components and when assembled

4.3.3.5 Sleep Questionnaire

A sleep quality questionnaire was completed during the hospital stay (day 7 ± 2 days). A large number of sleep questionnaires are available in the literature (*602*). However, most of these are designed for the assessment of sleep disordered breathing or for psychometric analysis; these comprise of sleep diaries / logs and evaluate long periods, i.e., intended for use over weeks or months. Most have not been validated. None of the published questionnaires assessed sleep quality during a hospital stay, compared with home. I, therefore, designed a bespoke sleep quality questionnaire. One version of the questionnaire is suitable for use by young children with no parent present overnight during the hospital admission. The questionnaires (Appendix Chapter IV Section 8.4.1) included a 'sleep questionnaire for children 12 years and younger', 'a sleep questionnaire for parent' and a 'sleep questionnaire for children 13 years and older' as appropriate. A visual analogue score (from 0-10) was used to describe the perception of the child and parent (when staying overnight) of sleep quality. The questionnaires also asked about the difference between the quality of sleep at the hospital compared to home. Visual analogue scores of 7-10, 4-6 and 0-3 were described as good quality, moderate quality and poor quality sleep respectively.

4.3.3.6 PK modelling

The PK analysis was performed using MW pharm software package, version 3.7 (Mediware a.s., Prague, Czech Republic) (*358*), based on single compartment model. Data on patient's age, sex, date of birth, height, weight, serum creatinine concentration, tobramycin dose, infusion timings, tobramycin sample timings and serum tobramycin levels were entered into the program by investigator Dr A Prayle. The original study protocol described the primary outcome measure as a comparison of renal elimination rate constant (kelr) between two

groups, which was later (after recruitment was completed) changed to total drug elimination; more details are given in the discussion section (Section 4.5). A Bayesian analysis method was used which uses population PK parameters as a starting point and then, adjusts these based on the serum levels taking into consideration the population PK values.

Following parameters were provided by software package:

1. Elimination rate constant (Kel) (hr^{-1})
2. Total Drug clearance (CL) (L/hr)
3. Volume of distribution/Kg (V_1) (L/Kg)
4. Volume of Distribution (Vd) (L)
5. Area under concentration time curve (AUC) (mg*h/L)

The details of PK analysis are described in Appendix (please refer to Appendix Chapter IV Section 8.4.2).

4.3.3.7 Analysis of circadian rhythm

For the study purposes, we defined DLMO as the time when the absolute salivary melatonin values increased to ≥ 4 pg/ml while rising from a lower baseline value (*598*). Patients were described as having normal circadian rhythm when DLMO was observed between 19:00 to 22:00 hrs., or when a rise in melatonin level was observed during the night with an evidence of a fall / nadir in the daytime (*595,603*).

The melatonin graphs were reviewed independently by two investigators (Dr A Prayle and me), and a consensus was reached. The decision on the type of rhythm was assessed and approved by an investigator who is an expert in the field (Dr Birgit CP Koch, Erasmus Medical Centre, Rotterdam, The Netherlands).

4.3.4 Statistical Methods

4.3.4.1 Primary outcome and Sample size justification

At the study initiation, the primary endpoint of the study was Kelr (h^{-1}/ml/min/1.73m^2). A difference of 0.000734 (h^{-1}/ml/min/1.73m^2) represented a difference of 30% of the values seen in the TOPIC study (*217*): based on 30% of the average of once daily and thrice daily paediatric PK analysis. The SD of Kelr is estimated to be 0.00034 (hr^{-1}/ml/min/1.73m^2), (using data from both once-daily and thrice-daily groups of TOPIC study). Taking a power of 95%, and performing a sample size calculation using Minitab version 15 (Minitab Inc., PA, USA), eight participants were required in each arm. The study aimed to recruit 10 participants in each arm to allow for a drop-out rate of 20%.

During the conduct of this research, data from CEFIT CF study became available (Chapter 2, Section 2.4.3) which indicated that the GFR estimated by the Schwartz formula did not accurately assess the GFR in patients with CF. Hence the primary outcome measure for the CRITIC study was changed post hoc (after recruitment and data collection was complete) to avoid having to use eGFR in the calculation. Instead, total body drug clearance was measured using a one-compartment model and a Bayesian analysis method. TBC incorporates both renal and metabolic clearance values.

Any patients who had data available for height, weight, tobramycin dose, serum creatinine and serum tobramycin levels for any one day of the therapy were included in the analysis.

Differences in the weight and lung function parameters from start to end of treatment were calculated and compared between the groups; however, the study was not powered to detect differences in the secondary endpoints.

4.3.4.2 Statistical tests

Results were analysed using SPSS version 22, (IBM Corp., Armonk, NY, USA) and graphs were prepared using GraphPad Prism Version 7.03. Salivary melatonin graphs were prepared using software R version 2.15.2 by Dr A Prayle. The parameters were assessed for normality by viewing a histogram. The groups were compared using an independent samples t-test for normally distributed parameters and a Mann-Whitney U test for non-normally distributed parameters. A P value of less than 0.05 was taken as statistically significant.

4.4 Results

Twenty-five eligible subjects were approached, and 18 of these consented to participate in the study. Nine subjects were randomised to the evening group and nine, to the morning group (please see Figure 4-3). Both groups were similar in their characteristics including age, sex, initial weight, height, serum creatinine and eGFR (Schwartz formula) (*604*) (please refer to Table 4-1)*.*

4.4.1 Pharmacokinetics results

All the patients received treatment according to the randomised groups (i.e. morning or evening). All eighteen randomised patients had samples taken for tobramycin pharmacokinetics, and hence included in the primary analysis. The dose of tobramycin was 10 mg/kg/24hours for all the patients. Median day of therapy when PK samples were taken was similar for both the groups (median 8 days, range 2-9 days). Two patients had their PK samples collected on day 2 of therapy, which is outside the range specified in the protocol (5-9 days). Both these children were randomised to the evening groups and had serum creatinine values in the upper normal range, 92 and 93 µmol/L respectively (reference range in 9-17 years of age: 46 to 94 µmol/L equivalent to 2.5 to 97.5 centile (*605*)). The responsible physician directed that the tobramycin levels should be taken early to ensure the trough tobramycin level had not become elevated. I felt that it was not justified to repeat the sampling on day 5 to 9, solely for research purposes.

The PK modelling results for IV tobramycin did not show a significant difference in PK parameters between the two groups (Table 4-2). The median value of total drug clearance for tobramycin was 4.1 L/hr (IQR 3.77 to 4.47) in the morning group compared to 3.54 L/hr (IQR 3.39 to 4.51) in the evening group (P value=0.86) (please refer to Figure 4-4).

The results for other parameters including Kel, half-life, AUC, and Vd were also similar for both the groups (Table 4-2). In addition, both the groups did not have any significant difference in Kelr (based on Schwartz formula), which was originally designated as the primary outcome measure.

4.4.2 Other clinical parameters

The total duration of the course of IV tobramycin was 14 days in 15 of 18 patients. In one patient from the morning group, the antibiotic course was stopped at 10 days due to IV access issues. In two other patients, tobramycin was changed to a different antibiotic by the clinicians; the causes included lack of sensitivity of the organism to tobramycin in one and concern regarding high initial serum creatinine value in the other.

Results, including both initial and end of therapy, for spirometry and weight, were available for 13 patients and 14 patients respectively. One patient had tracheostomy so spirometry could not be performed (and hence no lung function data). In two patients, no secondary outcome data were available as one patient completed their course at home after spending 5 days in the hospital and the other patient was transferred to their referring hospital (who was not participating in the study) after one week of treatment. Two patients from the evening group were no longer in the study at the end of the antibiotic course as tobramycin was replaced with another antibiotic at day 5 and day 3 of therapy as described in the paragraph above.

4.4.2.1 Weight

The change in the weight from the beginning to the end of the treatment was not significantly different between the two groups (Table 4-3).

4.4.2.2 Lung function tests

The change in lung function parameters, FEV_1 and FVC, from the beginning to the end of the therapy was not significantly different between the two groups (Table 4-3).

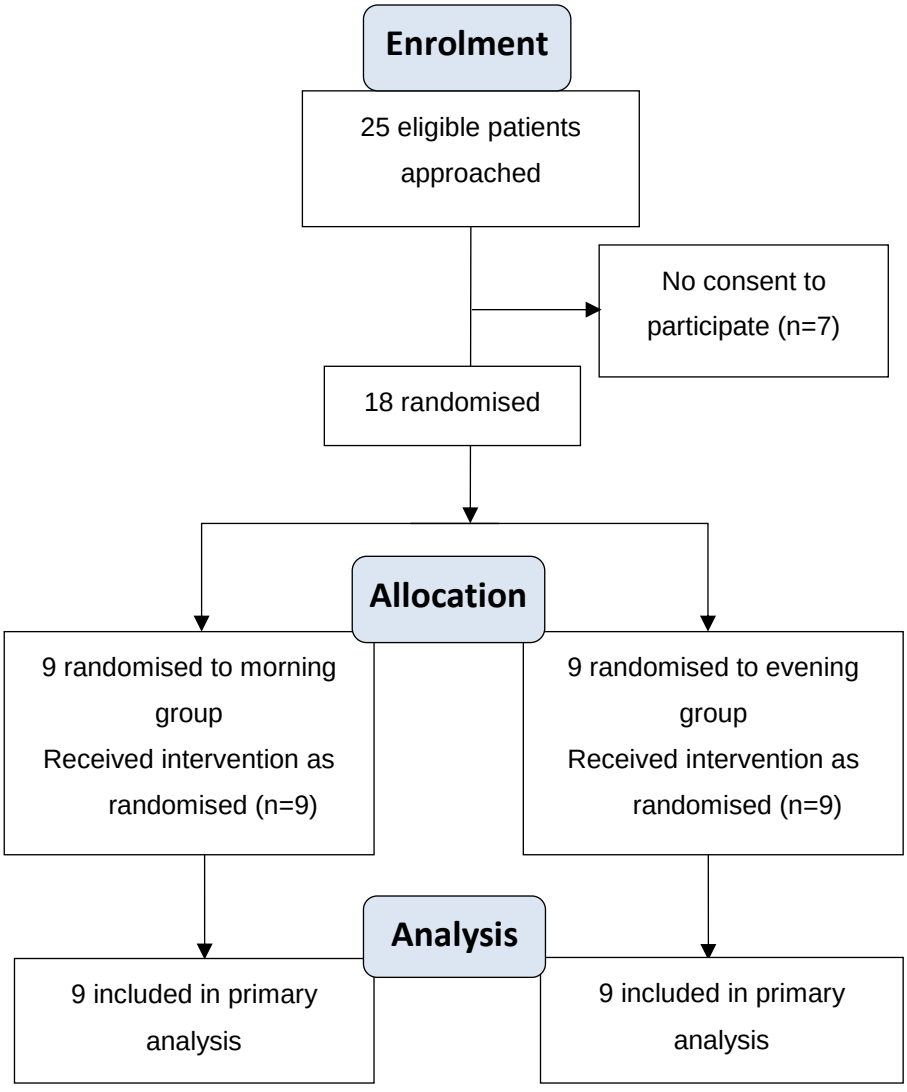


Figure 4-3 Consort diagram of the study participants

Table 4-1 Demographic Characteristics			
Demographic Characteristic	**Morning Group Median (IQR)**	**Evening Group Median (IQR)**	**P value (Mann-Whitney U test)**
Number	9	9	
Age (Years)	12.5 (12.3-15.1)	14.5 (12.8-14.9)	0.86
Weight (Kg)	39.2 (30.4-52.0)	42 (38.5-50.7)	0.73
Height (cm)	146.0 (137.0-165.0)	164.0 (162.0-167.0)	0.34
Estimated GFR ($ml/min/1.73^2$)	136.0 (123.4-165.1)	122.0 (100.9-159.0)	0.19
Serum Creatinine (mmol/L)	57.0 (40.0-60.0)	56.0 (50.0-79.0)	0.26

GFR: glomerular filtration rate; IQR: interquartile range

Table 4-2 Pharmacokinetics Results			
Pharmacokinetic Parameter	**Morning Group Median (IQR)**	**Evening Group Median (IQR)**	**P value (Mann-Whitney U test)**
Number	9	9	
Kel (h^{-1})	0.42 (0.37 to 0.44)	0.36 (0.34 to 0.46)	0.73
Clearance (L/h)	4.1 (3.77 to 4.47)	3.54 (3.39 to 4.51)	0.86
$T_{1/2}$(h)	1.66 (1.56 to 1.88)	1.89 (1.49 to 2.04)	0.73
AUC (h.mg/L)	4.15 (3.09 to 4.85)	3.72 (3.53 to 5.35)	0.73
V_1 (L/kg)	0.26 (0.22 to 0.31)	0.24 (0.21 to 0.32)	0.61
Vd (L)	10.2 (9.06 to 10.93)	9.72 (8.45 to 12.26)	0.93
Kelr (h^{-1}/ml/min/1.73m^2)	0.0026 (0.0024 to 0.0031)	0.0026 (0.0022 to 0.0035)	0.86

Kel: elimination rate constant; AUC: area under curve; $T_{1/2}$: half-life of tobramycin; V_1: volume of distribution/kg; Vd: volume of distribution; Kelr: renal elimination rate constant; IQR: interquartile range

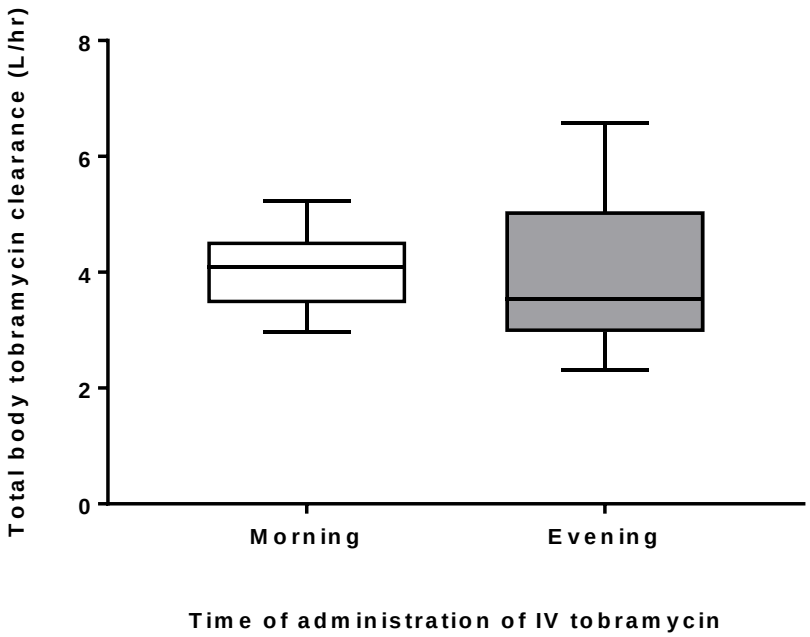


Figure 4-4 Box plot of total body clearance of tobramycin (for morning and evening administration groups); the box shows the interquartile range, the black line inside the box shows the median value and the whiskers show the maximum and minimum values of tobramycin clearance; the white and the grey boxes represent morning and evening groups respectively

Table 4-3 Clinical Parameters			
Clinical Parameter	**Morning Group Median (IQR)**	**Evening Group Median (IQR)**	**P value Mann-Whitney U Test**
Number (N)	7	6	
Δ Weight (Kg)	1.2 (0.65 to 1.53)	0.60 (0.30 to 0.70)	0.13
Δ FEV_1 (L)	0.19 (0.07 to 0.22)	0.23 (0.18 to 0.29)	1.0
Δ FVC (L)	0.32 (-0.01 to 0.38)	0.19 (-0.10 to 0.33)	0.46

Δ depicts a change in the parameter from treatment initiation to the end; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; IQR: interquartile range

4.4.3 Salivary melatonin levels

The results for salivary melatonin levels were available for 13 patients. Two patients had insufficient samples for analysis; hence, they were excluded from analysis. Two patients had all seven samples, 5 patients had six specimens, and 4 patients had 5 samples available for analysis. Midnight samples were not taken for 4 patients (deep asleep) and an afternoon sample could not be taken for one child. Melatonin profiles of 7 of 11 patients suggested a normal circadian rhythm whereas 4 of 11 patients showed an altered circadian rhythm (please refer to Figure 4-5 and Figure 4-6 respectively). One patient showed excessively high melatonin levels from evening until next afternoon without any rhythmic variation (graph T15). Another patient had very high concentrations all through the evening (from 18:00 onwards) and then rise in levels during the night (Graph T07). One patient showed no pattern at all (T18), and another one showed a fall in melatonin levels near midnight instead of the expected peak levels (T09).

Two patients with an abnormal rhythm were in evening group and two in the morning group. The numbers were too small to allow a comparison of the difference in the PK parameters. Three patients of the four whose midnight saliva samples were missing had good circadian rhythm.

The sleep quality questionnaire was completed by 10 children; seven children rated their sleep as good quality, three children rated it as moderate quality. Only two children rated their sleep in the hospital worse than at home whereas 5 of the 7 parents who completed the questionnaires perceived it is worse than at home. Only 2 of the 4 children with an altered melatonin rhythm completed the questionnaire, and both of them rated their sleep as good quality.

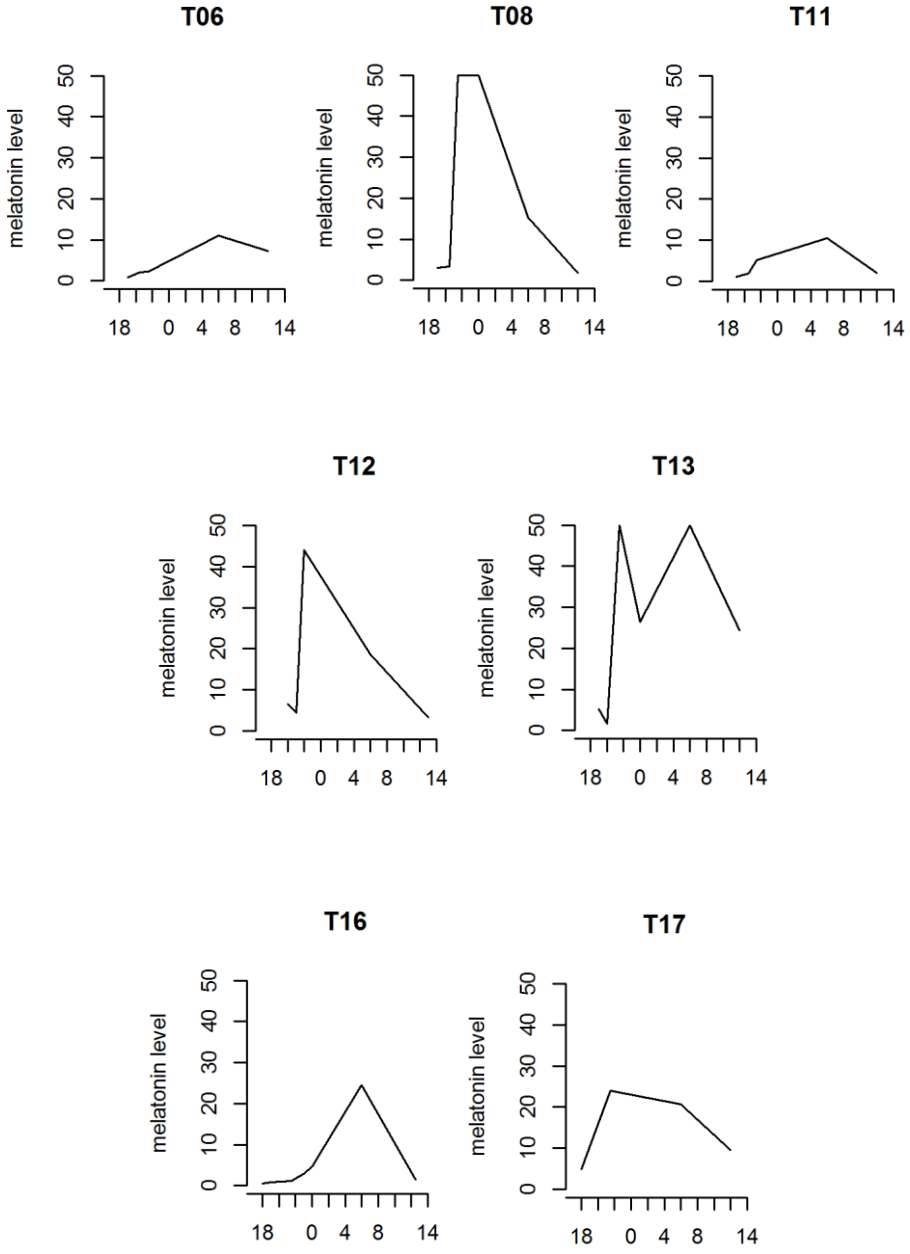


Figure 4-5 Normal Melatonin Profiles; the graphs show melatonin levels (Pg/ml) (y-axis)against the time (x-axis) when the sample was taken in 24-hour clock; the T numbers illustrate graphs for individual patients.

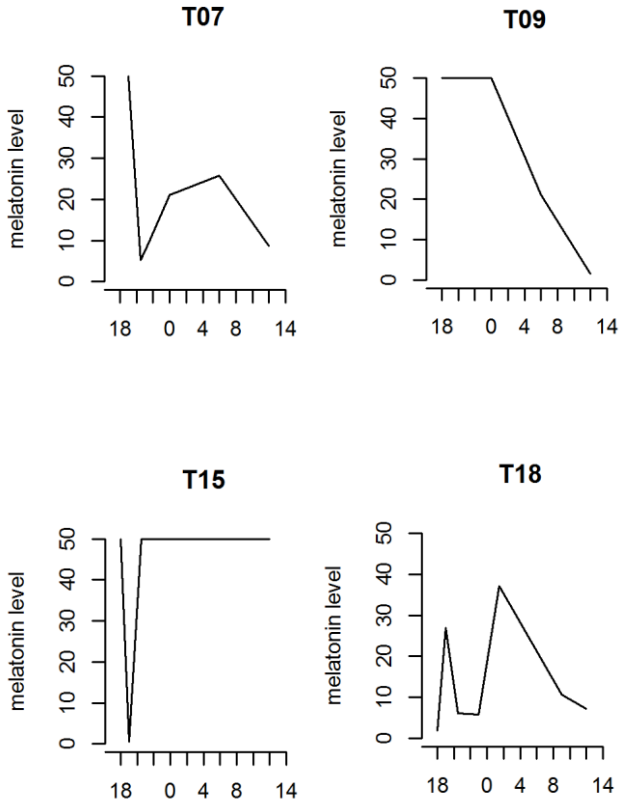


Figure 4-6 Abnormal Melatonin Profiles, the graphs show melatonin levels (Pg/ml) (y-axis) against the time (x-axis) when the sample was taken in 24-hour clock; the T numbers illustrate graphs for individual patients.

4.5 Discussion

This is the first randomised PK comparison investigating the circadian variation of aminoglycoside elimination in children with CF. This study did not demonstrate any difference between renal clearance of IV tobramycin administered in the morning or evening, during the first 10 days of therapy. In addition, time of day of administration did not affect any clinical parameters including changes in lung function and weight during the course of treatment. A normal circadian rhythm was illustrated in 7 of 11 children.

As once daily aminoglycoside regimens have become widely used, it has become clinically relevant to determine whether there is a circadian variation in nephrotoxicity. Kidneys are the principal route of excretion of aminoglycosides, which is dependent on glomerular filtration. Based on studies in uninfected animals and human volunteers, GFR generally falls during sleep (*577*) and should lead to reduced clearance; hence, decreased elimination of tobramycin during night seems plausible. Only a small proportion of the filtered drug is absorbed in the kidneys by a saturable process (*320*). Thus, reduced elimination could lead to greater nephrotoxicity.

Studies on experimental animal models have persistently shown reduced clearance and increased nephrotoxicity of aminoglycosides, including tobramycin when administered during the rest periods of animals (*575,576*). Studies on human subjects, however, showed variable results. Most studies that found reduced clearance during evening used multiple daily regimens (*579,581*) or continuous infusion for administration of aminoglycosides (*582*); some studies used the intramuscular route of administration (*585*) and were conducted in healthy volunteers. On the contrary, larger studies that compared once daily regimen in humans did not show any difference in

clearance with different times of administration (*580,583,584*). Although our results are similar to Prins et al. and Fauvelle et al. (*580,583*), it is important to highlight that their patient populations were different from ours. Patients with CF are shown to have higher renal clearance (*327*), whereas patients with sepsis are more prone to develop AKI (*606*). At the same time, our study used samples between one and three half-lives of tobramycin, which is the optimum time (*607*) to detect any differences in elimination rate of aminoglycosides compared to trough levels. Prins et al. (*580*) found increased risk of nephrotoxicity associated with aminoglycosides administered in the night; however, the patients were not randomly assigned to the groups, and it is possible that the patients admitted in the night (and hence receiving their dose in the night) were sicker than the patients admitted in the daytime.

Species-specific differences may explain the discrepancy between human and animal studies on tobramycin. Most experimental animal studies use significantly higher aminoglycoside doses, compared to those employed in human subjects and so the results of animal work may not be applicable to humans.

The PK results from the TOPIC study (*354*), which investigated once versus thrice-daily tobramycin dosing in CF, demonstrated nearly 30% higher elimination rate of tobramycin in the thrice-daily group compared to once daily group. The results from our study do not agree with the notion that this difference was due to circadian variation in the renal elimination of tobramycin. Our study was adequately powered to detect a difference of 30% in Kelr between the groups. It follows that, if there was a real variation, related to the time of day of administration, there was a 95% chance of it being detected. We had set out to use Kelr as a primary outcome measure. The PK analysis

software uses a creatinine-based formula to estimate GFR and to calculate Kelr. The CEFIT CF study (Chapter 2, Section 2.4.3) established that the Schwartz formula is not a reliable estimate of GFR in children with CF. Other previous studies have also raised questions regarding the reliability of eGFR for prediction of true GFR (*215,429*) in patients with CF. The revised primary outcome measure, total body drug clearance, is not dependent on eGFR and hence should be a more robust parameter to compare the pharmacokinetics. The primary outcome measure of the study was changed after the recruitment and data collection was complete. I performed a post hoc power analysis (G*Power (*608*)) to calculate the observed power (i.e. 1-β that is the probability of correctly rejecting the null hypothesis). The mean drug clearance in both the groups was 4.04 L/hr; taking that as a baseline value (pooled SD of both groups 1.1), aiming to identify a difference in total clearance of 30% with a P value of 0.05 and using our sample size (9 in each arm), the observed power of the study was 0.65. To achieve a power of 80%, while keeping all the other parameters same, the study would need to recruit 18 patients into each arm. This suggests a type II error, and another adequately powered study to assess the difference in renal elimination of tobramycin is recommended.

As the aminoglycoside toxicity is shown to increase with increasing duration of therapy, there may be a possibility that renal elimination declines with increasing duration of treatment, in which case, circadian variation in tobramycin elimination may be more likely in patients receiving two or three weeks therapy. This study cannot explore this possibility.

This study did not measure the difference in nephrotoxicity between the two groups; however, a parallel study (conducted by Dr A Prayle), compared the change in urinary Kidney Injury Molecule-1 (KIM-1)

concentration during the course of tobramycin therapy between the morning and evening groups from our study participants. KIM-1 is a cell membrane glycoprotein located in the renal proximal tubular cells. It has been described as a biomarker of renal tubular injury and repair processes (*609*). The mRNA and protein for KIM-1 are expressed at very low levels in normal kidneys, but the expression level is increased significantly along with increased urinary excretion following nephrotoxic insults, before the rise of serum creatinine (*609*). In a study on children with CF receiving tobramycin, there was significant elevation (3 fold increase, 95% CI 1.9-4.2) of KIM-1 levels at the end of IV course from the pre-treatment baseline (*610*). The analysis of KIM-1 levels from patients in the CRITIC study (*611*) showed an increase in urinary excretion of KIM-1 (from levels just before treatment initiation to the end of IV tobramycin treatment course) in the group receiving the drug in the evening whereas the levels remained almost static in the morning group (*611*). This suggests evidence of increased toxicity with the evening administration of tobramycin.

The lack of difference between the PK parameters for morning and evening administration conflicts with the finding of increased nephrotoxicity during evening administration. This may point towards an alternative mechanism that has diurnal pattern e.g. urinary pH (*612*) or effect of food intake (*613*). A circadian rhythm has been described for urinary pH in both experimental animals and humans (*612,614*). Urinary pH falls during the night (or during the rest period for animals) to a nadir at about 6 am followed by a rise in the morning. The renal toxicity of aminoglycosides is described to be higher in the periods with lower urinary pH (*588,613*). Aminoglycosides are positively charged molecules and bind to negatively charged acid phospholipids in the proximal tubular luminal cells leading to subsequent inhibition of phospholipases and accumulation of myeloid

bodies (*615*). There is a good correlation between phospholipidosis and aminoglycoside toxicity, and thus it has been speculated that the circadian variation in the aminoglycoside toxicity is related to (circadian) variation in the urinary pH (*588*). What drives the temporal changes in urinary pH is not very clear, and relation with food intake has been suggested (*613*). Low albumin levels and poor nutritional status have been associated with increased risk of nephrotoxicity (*202,344*). The feeding period has been shown to be crucial in the circadian variation of aminoglycosides and fasting has been shown to abolish the circadian variation in aminoglycoside-induced nephrotoxicity both in experimental animals (*588,616*) and human studies (*586*). Time-restricted feeding in experimental animals showed that irrespective of the dark-light cycle, the minimum toxicity of gentamicin injection was during the feeding period and maximum toxicity was during the fasting period (*617*). In a PK study, a period of protein loading significantly increased the TBC of gentamicin in human volunteers (*586*). Though urinary pH was shown to decrease just after main meals and to remain low for some time, overall the urinary pH is higher during periods of food intake (in the day for humans or active period for animals) compared to fasting (or rest) period; this may explain the phenomenon of reduced nephrotoxicity by aminoglycosides (*588*) during the active period.

To exclude the possibility of an altered circadian rhythm in relation to a pulmonary exacerbation in patients with CF, we measured salivary melatonin profiles to establish a circadian rhythm. This is the first time circadian rhythm has been investigated in this population. To allow for adjustment to the hospital environment, the melatonin profile was collected in the middle of the course of antibiotics. The results were available in 11 (60%) participants. A good rhythm was demonstrated in 7/11 children; an altered or no rhythm was observed in 4/11 patients.

There is very limited literature available regarding circadian rhythm and sleep disturbance during hospitalisation in children. Both parents and children experience reduced sleep quality during hospital stay (*618-620*). Various factors that can affect sleep include high noise levels (from other patients, staff and monitoring equipment), administration of medication and routine nursing observations (*618,620*). Highest prevalence of poor sleep (62%) was noted in children with chronic medical conditions whereas children with cancer had lowest prevalence of poor sleep (33.3%) (*618*). Children who had slept poorly in the week before hospital admission had poorer sleep during hospital stay compared to those children who had slept well during the week before hospital admission (*618*). Similar results of poor sleep quality during hospital stay were reported in other studies including children with cancer (*621,622*) and children in intensive care (*623,624*). Septic patients in intensive care unit were shown to have lost circadian rhythmicity of urinary 6-sulfatoxymelatonin levels (a metabolite of melatonin in urine) (*625*), which was not related to level of light exposure in intensive care unit (*626*). Nocturnal plasma melatonin levels were significantly higher in children with sepsis in septic shock state compared to the children with sepsis but not in septic shock state (*627*). In addition, there are suggestions that melatonin may play a role of immue buffer i.e. acting as an immunostimulant under immunosuppressive conditions and acting as an anti-inflammatory agent in the conditions with accelerated immune response e.g. acute inflammation (*628*). These findings suggest that circadian rhythm markers may be altered in disease states affecting interpretation of circadian phase studies during periods of acute illnesses. Due to the small number of patients, it was not possible to study the effects of disrupted circadian rhythm on tobramycin elimination. However, these results suggest that circadian rhythm is

disrupted in some hospitalised patients with CF. The saliva collection period was extended (one sample in the middle of the night and two samples on the following day), from the number required to identify DLMO, with an aim to avoid misdiagnosis of the type of circadian rhythm and identify circadian phase lag. Some caution needs to be exercised with these results as the saliva profile was incomplete (e.g. no overnight sample available) in some patients. There may be a potential bias involved, as it is more likely that the patients with altered circadian rhythm are awake in the middle of the night compared to children with good circadian rhythm (who were in a deep sleep at midnight, and hence missed giving a sample that time).

Many CF patients perceive their sleep to be of poor quality (*629-631*). There is also objective evidence of reduced sleep efficiency in adults and children with CF, even while not having an exacerbation (*630-632*). Sleep efficiency has been associated with severity of lung disease (*632-634*) and in some studies with nocturnal oxygenation (*630*) levels. In children with CF, two factors could influence the quality of sleep: effects of pulmonary exacerbation (e.g. a cough) and disruption of routine through hospital admission. In this study, the majority of the patients rated their sleep as good quality; however, this questionnaire was designed to give a reflection of only one night in the hospital. Objective measures such as actigraphy, polysomnography and validated questionnaires need to be used to assess the sleep quality in relation to melatonin levels. We did not measure oxygen saturations overnight as part of this study.

Our observation of a disrupted circadian rhythm in some children with an exacerbation of their CF, who have been admitted to hospital, could be explored further. In addition to regulation of biological rhythms, melatonin has multiple roles (*635*), which include modest

antioxidant properties (*636*), and has been proposed as a potential therapeutic agent (*637*) to combat oxidative stress in CF airway epithelial cells related to deficiency of glutathione reductase (*638*). Whether the altered melatonin rhythm and high levels in some of our patients are related to sleep inefficiency or reflect a response to inflammation or oxidative stress will need further investigation.

4.6 Conclusion

In summary, this randomised controlled study found no difference in the renal clearance for IV tobramycin after its administration in the morning versus evening in children with CF during the first 10 days of therapy. Based on our results it is not possible to recommend morning or evening as preferred time for administration of IV tobramycin; however, it is worthwhile exploring the difference in renal clearance by time of day of administration in an adequately powered study and with longer duration of treatment. The possibility of a circadian variation in nephrotoxicity that is independent of tobramycin elimination also needs to be explored. An altered circadian rhythm, suggested by aberrant melatonin levels, was observed in a large proportion of hospitalised children with CF.

Chapter-5 Bronchoscopy Guided Antimicrobial Therapies in CF

5.1 Introduction

[Note: This review has been published previously in the Cochrane database of systemic reviews with the latest update in January 2016 (*639*). I am thankful to John Wiley & Sons for granting me permission to reuse the content of the publication in my thesis/dissertation: licence number 3958231248646, Date 20/09/2016 and 4435960344862, Date 25/09/2018 (please refer to Appendix Chapter V Section 8.5.1).]

Respiratory disease is the cause of most mortality and is a major contributor towards morbidity related to CF. There are repeated episodes of pulmonary infections, termed as exacerbations, which manifest as acute deterioration of respiratory symptoms. These may be associated with worsening of lung function or loss of weight (*640,641*). Over a period of time, these exacerbations may lead to progressive structural lung damage and poor lung function (*642*).

Respiratory infections in CF are caused by a limited range of organisms. At an early age, the commonest organisms are *S. aureus* and *H. influenzae* which are followed very soon after by *P. aeruginosa* (*446*). Over a period, the initial intermittent infections by *P. aeruginosa* may develop into a chronic infection (*643*), which is present in more than 50% of the adult CF population (*446*). Chronic *P. aeruginosa* infection is associated with increased morbidity and mortality (*643-647*)

The management of CF-lung disease incorporates different aspects such as the prevention and treatment of infections, chest

physiotherapy and mucus clearance techniques. The isolation of infecting organisms is important as these are more readily eradicated at an early stage in the infectious process. For example, eradication of an initial infection with *P. aeruginosa* can be achieved (*648,649*) and is recommended in guidelines (*647*). However, chronic pulmonary infection with *P. aeruginosa* cannot be eradicated. Eradication has become a major treatment goal in people who are *P. aeruginosa*-naïve as it delays the onset of chronic infection (*650*). To achieve early identification of organisms, routine surveillance is conducted which includes regular two- to three-monthly microbiological cultures of respiratory specimens (*45,647*). These include either spontaneously produced or induced sputum. Very young children, many older children and even some adults are unable to expectorate sputum. In such cases, the standard practice has been to use upper respiratory swabs including throat or oropharyngeal swabs (a cotton-tipped swab is rotated in the back of the throat or tonsils) or 'cough swabs' (a cough swab is taken by holding a sterile swab at the back of the throat and asking the individual, who is often a child, to cough). The same methods are used to guide the treatment of infective exacerbations.

Bronchoscopy is an endoscopic procedure, which helps visualise the upper and lower airways using a bronchoscope. Flexible bronchoscopy was first introduced by Ikeda in 1968 (*651*) and was first reported to be used in children in 1978 (*652*). Since then the technique has been significantly improved, and safety of the procedure has been described (*653-655*).

Several special bronchoscopic procedures are used to aid diagnosis. Bronchoalveolar Lavage (BAL) and protected bronchial brush (PBB) sampling have been used to obtain specimens from the lower respiratory tract. The use of BAL involves the instillation of small

aliquots of saline into bronchi followed by its recovery by suction, whereas PBB sampling allows direct sampling from the airway mucosal surface.

The newborn screening programme was started for CF with one of its aims being the improvement of clinical outcome for people with CF by making the diagnosis of CF soon after birth and by providing early interventions. It has been shown to have an effect on nutritional status (*656*), but a direct evidence of a significant difference in the status of the lung disease (*657-659*) has not been found.

It is recognised that the onset of lung disease happens very early in the course of CF, and infection and inflammation have been documented in BAL in the first few months of life by many groups, including asymptomatic infection with *P. aeruginosa* (*660-662*). Early identification of the correct organism should allow appropriate treatment and the use of eradication regimens (where appropriate e.g. for *P. aeruginosa*), with the aim of avoiding chronic infection and the related morbidity. Improving the accuracy of microbiological testing is, therefore, a logical goal.

Traditional surveillance methods to guide the treatment of acute exacerbations and to prevent the development of chronic infection include the identification of pathological organisms from sputum and, in non-expectorating patients, of organisms from the oropharynx, e.g. from a throat swab or a 'cough swab'. However, the upper respiratory tract is inhabited by a large number of bacteria as normal commensals (passive inhabitants that do not cause active disease). This leads to contamination of the upper respiratory specimens and can affect the culture results. It has been shown that oropharyngeal cultures, particularly in very young children, have poor sensitivity for lower respiratory tract infection (*663*). The most reliable method for

obtaining lower respiratory secretions is BAL, and many studies have shown cultures based on these samples have a higher yield of organisms as compared to standard methods (*663-666*). BAL could potentially be useful for routine surveillance of people with CF, but it comes with the drawback of being an invasive procedure with the need for anaesthesia and a hospital visit.

There has been a change in practice in many CF centres, based on the need for early identification of infections; bronchoscopy has been used to collect lower respiratory specimens routinely from young children and from adults in some specific situations. Studies have suggested that this practice has led to the identification of new organisms and use of different antibiotics in situations where bronchoscopy has been performed both, in symptomatic individuals (*664*) and in asymptomatic individuals (*662*). No systematic review has been undertaken to assess the effect of routine bronchoscopy-guided antimicrobial management of acute and chronic respiratory disease in children and adults with CF compared to standard management (which is directed by culture of oropharyngeal swab or sputum, as described above).

5.2 Objectives

This review was conducted with an aim to evaluate the use of bronchoscopy-guided antimicrobial therapy in the management of lung infection in people (both adults and children) with CF.

5.3 Methods

I conducted this study with 2 co-authors (Dr. Claire Wainwright (CW, Department of Respiratory Medicine, Royal Children's Hospital, Brisbane, Australia) and Professor Alan Smyth (AS, Division of Child Health, Obstetrics & Gynaecology, School of Medicine, University of Nottingham, Nottingham, UK). I conducted the literature search with support from Mrs Nikki Jahnke, the Assistant Managing Editor of Cochrane Cystic Fibrosis and Genetic Disorders Group. I reviewed the abstracts followed by full texts of relevant studies while completing the "Study Selection, Quality Assessment & Data Extraction Form" (please refer to Appendix Chapter V Section 8.5.2).

5.3.1 Criteria for considering studies for this review

5.3.1.1 Types of studies

Randomised controlled studies

5.3.1.2 Types of participants

Children and adults with CF (according to standard definitions, clinical features of CF plus a positive sweat test or the presence of two genes known to be associated with CF (*458*).

5.3.1.3 Types of interventions

We included studies comparing outcomes following therapies guided by the results of bronchoscopy (with BAL or PBB sampling) with outcomes following therapies guided by the results of any other type

of sampling (including but not limited to cultures from sputum, throat swab and cough swab).

5.3.2 Primary Outcome Measures

1. Lung function
    a) Conventional spirometry
        i. Percent predicted FEV_1 change and absolute values in litres
        ii. Percent predicted FVC change and absolute values in litres
    b) Infant lung function-squeeze
        i. percent predicted $FEV_{0.5}$, forced expiratory flow from 25% to 75% of vital capacity (FEF_{25-75}) and FEF_{75} changes and absolute values in litres
        ii. percent predicted FVC change and absolute values in litres
    c) Lung clearance index (LCI)
2. High-resolution computed tomography (HRCT) appearances using a recognised scoring system (e.g. Brody Score (*667*), SALD (Severe Advanced Lung Disease) System (*668*))
3. Nutritional parameters
    a) Weight (in kg or percentile)
    b) BMI percentile

5.3.3 Secondary Outcome Measures

1. Number of positive isolates per child per year of follow-up (for BAL defined as a positive culture of the growths from 10^3 colony-forming units (CFU)/ml)
    a. P. aeruginosa
    b. S. aureus
    c. *H. influenzae*
    d. *B. cepacia* complex
    e. S. maltophilia
    f. *Achromobacter xylosoxidans* (*A. xylosoxidans*)
    g. non-tuberculous mycobacteria
    h. *Aspergillus* species
    i. any other organism
2. Clearance of the organism from the cultures
3. Time to the first infection with *P. aeruginosa**
4. Time to chronic infection using any recognised definition e.g. Leeds Criteria (*669*)*
    a. with *P. aeruginosa*
    b. with *S. aureus*
5. Complications and adverse effects related to bronchoscopy (e.g. fever, hypoxaemia and increased cough, unplanned admissions and other serious adverse events, etc.)
6. Quality of life (QoL) measured using a validated tool such as Cystic Fibrosis Questionnaire-Revised version (CFQ-R) (*670*) and Cystic Fibrosis Quality of Life Questionnaire (CFQoL) (*671*)
7. Hospitalisations
    a. number of hospitalisations per participant per year
    b. days as inpatient per participant per year
    c. the cost of care

8. Number of courses of antibiotics prescribed per participant per year (not including prophylactic antibiotics)
    a. Intravenous
    b. Oral
9. Number of pulmonary exacerbations (requiring oral or IV antibiotics) per participant per year (diagnosis based on clinical judgement or any approved or published definition (*641,672*)

* In the studies where the follow-up was started just after the birth, we considered the age of first acquisition of infection and age of establishing chronic infection. For studies in older people where follow-up is started later in life, we considered the time to acquire the first infection and time to acquire chronic infection.

5.3.4 Search methods for identification of studies

We identified relevant studies from the Cochrane Cystic Fibrosis and Genetic Disorders Group's Cystic Fibrosis Trials Register using the term: sampling techniques. The latest search was conducted on 28/08/2015.

The Cystic Fibrosis Trials Register is compiled from electronic searches of the Cochrane Central Register of Controlled Trials (CENTRAL) (updated with each new issue of *The Cochrane Library*), quarterly searches of MEDLINE, a search of Embase to 1995 and the prospective hand searching of two journals - *Pediatric Pulmonology* and the *Journal of Cystic Fibrosis*. Unpublished work is identified by searching the abstract books of three major CF conferences: the International Cystic Fibrosis Conference; the European Cystic Fibrosis Conference and the North American Cystic Fibrosis Conference.

I searched the ongoing study registers: clinicaltrials.gov (*600*) and International Standard Randomised Controlled Trial Number (ISRCTN)

Register (www.isrctn.org) (*673*) using the search terms: cystic fibrosis AND bronchoscopy, cystic fibrosis AND bronchoalveolar lavage, cystic fibrosis AND sampling technique. Date of the latest search of the ongoing study registers was 02/11/2015. We checked the reference lists of primary studies and review articles for additional references.

5.3.5 Data collection and analysis

We conducted the review according to the recommendations from the Cochrane Handbook for Systematic Reviews of Interventions (*674*). Data were recorded on "Study Selection, Quality Assessment & Data Extraction Form" (please refer to Appendix Chapter V Section 8.5.2).

5.3.6 Selection of studies

CW and I independently screened the titles and abstracts of all the studies identified by the primary search and then obtained the full text of relevant studies. Both of us independently went through the studies while considering inclusion criteria to decide whether to include or exclude the studies. We had planned to contact the investigators for more information for any study where the criteria for inclusion were unclear. We had planned to resolve any disagreement by discussion, referral to the third review author and consensus. I have described the reasons for exclusion of the studies, initially considered eligible for inclusion according to title or abstract.

5.3.7 Data extraction and management

I extracted the data from the only included study; the second review author (CW) was familiar with the data (being the author of the study). We completed a pre-decided data extraction form and resolved any disagreements by discussion, referral to the third review author and consensus. Extracted information included:

- administrative details including authors, year of publication, country of study;
- participant characteristics including the number of participants in each group, age, gender, weight, BMI, concomitant morbidities and other baseline characteristics mentioned in the studies;
- study characteristics including the design of the study, inclusion and exclusion criteria, duration of follow-up, co-morbidities, primary and secondary outcome measures;
- details of intervention techniques used for the collection of specimens and the description of any adverse effects;
- data for primary and secondary outcome measures

I created a study flow diagram (please refer to Figure 5-1)following the template described in the PRISMA statement (*675*). I also completed a table of 'characteristics of included studies' including information about study design, relevant information on the demographics and health of participants and a list of interventions and outcome measures (please refer to Table 5-1)

If suitable data were available, we would have undertaken a meta-analysis for each outcome. Where possible, the results have been presented in a graph (mainly for primary outcome measures or secondary measures with a significant effect), or otherwise in a narrative way.

We had planned to report data at two weeks (after finishing the antibiotic course), three months, six months, one year and annually thereafter. If there were studies reporting data at other time points, we would have considered reporting those as well.

5.3.8 Assessment of risk of bias in included studies

CW and I independently assessed the studies (that fulfilled the inclusion criteria) for risk of bias as per guidelines from the *Cochrane Handbook for Systematic Reviews of Interventions* (*676*). We assessed studies for risk of bias according to a standardised set of questions covering the following domains:

- random sequence generation;
- allocation concealment;
- blinding of outcome assessment for the primary outcomes for HRCT assessment and lung function parameters;
- incomplete data outcome;
- selective reporting bias;
- other sources of bias

We classified the responses from each category into one of three grades (low risk of bias, high risk of bias and unclear risk of bias) and generated a risk of bias graph (Figure 5-2).

5.3.9 Measures of treatment effect

I analysed binary data using risk ratio (RR) and 95% CI. I used the mean difference (MD) with 95% CIs to analyse continuous data. The MD measures the absolute difference between the mean values in two groups when outcome measurements in all studies use the same scale; we had planned to use the standardised MD when the studies were assessing the same outcome but measuring it in a variety of ways. We used time-to-event analysis using hazard ratios with 95% CIs, e.g. for time to acquisition of chronic infection with *P. aeruginosa*. With reference to count data, e.g. for the number of isolates of organisms, we treated the data as continuous data and measured the intervention effect using the MD and 95% CIs between the groups. By convention, the changes during the study were reported as the effect

measured post-intervention minus that measured pre-intervention; and differences between the study arms were reported as treatment arm effects minus control arm effects.

5.3.10 Unit of analysis issues

We planned to include cluster-randomised studies if the clustering had been taken into account and the intra-cluster coefficient was included. We planned not to include crossover studies in the review since CF is a chronic disorder with progressive worsening of lung disease following repeated infections. Some of the infections like *P. aeruginosa*, once established, cannot be eradicated and are associated with worse outcome. In addition, for outcome measures such as a change in lung function and structural lung damage, it may not be possible to revert completely to the same level, even with treatment. In view of this, we felt it was justified not to include crossover studies.

For events happening multiple times, such as pulmonary infections, the unit of analysis will be individual participants (except in some cases e.g. the number of isolates of organisms per year, where we will analyse the number of events).

5.3.11 Dealing with missing data

In the case of missing data, we aimed to contact the primary author of any such studies, and if we did not receive any responses, we had planned to perform an intention-to-treat analysis where possible.

5.3.12 Assessment of heterogeneity

If we had included more than one study in the review, we would have used the chi^2 test in the forest plot and also the I^2 test for assessing the heterogeneity of results (*677*).

A rough guide to interpretation of thresholds for the I^2 statistic is as follows, which is based on guidance from Chapter 9 of The *Cochrane Handbook for Systematic Reviews of Interventions* (*678*):

- 0% to 40%: might not be important;
- 30% to 60%: may represent moderate heterogeneity*;
- 50% to 90%: may represent substantial heterogeneity*:
- 75% to 100%: considerable heterogeneity*.

* The importance of the observed value of I^2depends on (i) the magnitude and direction of effects and (ii) strength of evidence for heterogeneity (e.g. P value of chi^2 test or a CI for I^2).

5.3.13 Assessment of reporting biases

AS and I examined the included study for any evidence of selective outcome reporting bias by comparing the outcomes described in the protocol with the results published. If we had included more than one trial, we would have constructed a matrix indicating which outcomes were recorded in each study to establish if any studies omitted any key outcomes. We had planned to compare the protocols (if available) or the 'Methods' section of the included articles with their 'Results' sections to establish selective reporting of outcomes that were pre-specified. If there was a suspicion or a direct evidence of selective outcome reporting, we would have contacted the study authors to provide the study protocol and full information for the outcomes reported inadequately.

If there were a sufficient number of studies included (at least 10), then I had planned to construct a funnel plot and assess this to help identify evidence of publication bias or any other type of bias.

5.3.14 Data synthesis

I followed the guidelines from the *Cochrane Handbook for Systematic Reviews of Interventions* to conduct the comparison between the bronchoscopy-guided and the standard treatment groups covering all the primary and secondary outcome measures (*674*).

If we had combined more than a single data set and had identified no heterogeneity or only mild to moderate heterogeneity based on I^2 test results (*677*), then we would have used a fixed-effect analysis model; if the value of I^2 had been between 50% and 75%, we would have performed a random-effects analysis. If the I^2 values had been greater than 75%, we would not have considered it suitable to pool the studies together.

For each outcome where I was able to extract suitable data, I constructed a forest plot to display effect estimates and 95% CIs using a fixed-effect model of analysis.

5.3.15 Summary of findings and quality of the evidence

In line with latest Cochrane guidance, I planned to summarise the evidence in a summary of findings table (Summary of findings, Table 5-2). The table presents the primary outcomes lung function, HRCT and nutritional parameters along with the secondary outcomes of cost of care after five years of follow-up. We determined the quality of the evidence using the GRADE approach. The evidence was downgraded for the outcomes for which the study had low statistical power, and more research was required to provide definitive answers. High-quality evidence was available for the effect of BAL-directed therapy on radiological features (HRCT) and cost of care.

5.3.16 Subgroup analysis and investigation of heterogeneity

If sufficient studies (at least 10) were included in the review, we had planned to undertake the following subgroup analyses:

- age groups: 0 to 5 years, 6 to 16 years and 17 years and above;
- chronic or intermittent isolation of *P. aeruginosa* versus no *P. aeruginosa*;
- Different methods of sampling, e.g. BAL versus PBB or sputum culture versus throat swab.

5.3.17 Sensitivity analysis

If sufficient studies (at least 10) were included, we had planned to undertake the following sensitivity analyses:

- to analyse the effect of bias including effects of sequence generation, allocation concealment, intention-to-treat analysis and reporting bias which are not resolved after contacting the authors;
- to assess the differences between fixed-effect and random-effects analysis on the results

5.4 Results

The searches identified 29 references to nine separate studies. Of these, only one study (nine references) met our inclusion criteria (*679-686*) and we excluded eight studies (20 references). The process of assessing the search results is shown in the study flow diagram (Figure 5-1).

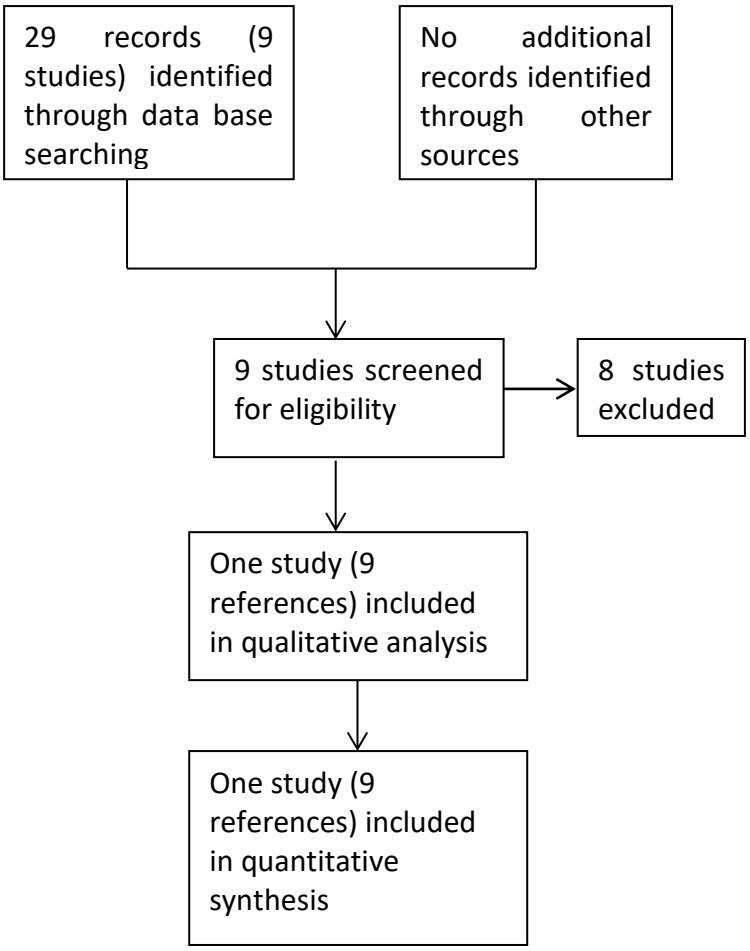


Figure 5-1 Systematic Review flow diagram

5.4.1 Included studies

5.4.1.1 Methods

The only included study was a multicentre, randomised controlled study of parallel design (*685*). This study was conducted across eight CF centres in Australia and New Zealand and recruited infants from June 1999 to April 2005. After consent, the participants were randomly assigned in a 1:1 ratio to two groups. The study was completed in December 2009.

5.4.1.2 Participants

Infants younger than six months, diagnosed with CF through the newborn screening program with a confirmed diagnosis of classic CF (two of the following: two CF mutations; sweat chloride level over 60 mEq/L; pancreatic insufficiency; or meconium ileus) were eligible for inclusion.

Of the total 267 infants eligible for the study, 170 infants were recruited. Eighty-four infants were randomised to receive BAL-directed therapy and 86 randomised to receive standard therapy. All the participants randomised to the BAL group received the intended therapy; however, 4 out of 84 participants did not complete the study and were excluded from the primary analysis. Of the 86 participants randomised to a standard therapy group, 84 participants received the intended therapy and 77 of these (92%) completed the study to be included in the primary analysis.

The mean (SD) age of the participants at enrolment was 3.8 (1.6) months in the BAL-directed therapy group and 3.7 (1.7) months in the standard therapy group. In both the groups, 44 participants were male. Mean (SD) weight at enrolment was 5.7 (1.40) kg in the BAL-directed therapy group and 5.6 (1.5) kg in the standard therapy group. The number of participants with the homozygous Phe508del mutation

was 57 (68%) in the BAL-directed therapy group and 54 (64%) in the standard therapy group. The number of participants with pancreatic insufficiency was 73 (87%) in the BAL-directed therapy group and 71 (85%) in the standard therapy group. The number of participants with meconium ileus was 17 (20%) in the BAL-directed therapy group and 16 (19%) in the standard therapy group. The number of participants born pre-term (under 37 weeks gestation) was eight (10%) in the BAL-directed therapy group and nine (11 %) in the standard therapy group. History of exposure to tobacco smoke during pregnancy was present in 22 (26%) and 13 (15%) participants from the BAL-directed therapy group and standard therapy group respectively. History of concurrent smoking in the household was present in 30 (36%) participants in the BAL-directed therapy and 23 (28%) of participants from standard therapy group.

5.4.1.3 Interventions

Participants received treatment of pulmonary exacerbations either directed by results of BAL or according to standard policy of treatment (based on clinical features and oropharyngeal cultures).

The standard therapy included oropharyngeal swabs at following time points:

- When a child was unwell with a change in respiratory symptoms from baseline (pulmonary exacerbation);
- At the end of the antibiotic eradication treatment for *P. aeruginosa.*

In addition, the participants in the BAL-directed therapy groups also underwent BAL at following times:

- Before six months of age when well;

- When hospitalised for a pulmonary exacerbation (unwell with change in respiratory symptoms from baseline);
- If *P. aeruginosa* was cultured from oropharyngeal specimens;
- Following *P. aeruginosa* eradication therapy.

At five years of age, all the participants underwent BAL, HRCT scan (to compute 'cystic fibrosis computed tomography' (CF-CT) scores), pulmonary function testing and anthropometric assessments.

5.4.1.4 Outcomes

The primary outcome measures were the prevalence of *P. aeruginosa* infection (defined as greater than or equal to 10^3 CFU)/ml in the BAL cultures) and evidence of structural lung disease assessed by total CF-CT score (as a percentage of a maximum score) on HRCT at age five years. Secondary outcome measures, also assessed at five years age, included z scores (standard score: shows how many SD away (above or below) the observed mean is from the population mean) for weight, height and BMI; lung function parameters; CF-CT components; respiratory exacerbation rate; number and duration of hospitalizations for respiratory exacerbations not associated with *P. aeruginosa* infection; number of episodes of *P. aeruginosa* infection per child per year; and final BAL microbiology and inflammatory indices.

5.4.2 Excluded studies

We excluded eight studies (20 references) after the screening. Four studies were excluded as they were investigating a different intervention, which was not relevant to this review: cough plates- Jyothish, 2005 (*687,688*); Cough plates - Maiya 2004 (*689,690*); induced sputum -Chmiel 2007 (*691*); and throat swabs and nasopharyngeal suction - Taylor 2006 (*692,693*). One study looked at the effect of dornase alfa on the surface of the lungs using BAL - Paul 2004 (*694-702*) and one study was to establish levels of tobramycin,

not a comparison of therapy depending on sampling technique - Rosenfeld 2006 (*703,704*). One study compared sputum induction, BAL and expectorated sputum to identify pathogens, but did not lead to a comparison of treatment - Henig 2001 (*705,706*). The remaining study was excluded as it had a crossover design to compare inflammatory marker in the samples obtained by sputum induction and bronchoscopy but did not follow any comparison of treatment - McGarvey 2002 (*707*).

Table 5-1 Characteristics of included study (*685*)		
Methods	Multicentre (8 CF centres in Australia and New Zealand), randomised controlled study.	
Participants	170 Infants < 6 months age, with a confirmed diagnosis of CF, diagnosed through newborn screening; 84 infants randomised to receive BAL-directed Rx (80 completed study) and 86 to receive standard Rx (77 completed study).	
Characteristic	**BAL-directed Rx**	**Standard Rx**
Mean age (SD)	3.8 (1.6) years	3.7 (1.7) years
Gender split	44 male/40 female	44 male/42 female
Mean (SD) weight at enrolment	5.7 kg (1.40)	5.6 kg (1.5)
No. with homozygous Phe508del	57 (68%)	54 (64%)
No. with pancreatic insufficiency	73 (87%)	71 (85%)
No. with meconium ileus	17 (20%)	16 (19%)
No. born pre-term (< 37 weeks)	8 (10%)	9 (11%)
Smoke exposure (pregnancy)	22 (26%)	1 (15%)
Concurrent smoking household	30 (36%)	23 (28%)
Interventions	BAL-directed Rx for pulmonary exacerbations until age 5 years (standard Rx directed by clinical features & oropharyngeal swabs for control group)	
BAL-directed Rx group-BAL at: 1. before 6 months age when well; 2. when hospitalised for a pulmonary exacerbation* 3. if *P. aeruginosa* was cultured from oropharyngeal specimens; 4. After *P. aeruginosa* eradication	**Standard Rx group-oropharyngeal swab at:** 1. Pulmonary exacerbation* 2. at the end of the antibiotic eradication treatment for *P. aeruginosa*	
Primary outcome measures (Age 5 yrs.) 1. Prevalence of *P. aeruginosa* on BAL cultures (defined as ≥ 10^3 CFU/ml) 2. Total CF-CT score (as percentage of the maximum score) on high-resolution chest CT scan	**Secondary outcome measures (Age 5 yrs.)** 1. Z Scores for Weight, Height & BMI 2. Spirometry measures (FEV_1, FVC) 3. CF-CT components 4. Respiratory exacerbation rate 5. No. & duration of hospitalisations for exacerbations (not *P aeruginosa*) 6. No. of episodes of *P. aeruginosa* infection per child per year 7. Final BAL microbiology and inflammatory indices	

Pulmonary exacerbation: (unwell with change in respiratory symptoms from baseline); CF: cystic fibrosis, BAL: bronchoalveolar lavage; Rx: Therapy, No.: number; P. aeruginosa: Pseudomonas aeruginosa; SD: standard deviation; CFU: colony forming unit; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; BMI: body mass index; CF-CT: cystic fibrosis computed tomography; z score: standard score (no. of SD the observed mean is from the population mean)

Table 5-2 Summary of findings				
Outcomes (at 5 years follow-up)	Illustrative comparative risks* (95% CI)		Number of Patients (studies)	Quality of the evidence (GRADE)
	Assumed risk	Corresponding risk		
	Standard Rx Mean (SD)	BAL-directed Rx Mean (SD)		
Z score FEV_1**	-0.41 (SD 1.23)	0.56 (SD 1.25) [**0.15 lower** (0.54 lower to 0.24 higher) than the standard Rx group]	157 (1 study)	⊕⊕⊕⊖ moderate#
Z score FVC**	0.01 (SD 1.2)	-0.04 (SD 1.31) [**0.05 higher** (0.44 lower to 0.34 higher) than the standard Rx group]	157 (1 study)	⊕⊕⊕⊖ moderate#
HRCT score (Brody-II)	2.83 (SD 3.5)	3.02 (SD 3.48) [**0.19 higher** (0.93 lower to 1.31 higher) than the standard Rx group]	152 (1 study)	⊕⊕⊕⊕ high
Z score weight***	0.21 (SD 0.82)	-0.15 (SD 0.88) [**0.06 higher** (0.21 lower to 0.33 higher) than the standard Rx group]	157 (1 study)	⊕⊕⊕⊖ moderate#
Z score BMI***	0.01 (SD 0.83)	0.03 (SD0.93) [**0.02 higher** (0.26 lower to 0.30 higher) than the standard Rx group]	157 (1 study)	⊕⊕⊕⊖ moderate#
Overall cost of care per participant (AUD)	90,958 AUD (SD 110,255)	92,860 AUD (SD 73,378) [**1902 AUD higher** (27,508.98 lower to 31,312.98 higher) than the standard Rx group]	157 (1 study)	⊕⊕⊕⊕ high

Assumed risk: median risk in the control groups across studies; Corresponding risk is based on assumed risk in the comparison group and the relative effect of the intervention; ** FEV_1 (forced expiratory volume at 1 second) and FVC (forced vital capacity): measured using standard spirometer after bronchodilation; z scores for FEV_1 & FVC: calculated from British reference values (708); the mean difference between the two groups represents the difference in the mean z scores for each parameter; *** z scores for weight and BMI (body mass index): calculated from the 2000 CDC Growth Reference Charts (709); the mean difference between the two groups represents the difference in their z scores for each parameter; # the study had low statistical power and research is needed to provide definitive answers; SD: standard deviation; CI: confidence interval; BAL: bronchoalveolar lavage; Rx: treatment; HRCT: high-resolution chest computed tomography; AUD: Australian Dollars; z score: standard score (no. of SD the observed mean is from the population mean)

5.4.3 Risk of bias in included studies

A summary of the risk of bias for the included study is shown *in* Figure 5-2*.*

5.4.3.1 Allocation (selection bias)

5.4.3.1.1 Sequence generation

A central computer-generated sequence was used to randomise the participants to either group in blocks that were stratified by CF centre and gender. A randomisation number was generated centrally, away from the local investigators. This domain was judged to have a low risk of bias.

5.4.3.1.2 Concealment of allocation

The randomisation key was concealed and held remotely; allocation was disclosed on the telephone after confirmation of recruitment to the study. The procedure was judged to have a low risk of bias.

5.4.3.2 Blinding (performance bias and detection bias)

The participants and the personnel were not blinded to the randomisation; however, the risk of bias was judged low for this item because the primary outcome measures were unlikely to be influenced by the lack of blinding. These were evaluated by the assessors who were blinded to the treatment allocation and were not directly involved in the care of the participants (a microbiologist assessed BAL and an expert scientist scored the HRCT).

5.4.3.3 Incomplete outcome data (attrition bias)

Although the study was planned to be analysed on an intention-to-treat basis, the primary analysis was based only on the participants who provided final outcome data, analysed according to randomised groups. The risk of bias was considered moderate to low as less than 10% of the data were missing, and the number of participants with

missing outcome data and the reasons for their exclusion were balanced across both treatment arms. In the BAL-directed therapy group, 4 out of 84 participants did not complete the study (three protocol violations and one withdrawal); of the 86 participants randomised to the standard therapy group, 77 participants (92%) completed the study (four protocol violations and three withdrawals).

5.4.3.4 Selective reporting (reporting bias)

The outcomes reported in the study protocol were compared with the reported results. The difference in cost between treatment arms was not reported with the primary study; however, an economic analysis was reported in a separate paper. No reporting bias was identified in the included study.

1.1.1.1 Other potential sources of bias

No other potential sources of bias could be identified for the study.

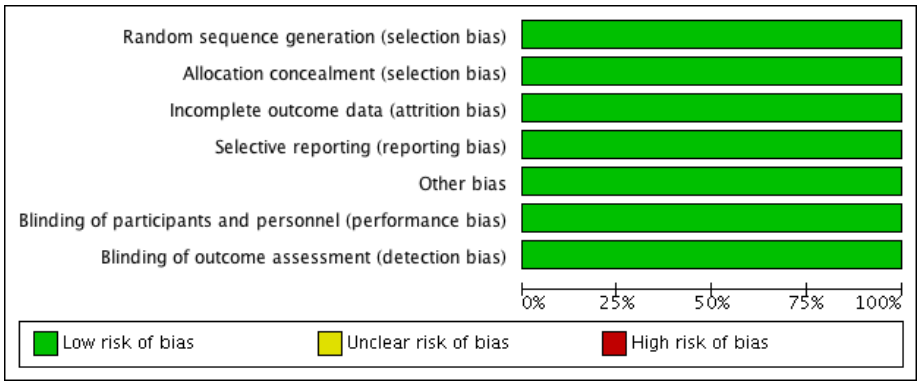


Figure 5-2 Risk of bias graph; Review authors' judgements about each risk of bias item is presented as percentages across all included studies.

5.4.4 Effects of interventions

5.4.4.1 Primary outcomes

5.4.4.1.1 Lung function

5.4.4.1.1.1 *Conventional spirometry*

Percent predicted FEV_1 (change and absolute values in litres)

Wainwright et al. reported z score for FEV_1 and no significant difference was found between the groups for this outcome, MD -0.15 (95% CI -0.54 to 0.24) (Figure 5-3) (Summary of Findings Table 5-2).

Percent predicted FVC (change and absolute values in litres)

The study reported z score for FVC. There was no significant difference between the groups, MD -0.05 (95% CI -0.44 to 0.34) (Figure 5-4) (Summary of Findings Table 5-2).

5.4.4.1.1.2 *Infant lung function-squeeze*

Percent predicted $FEV_{0.5}$, FEF_{25-75} and FEF_{75} (changes and absolute values in litres)

Infant lung function was not assessed in this study.

Percent predicted FVC (change and absolute values in litres)

Infant lung function was not assessed in this study.

5.4.4.1.1.3 *Lung clearance index (LCI)*

This outcome was not measured in this study.

5.4.4.1.2 HRCT appearances

Wainwright et al. scored HRCT appearances according to an upgraded version of Brody-II score (referred to as CF-CT score) *(685)*. Five different components were used to calculate the total CF-CT score; four were calculated on inspiratory images (bronchiectasis score, parenchymal disease score, mucus plugging score, airway wall thickening score) and air trapping score was assessed on expiratory

images. The results were reported as percentages of the total possible score for each component and for the maximum total score. The components were scored according to the distribution of changes into the following groups: 0%, 0% to 5% and more than 5% for inspiratory images; 0%, 0% to 20% and more than 20% for expiratory images.

There was no significant difference between the groups for total CF-CT score, MD 0.19 (95% CI -0.93 to 1.31) (Figure 5-5) (Summary of Findings Table 5-2). The scores across different components did not show any significant difference between the two groups with the risk of having a score of more than 5% for inspiratory images and more than 20% for expiratory images being the following: bronchiectasis score, MD -0.10 (95% CI -1.53 to 1.33); parenchymal disease score, MD -0.49 (95% CI -1.36 to 0.38); mucus plugging score, MD 1.38 (95% CI -0.36 to 3.12); airway wall thickening score, MD 0.71 (95% CI -0.16 to 1.58); air trapping score, MD 0.43 (95% CI -4.82 to 5.68) (Figure 5-6).

5.4.4.1.3 Nutritional parameters

5.4.4.1.3.1 *Weight (in kg or percentile)*

Wainwright et al. reported z score for weight *(685)*. There was no significant difference between the groups, MD 0.06 (95% CI -0.21 to 0.33) (Figure 5-7) (Summary of Findings Table 5-2).

5.4.4.1.3.2 *BMI percentile*

The study found no significant difference in the z score for BMI between the two groups, MD 0.02 (95% CI -0.26 to 0.30) (Figure 5-8) (Summary of Findings Table 5-2).

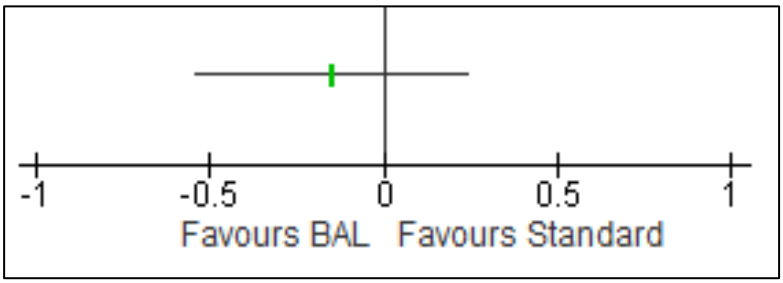


Figure 5-3 Forest plot for comparison of z score of FEV_1 between BAL-directed therapy group and standard therapy group, mean difference -0.15 [95% confidence interval -0.54 to 0.24]; BAL: bronchoalveolar lavage; FEV_1: forced expiratory volume in one second; z score: standard score (shows how many standard deviation away (above or below) the observed mean is from the population mean)

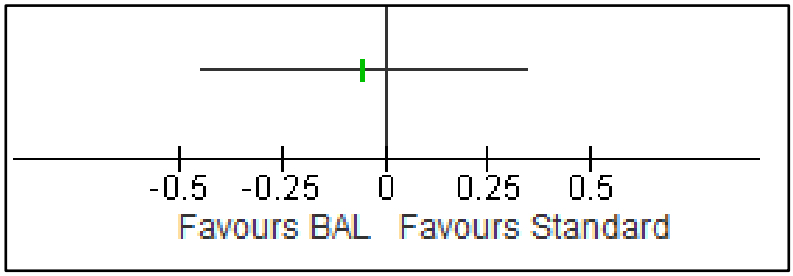


Figure 5-4 Forest plot for comparison of z score of FVC between BAL-directed therapy group and standard therapy group, mean difference -0.05] [95% confidence interval -0.44 to 0.34] ; BAL: bronchoalveolar lavage; FVC: forced vital capacity; z score: standard score (shows how many standard deviation away (above or below) the observed mean is from the population mean)

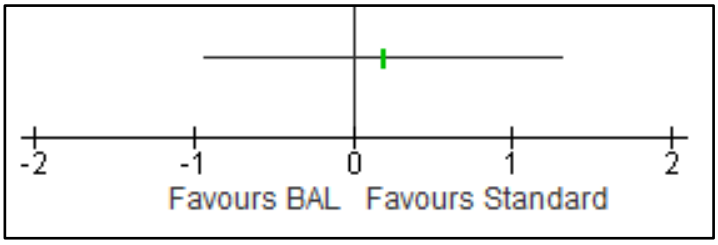


Figure 5-5 Forest plot for comparison of z score of total CF-CT score (Brody II) between BAL-directed therapy group and standard therapy group; mean difference 0.19 [95% confidence interval -0.93 to 1.31]; BAL: bronchoalveolar lavage; CF-CT score: cystic fibrosis computed tomography score; z score: standard score (shows how many standard deviation away (above or below) the observed mean is from the population mean)

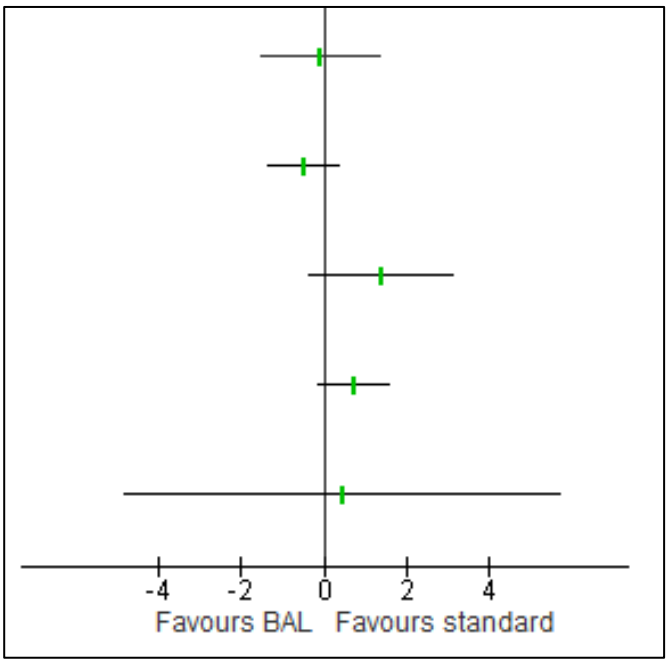


Figure 5-6 Forest plot for comparison of z scores of individual CF-CT scores (Brody II) between BAL-directed therapy group and standard therapy group, from top to bottom: Bronchiectasis score, Parenchymal disease score, Mucous plugging score, Airway wall thickening score, Air trapping score; BAL: bronchoalveolar lavage; CF-CT score: cystic fibrosis computed tomography score; z score: standard score (shows how many standard deviation away (above or below) the observed mean is from the population mean)

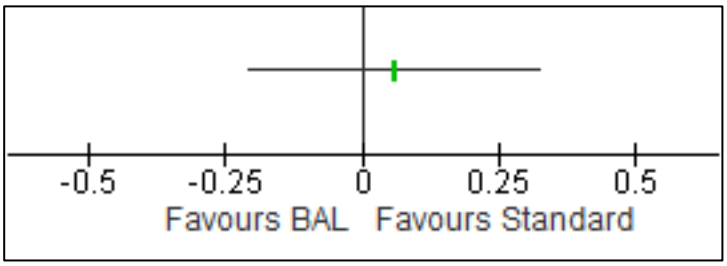


Figure 5-7 Forest plot for comparison of z score of weight at 5 years agebetween BAL-directed therapy group and standard therapy group, mean difference 0.06 [95% CI -0.21 to 0.33]; BAL: bronchoalveolar lavage; z score: standard score (shows how many standard deviation away (above or below) the observed mean is from the population mean)

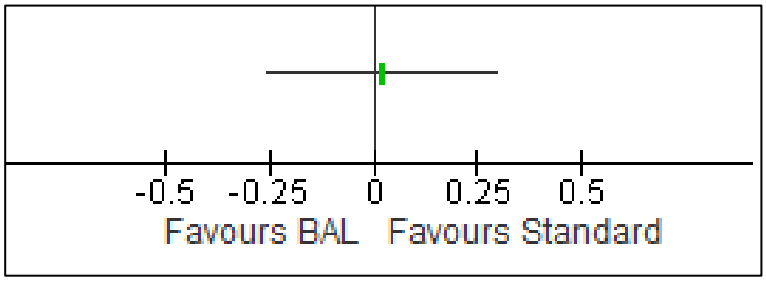


Figure 5-8 Forest plot for comparison of z score of BMI at 5 years age between BAL-directed therapy group and standard therapy group, mean difference 0.02 [95% CI -0.26 to 0.30]; BAL: bronchoalveolar lavage; BMI: body mass index; z score: standard score (shows how many standard deviation away (above or below) the observed mean is from the population mean)

5.4.4.2 Secondary outcomes

5.4.4.2.1 Number of positive isolates per child per year of follow-up

5.4.4.2.1.1 *P. aeruginosa*

The number of positive isolates of *P. aeruginosa* per participant per year of follow-up was reported using the intervention specific to the randomisations, i.e. using BAL cultures for the BAL-directed group and oropharyngeal cultures for the standard therapy group. There was no significant difference between the groups, RR 0.77 (95% CI 0.52 to 1.16).

At age five years, *P. aeruginosa* infection was diagnosed in BAL of 8 out of 79 (10%) participants and 9 out of 76 (12%) participants from BAL-directed therapy group and standard therapy groups respectively, RR 0.86 (95% CI 0.35 to 2.10). I conducted a sensitivity analysis after including the missing data in the respective groups and assessed the influence on *P. aeruginosa* prevalence considering hypothetical situations where the risk of excluded participants being positive at five years was 40% in the BAL-directed therapy group and 5% in the standard therapy group and vice versa. In both cases, the result was not significant, RR 1.11 (95% CI 0.48 to 2.60) and RR 0.67 (95% CI 0.29 to 1.55) respectively.

5.4.4.2.1.2 *Other organisms*

Data specific to the number of positive isolates per participant per year of follow-up were not available for *S. aureus, H. influenzae, B. cepacia, S maltophilia, A. xylosoxidans*, non-tuberculous mycobacteria, *aspergillus* species or any other organisms.

5.4.4.2.2 Clearance of the organism from the cultures

There was no significant difference between the two groups in the proportion of children who were cleared of *P. aeruginosa* infection following one or two courses of eradication treatment, RR 1.08 (95% CI 0.96 to 1.21).

5.4.4.2.3 Time to the first infection with *P. aeruginosa*

There was no statistically significant difference in the age of first acquisition of *P. aeruginosa* infection between the two groups, RR 0.81 (95% CI 0.53 to 1.23).

5.4.4.2.4 Time to chronic infection

5.4.4.2.4.1 *With P. aeruginosa*

There was no statistical analysis of the number of children who developed a chronic infection in either group. Only one child in the BAL-directed therapy group developed chronic infection (confirmation by BAL) at 56 months of age. In the standard therapy group, four children developed chronic infection (confirmed by cough swab) between 33 months and 61 months of age (however, the BAL at five years age was negative for *P. aeruginosa* in all four participants).

5.4.4.2.4.2 *With S. aureus*

Data specific to the time to chronic infection with *S. aureus* were not available in this study.

5.4.4.2.5 Complications and adverse effects

No data were available for adverse events except those relating to BAL or use of medications. These data were collected and were recorded, but were not published.

A number of adverse events could be attributed to both bronchoscopy and general anaesthesia. Significant clinical deterioration was noted during or following the BAL in 25 of 524 procedures (4.8%). During the procedure, seven children developed haemoglobin desaturation to

less than 90%, which lasted for more than 60 seconds and needed intervention. Post-operatively, six children required supplemental oxygen; one required non-invasive ventilation and three developed stridor. The most common adverse event was transient worsening of cough noted in 96 children after 151 of 524 procedures (29%); however, this was a temporary effect and did not lead to hospitalisations. Fever with a temperature higher than 38.5°C, which occurred within 24 hours of BAL was reported following 40 (7.6%) procedures and fever with a temperature below 38.5°C following 52 (9.9%) procedures. In the post-BAL period, 12 (2.3%) participants required unplanned hospital admissions. Contamination of bronchoscope was reported on two incidences. Overall, the procedure was well tolerated with mild adverse effects to the participants; however, these were in addition to the inconvenience associated with hospitalisations and fasting pre- and post-procedure.

5.4.4.2.6 QoL

Data specific to QoL were not available for this study.

5.4.4.2.7 Hospitalisations

5.4.4.2.7.1 *Number of hospitalisations per participant per year*

The total number of hospital admissions per participant per year was higher in the BAL-directed therapy group compared to the standard therapy group, RR 1.40 (95% CI 1.08 to 1.82) (Figure 5-9) (data not published but provided by the authors) (Summary of Findings Table 5-2). For non-*Pseudomonas aeruginosa* exacerbations, the children in BAL-directed therapy group had 218 hospitalisations (0.57 per person-year). This was greater than the standard therapy group who had 140 hospitalisations (0.37 per person-year), RR 1.52 (95% CI 0.98 to 2.35) (Figure 5-10).

a. Days as inpatient per participant per year

The mean duration of hospital admissions for non-*Pseudomonas aeruginosa* respiratory exacerbations was reported to be significantly shorter in the BAL-directed therapy group compared to standard therapy, MD -3.50 days (95% CI -5.67 to -1.33) (Figure 5-11)**.** However, there was no significant difference between the hospital admission days per participant per year for each group, risk difference 0.08 (95% CI -3.31 to 3.47) (Figure 5-12)*,* (data not published, provided by the authors).

b. Cost of care

There was no difference in the total cost of care for the children in each group over the study duration (five years), MD 1902.00 Australian dollars (AUD) (95% CI 27508.98 to 31312.98) (Figure 5-13). There was no difference in the total cost of hospital admissions per child over the five-year study duration *(681,685)*, MD -9288.00 AUD (95% CI 34996.37 to 16420.37) (Summary of Findings Table 5-2).

5.4.4.2.8 Number of courses of antibiotics per participant per year (not including prophylactic antibiotics)

5.4.4.2.8.1 *Number of hospitalisations per participant per year*

Data for the number of courses of IV antibiotics prescribed per participant per year were not available.

5.4.4.2.8.2 *Oral antibiotic courses*

Data for the number of courses of oral antibiotics prescribed per participant per year were not available.

5.4.4.2.9 Number of pulmonary exacerbations (requiring oral or IV antibiotics) per participant per year

There was no significant difference in the number of pulmonary exacerbations diagnosed in either group, RR 1.01 (95% CI 0.85 to 1.19).

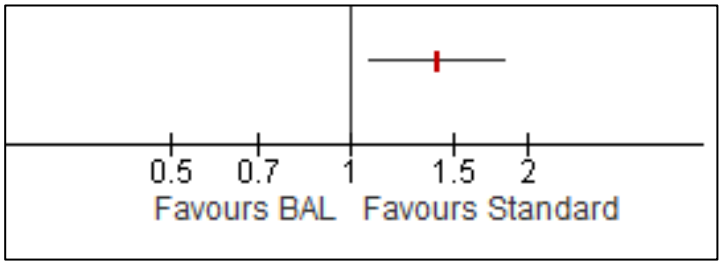


Figure 5-9 Forest plot for comparison of total number of hospital admissions per person per year between BAL-directed therapy group and standard therapy group, risk ratio 1.40 [95% confidence interval 1.08 to 1.82]; BAL: bronchoalveolar lavage

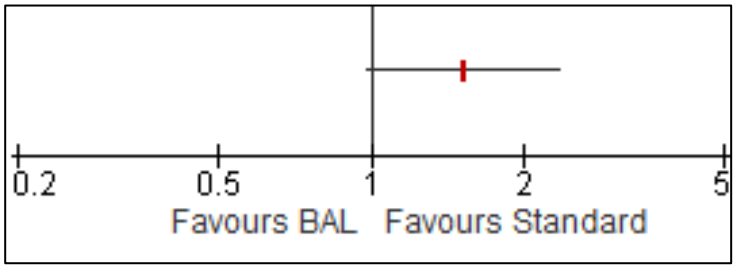


Figure 5-10 Forest plot for comparison of number of hospital admissions per patient per year (for non-Pseudomonas aeruginosa exacerbations) between BAL-directed therapy group and standard therapy group, risk ratio 1.52 [95% confidence interval 0.98 to 2.35]; BAL: bronchoalveolar lavage

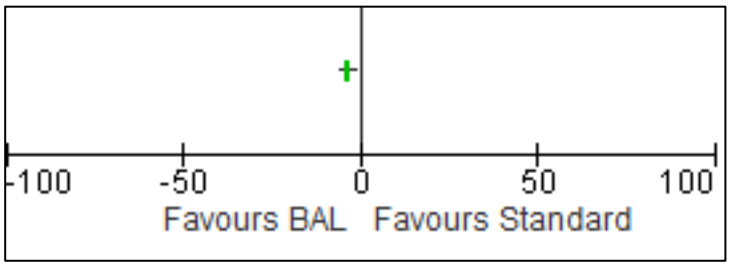


Figure 5-11 Forest plot for comparison of the duration of hospital admissions due to non-Pseudomonas aeruginosa exacerbations between BAL-directed therapy group and standard therapy group, mean difference -3.5 [95% confidence interval -5.67 to -1.33]; BAL: bronchoalveolar lavage

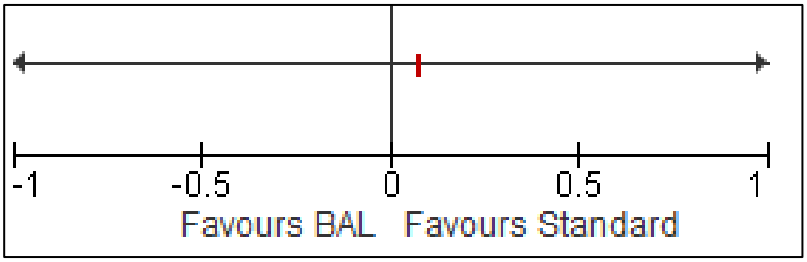


Figure 5-12 Forest plot for comparison of number of days as hospital inpatient per patient per year between BAL-directed therapy group and standard therapy group, risk difference 0.08 [95% confidence interval -3.31 to -3.47]; BAL: bronchoalveolar lavage

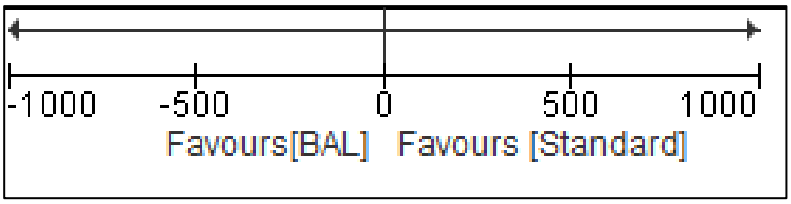


Figure 5-13 Forest plot for comparison of total cost of care per participant between BAL-directed therapy group and standard therapy group, mean difference 1902.00 [95% confidence interval -27508.98 to 31312.98] Australian Dollars; BAL: bronchoalveolar lavage

5.5 Discussion

This review found no significant difference in clinical outcomes and cost of care following treatment of pulmonary exacerbations in infants and children diagnosed by BAL compared to the standard management practice reliant on oropharyngeal cultures. As the review included only one relevant study, which although was a well-designed trial, a meta-analysis could not be conducted limiting the strength of the evidence. Furthermore, the only included study had insufficient power for detection of difference in prevalence of *P. aeruginosa* infection, which reflects need for use of other highly sensitive parameters in any further studies undertaken to address this issue.

5.5.1 Summary of main results

One randomised controlled study comparing outcomes following BAL-directed therapy with standard therapy was included. At age five years, there was no clear benefit of BAL-directed therapy on lung function or nutritional parameters; and there was no significant difference between the two groups regarding evidence of structural lung disease diagnosed by HRCT scan. The number of positive isolates for *P. aeruginosa* per child per year and the age at development of the first infection were similar in the two groups. In addition, the overall morbidity, measured by the number of pulmonary exacerbations was similar, and there was no difference in the proportion of participants who cleared *P. aeruginosa* infection after one or two courses of eradication therapy. A higher number of hospital admissions in the BAL-directed therapy group was balanced by the shorter duration of hospitalisations in this group. There was no difference in the overall cost of care and cost of hospital admissions.

5.5.2 Overall completeness and applicability of evidence

As only one study could be included in the review, a meta-analysis could not be conducted; however, this study provided highly relevant evidence to answer the review question. Although no evidence was available for adults with CF, this study did include infants and young children, which is the group mostly unable to provide sputum samples. Moderate quality evidence was available for most outcomes; however, regarding the microbiological outcomes, we should be aware that two different treatment goals were compared (e.g. clearance of *P. aeruginosa* from throat swab versus clearance from BAL). None of the four children, who were defined to have developed chronic *P. aeruginosa* infection by sampling via throat swabs, had the organism isolated from the BAL at the five-year time-point. While this highlights the difference in sensitivities of the methods involved, it suggests that we should be relying more on highly sensitive clinical parameters (such as LCI, HRCT) rather than microbiological outcomes for such studies. Most adults who do not expectorate spontaneously and many, quite young, children respond to hypertonic saline to provide satisfactory sputum samples (induced sputum). This procedure is less invasive as well as more acceptable than repeated BAL. No randomised controlled studies were available which compared treatment outcomes following induced sputum with bronchoscopy and BAL from single or multiple lobes. The available evidence was mainly relevant to *P. aeruginosa* infection. This is the most significant infection associated with CF lung disease; however, many other organisms are also increasingly being implicated in the disease progression. No study is available regarding the effect of the intervention on other organisms.

As there is no difference in the number of pulmonary exacerbations and the number of *P. aeruginosa* isolates per participant per year between the two treatment arms, the significant difference observed with regards to the number and duration of hospital admissions appears to be mainly protocol-driven and related to short admissions for BALs at different stages of the study. Data on the economic impact of conducting repeated bronchoscopies and frequent hospital admissions, however, did not show any difference between the two groups.

5.5.3 Quality of the evidence

The main strengths of the study were the quality of methods and the low attrition rate (despite a prolonged follow-up period and invasive intervention procedure). Though the study was set up for analysis on an intention-to-treat basis, it was analysed on an available-case basis, as the outcome data were available for more than 90% of participants. A sensitivity analysis for the prevalence of *P. aeruginosa* at five years of age, based on best and worst-case scenarios, led to similar results. The prevalence of *P. aeruginosa* and the establishment of chronic infection are clinically relevant outcomes considering the influence that chronic infection with this organism can have on progressive CF lung disease. However, this study highlights the difficulties with prolonged longitudinal studies, particularly when the outlook is changing consistently as with CF.

The main limitation of the included study was its reduced statistical power for its primary microbiological outcome. The study was designed with an anticipated prevalence of *P. aeruginosa* infection at five years of age to be 30% in the control group. The sample size of the study was revised during the study. The current sample size was predicted to provide adequate statistical power for HRCT scoring. It

was also regarded to provide sufficient power for *P. aeruginosa* prevalence end point based on the predicted controlled group prevalence of *P. aeruginosa* of 40% derived from the rates of infection observed at 2 years follow-up. However, the control group prevalence of *P.aeruginosa* was found to be only 12% at five years age. Other centres have also reported reduced prevalence of *P. aeruginosa* infection and a delay in the onset of chronic infection following improved management practices and the institution of early eradication therapy against *P. aeruginosa (650)*. Defining time to onset of chronic infection was difficult in this study as the comparison was between different methods of diagnosis, i.e. BAL-based cultures versus oropharyngeal cultures. The participants diagnosed with a chronic infection (by using only oropharyngeal cultures to define it) were very low in number, too low to allow any meaningful comparison. Only one participant in the BAL-directed therapy group developed chronic infection compared to four in the standard therapy group. The overall quality of evidence for the key parameters was graded as moderate, except HRCT scoring and cost of care analysis, which were graded as high quality (Summary of Findings Table 5-2).

Only 64% of the eligible participants could be recruited to the study; however, the age and sex distribution of the eligible participants who could not be enrolled in the study was similar to those who participated. In addition, the prevalence of bronchiectasis in the study participants was similar to that reported in other parts of Australia (*710*). These points suggest generalisability of the results to the Australian CF population.

A crucial point that can act as a confounding factor while carrying out multicentre studies is the practice of having different treatment protocols at different centres. As the issue of an effect of anti-

staphylococcal prophylaxis on the acquisition of *P. aeruginosa* infection is not yet resolved, it is important to mention that participants from three of the eight centres were using oral flucloxacillin prophylactic therapy until their first birthday. However, this study randomised and stratified the participants by the site to equalise the bias for both the groups; hence, the aforementioned difference is unlikely in practice to have affected the validity of the comparison.

A very detailed economic analysis of the cost of care for participants in the two groups was presented (*681*) which took into account various aspects such as pharmaceutical cost (both hospital and home treatment), the cost of procedures and investigations, the cost of hospital admissions and professional attendances. Acknowledging the difficulties of obtaining such large amount of data from various centres for a period spanning half a decade, a potential confounding factor in the economic analysis was the use of assumption and estimates for costs of some aspects of care (such as the cost of professional attendances or consultations from some centres). However, this was a small part of the overall picture and is likely to have been balanced over the two arms.

5.5.4 Potential biases in the review process

One of the review authors was also the lead author of the only included study. To avoid any influence of this fact on the interpretation of bias in the study, the remaining two review authors (KJ and AS) assessed the study for all potential sources of biases and agreed on the interpretations used to generate a risk of bias graph and any related comments.

5.5.5 Agreements and disagreements with other studies or reviews

In many non-randomised studies, bronchoscopy-based cultures have been found to give a higher yield of microorganisms. However, this review, which is limited to the evidence from a single study, raises several questions.

Oropharyngeal cultures have been shown to have a low positive predictive value but a high negative predictive value for a lower airway *P. aeruginosa* infection (*663*). This may imply that by taking oropharyngeal swabs more frequently, we are successful at ruling out positive cases while treating (or may be over-treating) some people who are negative.

On the other hand, although BAL has been described as a 'gold standard' method to diagnose lower airway infections, there is still no evidence-based answer to the question of what constitutes an infection for a BAL culture, and whether the presence of any number of organisms in BAL specimens from young people who are *P. aeruginosa*-naïve should be considered as evidence of infection. This study used a cut-off point of greater than 10^3 organisms, which was a balanced approach between the two extremes of detection of any organisms and greater than 10^5organisms (*663,711*). In children younger than five years age, the sensitivity of oropharyngeal cultures to detect lower respiratory infections improves as the lower limit of bacterial density (in a simultaneously collected BAL, used to define a positive culture) is increased (*663*).

Could the lack of effect be related to the sensitivity of BAL for diagnosing lower airway infections? There is evidence to suggest that sampling two lobes for BAL is better than sampling only one (*712*); however, the microbiological yield is shown to improve further if

samples are taken from all the lobes (*713*). It is uncertain whether this will make a real difference to the overall outcome for all people with CF. Analysis of the microbiology of airway cultures during pulmonary exacerbations suggests negative CF-related bacterial cultures in a significant proportion of exacerbations (*714*). Similarly, there may not be any change in the bacterial density within the respiratory secretions during a pulmonary exacerbation (*715*). A negative BAL culture may indicate no infection or signify the lack of ability to detect the complex range of organisms comprising the CF airway microbial community (*716*).

Eradication therapy for *P. aeruginosa* in CF is highly effective. To achieve even greater efficacy in eradication, more sensitive diagnostic tests for *P. aeruginosa* will be needed, particularly for children and individuals who do not produce sputum. The Wainwright study (*685*) used HRCT score as one outcome measure, which is shown to be a highly sensitive parameter for detecting structural lung disease (*717-719*), albeit at the cost of increased radiation exposure. Though pre-school children have been shown to provide reliable lung function results (*720*), the LCI detects abnormal lung function more readily compared to spirometry (*721*). It is shown to act in a complementary fashion to HRCT (*722*) and can be a better alternative to spirometry to investigate early differences in lung function (*717*). However, even if a minor improvement in outcome was to be disclosed with these measures, it has to be enough to justify subjecting all patients to an invasive procedure with the potential risk of mild adverse effects.

5.6 Conclusion

5.6.1 Implications for practice

This review, limited to only one included study, shows that there is insufficient evidence to support the use of BAL routinely for the diagnosis and management of pulmonary infections in pre-school children with CF compared to the standard practice of providing treatment based on results of oropharyngeal cultures and clinical symptoms. There are no data available with regard to the adolescents and adult populations.

5.6.2 Implications for research

More research needs to be oriented towards the effect of incorporating BAL at certain stages in the management of CF, e.g. in asymptomatic patients at diagnosis of CF. A comparison of outcomes following the use of other less invasive alternatives, such as induced sputum, needs to be made. Highly sensitive outcome measures like HRCT and LCI individually or a composite score of these need to be explored further. Considering the improved health of young people with CF, in order to identify any difference in outcomes, larger studies will need to be conducted for a longer duration of follow-up.

Chapter-6 Arterial Stiffness in CF

6.1 Introduction

Ischaemic heart disease is reported very infrequently in patients with CF (*723,724*). To some extent, this may be due to reduced survival in CF, compared to the general population. Historically, adults with CF were shown to have lower blood pressure (*725*) and a favourable lipid profile with lower mean serum cholesterol levels compared to controls (*726*). With the increasing median age of patients with CF, the incidence of coronary artery disease is likely to increase, which is even more relevant considering the prolonged exposure of the ageing CF population to persistent inflammatory state and CFRD. In recent years, a potential increased risk of vascular complications in CF has been contemplated. Large artery haemodynamics of adult CF patients have been shown to be almost a decade advanced than expected for the chronological age (*160*).

6.1.1 Overview of Arterial Stiffness and Wave Reflection

In simple terms, arterial stiffness can be described as the resistance of blood vessels to deformation by pressure changes. The vascular stiffness of large arteries increases with increasing age (*161,727,728*). Aortic stiffness is recognised as an independent predictor of future risk of cardiovascular events and all-cause mortality (*9,728,729*). In addition, increased arterial stiffness is also a hallmark of CKD and a marker of progression to ESRD (*730*). Arterial stiffness, measured as pulse wave velocity (PWV), increased in a stepwise manner corresponding to the stage of CKD from one to five (*731*). There is also a concern regarding a vicious cycle i.e. increased arterial stiffness playing a causative role in end organ damage. Greater PP surges

associated with aortic stiffness produce reactive changes in the microvasculature of end organs such as kidneys, brain and heart (*732*).

6.1.1.1 Vascular Haemodynamics

The arterial pulse wave consists of two components: the pulsatile component, caused by ventricular ejection, and the steady component, present during the diastolic phase. The pulsatile flow is cushioned by the compliance of large vessels. Compliance of a vessel is the ability of the vessel to expand or contract in response to change in pressure within the vessel (i.e. change in volume per unit change in pressure), which is the inverse of the stiffness. Viscoelasticity of the vessel wall varies along the arterial tree (*733*). The aorta is a highly compliant elastic conduit; however, the compliance decreases in the distal, more muscular vessels increasing the stiffness. The young, healthy aorta transforms the pulsatile flow from the left ventricle to a near-steady flow, which helps protect the peripheral microcirculation from pressure-induced changes and supports diastolic coronary perfusion. Various indices used to describe arterial stiffness and related terminologies are presented in Appendix (please refer to Appendix Chapter VI Table 8-2 and Table 8-3). The speed of travel of blood along any blood vessel is defined as pulse wave velocity (PWV) of that vessel. Carotid-femoral PWV (cfPWV) is a non-invasive, robust and reproducible measure (*9*) of arterial stiffness determined by the time taken by the arterial pulse to travel from carotid to femoral artery.

The forward propagating pressure wave encounters increasing impedance (described as total resistance to the flow offered by any vessel) in the peripheral vessels due to the branching of the arterial tree and variation in the wall properties (including structure, diameter and compliance). The stepwise increase in impedance (described as

impedance mismatch) offered by the peripheral vessels summates to generate a pressure wave reflection that travels back towards central vasculature and augments the forward pressure wave (Augmentation Pressure, AP). In healthy circulation, this reflected wave meets the forward wave in late systole / early diastole to generate a secondary diastolic pressure rise that helps enhance the diastolic perfusion pressure in the coronary arteries. In addition, it protects the peripheral microcirculation by deflecting the pulsatile energy. This phenomenon also explains the finding of higher PP in peripheral vessels compared to central PP, and why they should not be used interchangeably (*9*).

Augmentation index (AIx) describes the pressure augmentation by the reflected wave (i.e. AP or (P2-P1)) as a percentage of central PP.

$$AIx = \frac{P2 - P1}{central\ PP}\ X\ 100\ \%$$

Equation 6-1

AIx=Augmentation Index (Please refer to Figure 6-1)

P1=Initial Peak Pressure, P2=Pressure at point of inflection,

central PP=central pulse pressure

AIx is a measure of amplitude and site of wave reflection that includes arterial stiffness and has a complex relationship with it. As PWV and AIx provide different information related to arterial stiffness, it is recommended to measure both to assess the impact of risk factors on vascular haemodynamics (*734*).

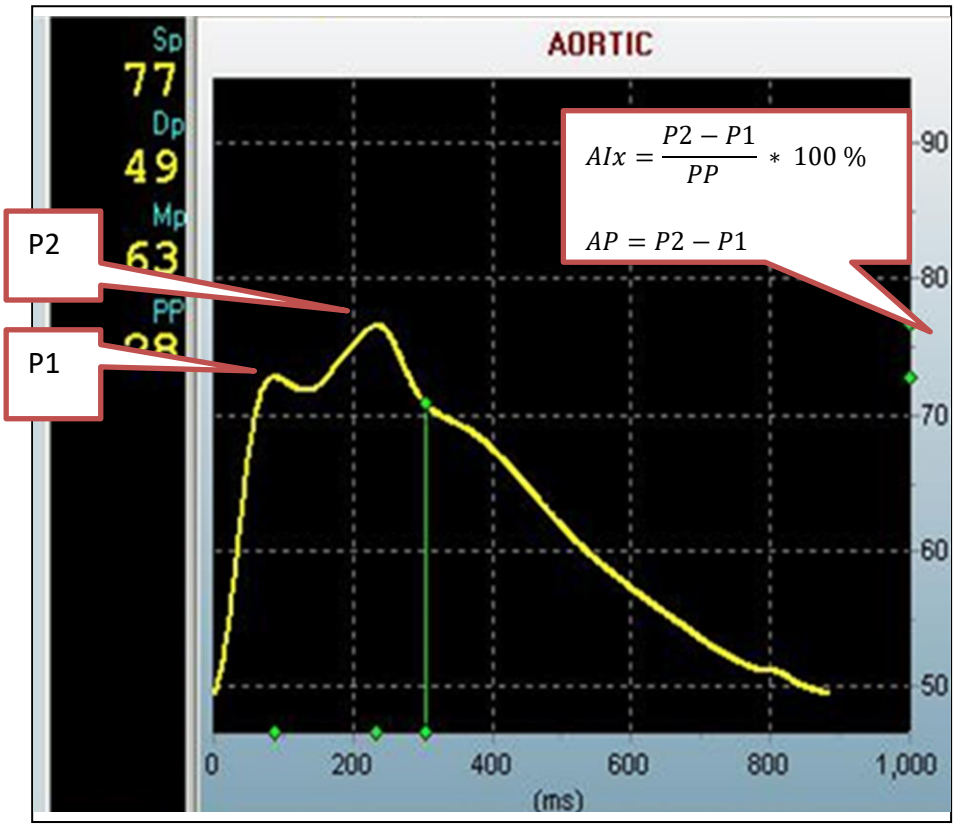


Figure 6-1 Augmentation Pressure (AP) and Augmentation Index (AIx); time (milliseconds) on x-axis and aortic pressure (mmHg) on y-axis; P1: initial peak pressure; P2: pressure at point of inflection of wave reflection, PP: pulse pressure; Sp: systolic pressure; Dp: diastolic pressure; Mp: mean arterial pressure

6.1.1.2 Effects of Vascular ageing

With ageing, there are structural and muscle tone related changes in the endothelial walls; the intima thickens (*735*), and the vessel diameter increases (*736*). The vascular compliance decreases and arterial stiffness progressively increases. Aortic PWV increases only modestly up to first 60 years of life, after which it increases dramatically. On the other hand, AIx is a more sensitive marker of cardiovascular risk in young age up to about 50-60 years; it increases significantly up to about age 60 after which it tends to plateau (Figure 6-2). The age-related stiffening is more marked in aorta compared to large muscular arteries (*9,735,736*). With the increasing age and increasing stiffness of the elastic arteries, there is a progressive loss of their cushioning effect and the reflected wave returns quickly to arrive earlier in systole (*732*). This increases the systolic pressure (increased AIx) and the PP while reducing the diastolic coronary perfusion. When the aortic stiffness approaches and exceeds the peripheral muscular arterial stiffness, the stiffness gradient at this interface is lost (or reversed) resulting in flattening or reduction of AIx (*9,734*).

Although no changes are evident in peripheral blood pressure measurement, early pressure changes affect central systolic pressure and increase central PP (*732*). It can increase haemodynamic stress to the organs directly supplied by central circulation (heart and brain). Increased central PP increases the systolic workload of the left ventricle (*737,738*) and predisposes to left ventricular hypertrophy; eventually, this predisposes to increased myocardial oxygen consumption and myocardial ischaemia. While the debate is still continuing regarding the association of low diastolic blood pressure with reduced coronary perfusion (*739,740*), there is evidence suggesting an association between increased arterial stiffness and reduced coronary perfusion and coronary reserve (*741,742*). These

vascular haemodynamic changes are intensified by various cardiovascular risk factors including hypertension, smoking, atherosclerosis, systemic inflammation, etc. A study on a large population cohort showed that there is a differential effect of risk factors on different parameters based on age. There is a stronger association of risk factors with aortic PWV in older individuals, and the same applies to the association of risk factors with AIx mainly in the younger population (*743*). As isolated systolic hypertension and increased PP are late surrogate markers of increased vascular stiffness; early identification of central pressure changes may provide an opportunity to take preventative actions and implement therapeutic measures. A new pathophysiological model of cardiovascular risk stratification has been developed which incorporates the concept of early vascular ageing (EVA), defined as PWV z score values of >95th percentile for that population (*744*).

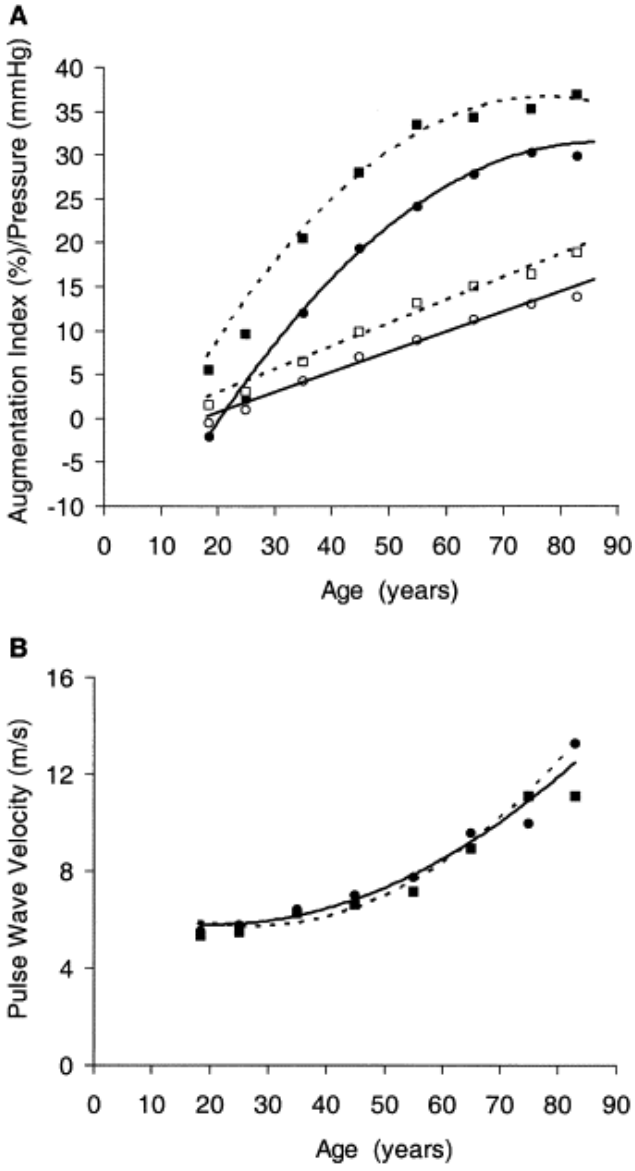


***Figure 6-2 Regression curves showing the effect of age on parameters of arterial stiffness for males (circles and solid lines) and females (square and dashed line); Panel A depicts changes in Augmentation pressure (open circle and squares) and Augmentation Index (closed circles and squares), Panel B illustrates aortic pulse wave velocity, (Image reference: (734)*)**

6.1.2 Arterial stiffness and microcirculation in the end organs

With attenuation of the peripheral wave reflection, the microcirculation in the end organs is directly exposed to the central pulsatile energy (*745*). The microvasculature of high blood flow organs like brain, retina and kidney are very sensitive to pressure pulsatility resulting in structural and functional remodelling of the vessels. Higher PP was shown to be associated with increased brachial artery vascular resistance in both resting and hyperaemic phases (*746*). Increased arterial stiffness was associated with impaired microvascular reactivity in the form of elevated flow velocity at baseline and during reactive hyperaemia after ischemia (*745*) as shown on the forearm vessels. This can lead to long-term, insidious damage following recurrent transient ischemia. Increased carotid flow pulsatility and cfPWV were associated with diffuse microvascular brain lesions and reduced scores in multiple cognitive domains (*747*). In addition, impaired peripheral vascular reactivity and increased microvascular resistance can affect central arterial stiffness that in turn, exaggerates the former, thus leading to a vicious cycle of increasing microvascular damage and arterial stiffness (*732*).

6.1.2.1 Arterial stiffness & CKD: A vicious circle

6.1.2.1.1 CKD propagating vascular changes

Cardiovascular disease is an important cause of morbidity and mortality associated with CKD (*748*). There is a graded increase in the risk of death, cardiovascular events and hospitalisation with reducing eGFR, which is independent of other risk factors (*181*). The effect has been noted from early stages of CKD with relatively high GFR of 90 ml/min/1.73m^2 (*749*). Increased arterial stiffness has been linked to cardiovascular comorbidity in CKD (*750,751*). The mechanisms have not been clearly identified [33]. One hypothesis suggests that

hyperphosphatemia, consequent to chronic renal disease, predisposes to the mineralisation of vascular smooth muscles, leading to vascular calcification (*752,753*), which has been associated with increased arterial stiffness (*750*). There is evidence to suggest increased PWV in advanced renal disease (*731,754-756*) but there are conflicting data regarding its relationship with mild renal insufficiency (*757-764*); the studies are summarised in the Appendix (please refer to Appendix Chapter VI Table 8-4, Table 8-5,Table 8-6,Table 8-7 and Table 8-8). The majority of the evidence is from cross-sectional studies on a middle-aged to elderly population, and different approaches have been used to assess arterial stiffness in each study. Only study so far, correlating arterial stiffness parameters with mGFR (gold standard ^{51}Cr-EDTA method) did not find an increase in aortic stiffness with the progression of CKD. However, carotid artery stiffness significantly increased during the 3-year follow-up period (*765*). There was a consistent positive association of vascular parameters with albuminuria, which was true even below the levels that are used to define microalbuminuria (*760*).

6.1.2.1.2 Vascular haemodynamics driving CKD

On the other hand, there is now evidence suggesting that increased arterial stiffness worsens renal impairment (*759,766,767*) and this propagates a vicious cycle of events. The pathogenesis is not completely understood, but there are several shared risk factors between arterial stiffness and CKD, which might play a part. Impaired vascular reactivity of the glomerular capillary bed may be an associated factor.

Glomerular capillaries are situated between afferent and efferent arterioles, and the resistance in the efferent arterioles is greater than the afferent arterioles to allow for filtration of large volumes. As a

result, glomeruli are highly vulnerable to variable pulsatile pressures. Impaired microvascular reactivity associated with a high baseline flow has been compared to a state of hyperperfusion (and so hyperfiltration) in the kidneys (*746*), which may propagate as glomerular damage leading to loss of renal function. Recent evidence from a study on hydronephrotic rat model suggested that renal autoregulation is a mechanism to protect glomeruli from pressure variations and afferent arterioles vasoconstriction in response to the arterial pulsatility rather than the mean arterial pressure (MAP) (*768*). Impaired autoregulation has been associated with increased glomerular capillary pressures in hypertensive rats (*769*).

Clearly, the relationship between arterial stiffness and CKD is quite complex with many unanswered questions and unexplained mechanisms with evidence from some studies pointing towards a vicious cycle of events, predominantly in the advanced stages of CKD. This can be highly relevant in older CF population particularly concerning the reduction in GFR, seen following lung transplant. It is vital to identify risk factors early to allow implementation of preventative strategies.

6.1.3 Arterial stiffness and chronic systemic inflammation

Arterial stiffness has been linked to the presence of systemic inflammation and its markers. Although there are several studies involving asymptomatic or hypertensive subjects that explore the relationship of arterial stiffness with the circulating inflammatory markers, e.g. CRP, there is no conclusive evidence. While some studies show a positive association of CRP with both PWV and AIx (*770*), others show correlation with only one of these parameters (*771-775*). A population-based study including male subjects, followed over 20

years (*770*), revealed that AIx was strongly related to current levels of CRP whereas PWV had a significant correlation with cumulative exposure to CRP, i.e. arterial stiffness was influenced by repetitive cyclical stress and inflammation (which is also a feature of CF).

Increased arterial stiffness has been demonstrated in patients with various systemic inflammatory disorders including systemic lupus erythematosus and rheumatoid arthritis (*776*). Some of these studies included adolescent and young adult subjects (*777,778*). This effect was shown to be independent of the presence of traditional risk factors (*776*). Young's elastic modulus (a measure of vascular stiffness) was shown to correlate with duration of inflammatory disease, the level of inflammatory markers (CRP and interleukin 6 (IL-6)) and fasting serum cholesterol (*776*). Treatment with anti-tumour necrosis factor-α was shown to reduce aortic inflammation in patients with rheumatoid arthritis (*779*). The beneficial effect of anti-inflammatory therapy to reduce arterial stiffness was demonstrated (*779-782*) in some studies but not in others (*783*).

Early identification of markers of cardiovascular diseases including parameters of arterial stiffness may provide a window of opportunity to institute early intervention measures or implement preventative strategies. Longitudinal studies will be needed to assess the progression of changes and effects of interventions.

6.1.4 Vascular haemodynamics: relevance in CF

Cardiovascular involvement has not been a component of CF multisystem spectrum of diseases. Traditionally, patients with CF were considered to have a reduced risk of developing cardiovascular diseases consequent to protective lipid profile and lower blood pressure values compared to subjects without CF (*725,726*). However,

these concepts have been reviewed in the light of increased survival reaching up to or beyond adulthood.

The factors contributing to the concern regarding cardiovascular diseases in CF are related to prolonged exposure to multiple risk factors in addition to the usual risk factors including ageing in general. The presence of persistent or recurrent inflammation, complications of long-standing diabetes, repeated exposure to nephrotoxic drugs with the potential to develop CKD are some of the common issues in CF population, which might contribute to increased risk of haemodynamic alterations including increased arterial stiffness.

Increased triglyceride to high-density lipoprotein cholesterol levels have been associated with increased arterial stiffness in children, adolescents and young adults (*784*). Patients with CF show abnormal lipid profiles including hypertriglyceridaemia (*159,785,786*) and lower high-density lipoprotein cholesterol levels (*785,786*). Pancreatic insufficient patients tend to have hypocholesterolaemia whereas pancreatic sufficient patients are more likely to have high cholesterol levels (*786*). Total cholesterol levels and triglyceride levels increase with increasing age and increasing BMI (*786*), which is likely to increase the risk of cardiovascular complications.

Animal studies on the mouse model of CF (age 16-20 weeks, adult mice) have shown that aortas of mice with Phe508del mutation have decreased internal diameters, reduced distensibility and are stiffer compared to wild-type mice (*787*). These findings were in the absence of any concomitant lung disease (airway transport defect, characteristic of CF airways in humans, is not observed in the airways of mouse model of CF (*522*)). A number of human studies including a study of adult patients with CF showed increased AIx compared to controls (*160*). AIx was greatest in the subgroup with CFRD. AIx was

related to circulating CRP and FVC. Paediatric patients with CF were shown to have significantly lower aortic compliance than the control group (*788*). Severe lung disease (FEV_1 <40%) was associated with reduced distensibility (*788*). Digital volume pulse (DVP) analysis to compute stiffness index (SI_{DVP})and cfPWV in children with CF (median age 12 (IQR 9.3-14.8) years) showed increased SI_{DVP} compared to controls (*789*).

6.2 Hypothesis & Aims

6.2.1 Rationale for the study

Advanced vascular ageing has been demonstrated in adult patients with CF; this was associated with systemic inflammatory status and FVC (*160*). Log CRP levels predicted nearly 50% of the variation in AIx in conjunction with age and height (*160*). Inflammation and chronic pulmonary infections have been documented at an early age in CF (*661,662,790*); Systemic inflammatory markers are raised in individuals with CF even when well (*791*). Chronic systemic inflammation has been linked with increased arterial stiffness (*772,776,792*). It is possible that the onset of vascular alterations in CF happens before adulthood. Vascular haemodynamic disturbances have also been associated with changes in eGFR in several studies including both mild and advanced CKD; however, any relationship with mGFR in children has not been studied so far.

I hypothesised that:

1. Vascular haemodynamics are altered in children with CF.
2. This alteration is associated with the current level of inflammation (increased circulating inflammatory markers), altered renal function (decreased GFR) and reduced lung function (FEV_1 and FVC).

6.2.2 Objectives

This study was undertaken with the following objectives:

6.2.2.1 Primary objectives

1. To measure vascular haemodynamic parameters in children with CF and compare with the previously published values in healthy children of similar age, specifically:
    a. AIx
    b. cfPWV
2. To assess relationship of AIx and cfPWV with mGFR (^{51}Cr-EDTA GFR)

6.2.2.2 Secondary objectives

1. To evaluate relationship of AIx and CfPWV with the following:
    a. Lung Function (spirometry)
    b. Circulating Inflammatory markers (high sensitivity CRP (hsCRP) and IL-6)
    c. Chronic *P. aeruginosa* colonisation

6.3 Material and Methods

6.3.1 Study design and setup

This study had an observational, cross-sectional design. Study was designed by Professor Alan Smyth, Dr Charlotte Bolton and I. It was conducted alongside CEFIT CF study, a larger study aimed at measuring GFR by the ^{51}Cr-EDTA method, see Chapter 2. The study was set up to include patients from multiple centres across the Midlands region of the UK. Patients were recruited from Nottingham Children's Hospital, and its CF shared care centres, University Hospitals Leicester, and Birmingham Children's Hospital. Experiments were carried out at Children's Research Facility at Queen's Medical Centre, Nottingham and at Wellcome Trust Children's Research Facility at Birmingham Children's Hospital, UK. Medicines for Children's Research Network nurses provided some administrative and nursing support.

The corresponding CF clinical teams identified potential participants. The eligible children and the families were approached while attending CF clinics or while attending hospital for other reasons as part of the broader research project, the CEFIT CF study. Those children who were between 8-14 years old were also provided with information regarding the arterial stiffness study. Informed consent was obtained from parents/legal guardians who were able to consent for the children. Informed assent was taken from the participating children.

Nottingham Research Ethics Committee granted an amendment to offer full ethical approval of the study (Reference Number: 09/H040723). Ethical approval also included taking consent for the review of medical notes and acquisition and retention of clinical samples in accordance with Human Tissue Act 2004, (*457*), licence number 12265: granted to the University of Nottingham's Faculty of

Medical and Health Sciences and licence number 11005: granted to Birmingham Children's Hospital (*457*).

The participants were invited to the study when they were clinically well (not hospitalised or having IV antibiotics) and not had IV antibiotics for at least 2 weeks prior to the study. They were asked to avoid caffeine-containing drinks from up to four hours before assessment. If taking salbutamol inhaler regularly, the participants were advised to avoid taking the morning dose or take it at least four hours prior to the study. The participants were advised not to speak or sleep during the study and stay still as possible.

The CEFIT CF study was already ongoing when this study was planned, and approvals were obtained. The patients who had already had their GFR measured prior to the ethical approval of arterial stiffness measurement were considered eligible if their age on 31/10/2011 was between 8-14 years. I approached these patients by phone or during hospital attendances to explain the arterial stiffness measurement. A separate appointment was then arranged for those patients who were interested in participating. Consent was obtained from the parents / guardians. For those patients who had their GFR measured after the approval of the amendment, arterial stiffness was measured on the same day as GFR measurement. These patients were considered eligible if their age on the day of GFR measurement fell between 8-14 years.

A standard study proforma was used to record data on the patients. The included fields were as following:

1. Demographics including age, sex, ethnicity
2. Height and weight
3. Time of the last dose of bronchodilator

4. Family history (first-degree relatives) of cardiovascular illness, hypertension, diabetes, cerebrovascular accidents and high cholesterol levels
5. History of concurrent smoking in the household or smoking during pregnancy
6. Comorbidities including diabetes, chronic *P. aeruginosa* infection were recorded from the medical notes or from the UK CF Registry database.
7. Concurrent medications were recorded from consulting patient's medical notes.

6.3.2 Clinical assessment

The weight was measured on electronic scales wearing indoor clothes and without shoes. The height was measured using a fixed stadiometer with socks on. After the children were rested for about 10 minutes, right brachial artery seated blood pressure was assessed using an oscillometric sphygmomanometer (Spot Vital Signs® Lxi Device, Welch Allyn®, New York, USA) in triplicate, using cuff sizes appropriate for the arm. This was followed by supine blood pressure measurement in the same arm. A mean of the three readings was taken.

6.3.3 Haemodynamic assessment

I received 2 days training in the technique of pulse wave analysis (PWA) and PWV measurement in the University of Cambridge, cardiovascular medicine department (Dr C. McEniery and Ms J. Woodcock-Smith). I performed the vascular haemodynamic assessment for all the children.

6.3.3.1 Pulse wave analysis (PWA)

The radial artery waveforms were recorded using a mikro-tip tonometer (Millar Instruments, Texas, USA) attached to an electronics

module (Sphygmocor software). The Sphygmocor device (AtCor Medical, Sydney, Australia) uses the applanation tonometry method. Applanation stands for flattening and tonometry means the measurement of pressure. The tonometer is applied to the radial artery with mild pressure sufficient to flatten the artery partially, which allows transfer of pressure to the sensor in the tonometer. This pressure is recorded digitally and presented as a waveform on the Sphygmocor screen. Using these measurements, the Sphygmocor software estimates the calibrated ascending aortic blood pressure waveform by generalised transfer function and calculates various parameters including central systolic/diastolic blood pressure, central mean blood pressure, and AP and AIx (please refer to Section 6.1.1.1 for definitions) (*793,794*).

The AP represents the increase in the systolic pressure generated by wave reflection and is calculated as the pressure difference between the first systolic peak (P1) and the second systolic peak (P2, produced by the reflected wave) i.e. P2-P1. The AIx is a ratio of AP to PP (details in the Section 6.1.1.1*,* Chapter 6).

The PWA measurements were undertaken in both sitting and supine positions using the respective peripheral blood pressure values. Overall quality of the captured signals was indicated by operator index (OI) which is calculated by Sphygmocor software by assigning a weighting to various quality control indices and adding them to give a percentage. These indices include average pulse height, pulse height variation, diastolic variation, shape deviation and maximum rate of rise of the peripheral waveform. I recorded readings with OI of ≥80%. I recorded the first two readings (OI ≥80%) unless the AIx adjusted to heart rate 75 beats/min (HR75) (described in next paragraph) differed by >4%. In this case, a third reading (OI ≥80%) was taken, and the

mean of the closest two readings was used. For those patients, where I did not manage to get OI >80%, an independent expert observer, Dr J H. Hull, (Respiratory Biomedical Research Unit, Royal Brompton & Harefield NHS Foundation Trust, London) reviewed the waveform traces for their quality and selected two suitable traces. These traces were then used to calculate the mean values (Please refer to Figure 6-3 for an example).

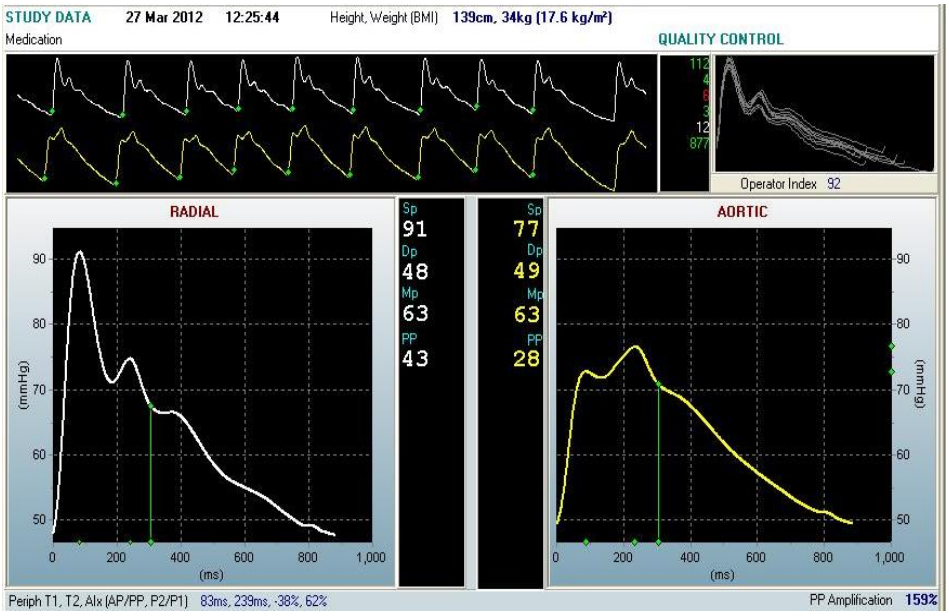


Figure 6-3 Waveform traces for pulse waves on the Sphygmocor screen; the frame on bottom left shows radial waveform captured by the tonometer and peripheral blood pressure parameters in the adjacent box; the frame on bottom right shows aortic waveform and central pressure parameters in the adjacent box (estimated by Sphygmocor software using generalised transfer function); Sp: systolic pressure; Dp: diastolic pressure; Mp: mean arterial pressure; PP: pulse pressure

6.3.3.2 AIx adjustment for heart rate

As AIx varies significantly with variation in the heart rate (HR), the Sphygmocor software adjusts the calculated AIx values to a random heart rate 75 beats per minute (bpm). Based on the linear regression results (β=slope, change in AIx per unit change in HR), I adjusted AIx values to heart rate 75 bpm (HR75) by using the following (*795*) (Equation 6-2).

$$\boldsymbol{AIx\ \ seated\ adjusted\ at\ HR75 = AIx - \beta * (HR - 75)}$$

Equation 6-2

6.3.3.3 Pulse wave velocity

The PWV system measures the velocity of the blood pressure waveform between any two superficial artery sites; it divides the PWV distance by the difference in the time between R wave in ECG and pulse onset at a peripheral site (Δt site A- Δt site B).

$$PWV = \frac{PWV distance}{\Delta T}$$

$$\Delta T = \Delta t\ site\ A - \Delta t\ site B,$$

Equation 6-3

Δt=difference in time between R wave in ECG and pulse onset at peripheral site (carotid or femoral)

A tonometer is used to record the pressure pulse waveforms transcutaneously at the two chosen sites consecutively. The distance between peripheral artery location and heart (referenced as the suprasternal notch) is measured. PWV distance is described as the difference between the distances of two sites from heart.

Sphygmocor uses 'intersecting tangent' algorithm to determine the time of respective pulse onset. Simultaneous to this, an ECG is

recorded to provide an R-wave timing reference. Sphygmocor software then uses the reference points on each set of pressure pulse and ECG waveform data to calculate the mean time difference between the R-wave and the pressure wave which is used to calculate the time delay or transit time (ΔT) between the 2 waveforms.

I used the 'gold standard' carotid-femoral (aortic) PWV. The electrodes were placed for ECG. Distances were measured between suprasternal notch to carotid and suprasternal notch to femoral (via umbilicus) using a tape measure. PWV was measured at the two sites in the supine position. An average of first three PWV readings with standard error means (± SEM) ≤1 and variation ≤0.5 m/s was taken. If the variation was >0.5 m/s, I selected those two values which were ≤0.5 m/s apart or closest and took an average and discarded the third value.

6.3.4 Spirometry

After the haemodynamic measurements, a forced expiratory manoeuvre was performed to measure FEV_1, FVC and Peak expiratory flow (PEF). The spirometry was performed using a Microlab MK-8 spirometer (ML3500), (Care Fusion, Germany) for all patients by me except those who had the study done in Birmingham, where Master lab (Viasys® Health Care, Germany) was used by the lung function technician, Ciaran McArdle.

The spirometer calibration check was performed at the beginning of each test day. All children had experience of performing lung function tests from previous clinic attendances. Prior to the test, the lung function technician or I coached the children for the procedure. The best of minimum three acceptable and maximum of up to eight acceptable attempts was chosen in accordance with ERS/ATS task force recommendation to fulfil the repeatability criteria (*601*). The test

was performed in standing position, and a nose clip was used. Incentive devices were used according to patient preference. Bronchodilator use was restricted up to 4 hour before the test. The percent predicted values were calculated based on reference equation by Zapletal et al. (*796*). The results were printed and stored in the case report form.

6.3.5 Inflammatory markers

While inserting cannula for measuring ^{51}Cr-EDTA, a 2-4 ml blood sample was collected in a serum separating tube. The collection of blood sample was done by investigators (Dr A Prayle or me). The sample was allowed to clot at room temperature for 30 min. The sample was then centrifuged at 1000g for 15 min to separate the serum. The serum was stored at -80° Celsius until analysis in accordance with Human Tissue Act, 2004 (*457*). The samples were sent to Immunology lab in Northern General Hospital, Sheffield for measurement of inflammatory markers. The samples were transported in accordance with The Carriage of Dangerous Goods and Use of Transportable Pressure Equipment Regulations 2009, No. 1348 (*797*), meeting UN3373 requirements. IL-6 and hsCRP were measured by Electrochemiluminescence (ECL) immunoassay (COBAS6000-e601 module, Roche Diagnostics, USA) and Nephelometry assay (BNII analyser, Siemens Healthcare Diagnostics, UK) respectively. Compared to conventional CRP assay, hsCRP assay has a significantly lower detection limit (<0.3 mg/L) and has been developed specifically to detect subclinical inflammatory states (*798*).

6.3.6 Statistical methods

IBM SPSS, Statistics for Windows, version 22 (IBM Corp., Armonk, NY, USA) was used to perform statistical analysis. The outcome variables were assessed for normality by viewing histograms. Age was divided into 4 age groups (<9.00yr, 9.01-11.00, 11.01-13, ≥13.01 years) for correlation with supine and seated AIx and used as a continuous variable for correlation with PWV (non-parametric). For normally distributed variables, general linear model regression analysis was done to calculate regression coefficient, R^2 values and adjustment was done for confounding factors, which are described in each section. For the non-normally distributed PWV, correlations were done by Spearman's Correlation and within-group comparisons for PWV were done using appropriate non-parametric tests. Test result values below the detection limit (0.173 mg/L for hsCRP and 1.5 pg/ml for IL-6) were excluded from analysis.

6.4 Results

A total 34 patients were eligible for the study from the age criteria, and 28 patients participated in the study. Of the 6 patients who could not be included, one patient did not consent; two patients agreed but no suitable dates could be arranged before the study closure (due to need to miss school/parents needing to take time off for half a day); one family could not be contacted. Two eligible patients did not attend the hospital and were included in CEFIT CF study based on clinical GFR results; hence, they could not be included in the arterial stiffness study.

Twelve patients had the study done in Birmingham, and 16 patients had it done in Nottingham and shared care centres. Arterial stiffness was measured on the same day as GFR measurement in 14 patients. A separate appointment was arranged in the rest. The median duration between GFR and arterial stiffness measurements was 3.5 months, range (0-27 months).

6.4.1 Patient characteristics

Patient characteristics are presented in Table 6-1.

6.4.1.1 Demographic details

The median age of participants was 11.5 (IQR 4.1) years. There were 13 males (46%). The majority of the patients (26/28, 92.8%) were of Caucasian origin, and two patients were of Asian descent.

6.4.1.2 Comorbidities

Two patients had a cardiovascular problem in the past for which they were still under follow-up/treatment. One patient had an atrioventricular septal defect for which corrective surgery was done in neonatal period. One patient had two episodes of paroxysmal supraventricular tachycardia, which was now under control on

medication. Only one patient had been diagnosed with CFRD and was on insulin treatment. Chronic *P. aeruginosa* infection was diagnosed by the treating physicians in 14 patients (50%).

6.4.1.3 Nutritional parameters

The mean BMI of the group was 17.5 (SD 3.1) kg/m^2 and the mean z score for BMI was -0.23 (SD 1.06).

6.4.1.4 Concurrent Medications

Ten patients were on long-term azithromycin treatment as part of anti-inflammatory treatment for CF. No patients were on oral hypoglycaemics, cholesterol lowering agents or bisphosphonates. Thirteen patients were on inhaled corticosteroid treatment including six patients who were also receiving low-dose alternate day oral steroid treatment simultaneously. One patient was receiving only oral steroids.

6.4.1.5 Family History

None of our patients smoked, however, one or both parents of 10 participants were current smokers. A history of maternal smoking during pregnancy was present for six of 23 participants, where a smoking history was available. A family history of cardiovascular disease in first-degree relatives was present in 2 patients.

Table 6-1 Patient Characteristics	
Characteristics	N=28
Sex: Male, n (%)	13 (46.4)
Age (years), median (IQR)	11.5 (4.1)
Weight (Kg), mean (SD)	36.2 (11.6)
Weight z score, mean (SD)	-0.47 (1.03)
Height (cm), mean (SD)	142.2 (15.15)
Height z score, mean (SD)	-0.59 (1.10)
BMI (Kg/m2), mean (SD)	17.5 (3.1)
BMI z score, mean (SD)	-0.23 (1.06)
Ethnicity, Mother: White, n (%)	26 (92.8)
Active cardiovascular problem n (%)	2 (7.1%)
CF-related Diabetes n (%)	1 (3.6%)
Prolonged azithromycin treatment n (%)	10 (35.7%)
Chronic *P.aeruginosa* infection n (%)	14 (50%)

IQR: interquartile range; SD: standard deviation; BMI: body mass index; CF: cystic fibrosis; P. aeruginosa: Pseudomonas aeruginosa; z score: standard score (shows how many standard deviation away (above or below) the observed mean is from the population mean)

6.4.2 Lab results

GFR was available in 27 patients. In one patient, the required number of blood samples for GFR measurement could not be collected, as their TIVAD stopped bleeding back after obtaining the initial sample. Mean GFR for the group was 124.3 ml/min/1.73m^2 (SD 22.8). The results of inflammatory markers, spirometry and GFR are presented in Table 6-2.

The results for inflammatory markers were available for all 28 patients. The minimum detection value of hsCRP and IL-6 were <0.173 mg/L and <1.5 pg/ml respectively. Where a value was below the detection limit, (10 subjects for hsCRP and 7 subjects for IL-6, it was excluded from the analysis. One extreme outlier for hsCRP and IL-6 each at higher end was excluded from the analysis. The highest values of hsCRP and IL-6 included in the analysis were 4.23 mg/L and 20.89 pg/ml respectively.

6.4.3 Spirometry results

The mean FEV_1 and FVC were 1.73 (SD 0.53) and 2.18 (SD 0.67) Litres respectively. The percent predicted values for FEV_1 and FVC ranged from 46% to 121% and 58% to 121% respectively. The results of spirometry are presented in Table 6-2.

Table 6-2 Laboratory and Spirometry Results					
Variable	**No. of patients**	**Mean / Median**	**SD/ IQR**	**Min.**	**Max.**
GFR (ml/min/1.73m^2)	27	124.33	22.84	74	173
FEV_1 (L)	28	1.73	0.53	0.89	3.1
FEV_1 (%)	28	85.54	21.15	46	121
FVC (L)	28	2.18	0.67	0.92	3.87
FVC (%)	28	89.93	18.68	58	121
FEV_1/FVC	28	80.07	8.4	61	99
PEF (L/min)	28	251.11	85.12	84	410
PEF (%)	28	83.39	22.26	39	116
Circ hsCRP (mg/L)*#	17	0.61*	2.22*	0.19	4.23
Circ IL-6 (pg/ml)*#	20	3.36*	3.98*	1.55	20.89

(*): Median/IQR; (#): one extreme outlier for each (hsCRP and IL-6) and all values below detection limit of the assay were excluded leaving N=17 for hsCRP and N=20 for IL-6; IQR: interquartile range; SD: standard deviation; Min: minimum; Max: maximum; GFR: glomerular filtration rate; FEV_1: forced expiratory volume in one sec; FVC: forced vital capacity; PEF: peak expiratory flow; FEV_1/FVC/PEF (%): percent predicted values; Circ: circulatory; hsCRP: high sensitivity C-reactive protein; IL-6: interleukin 6

6.4.4 Seated Haemodynamic results

6.4.4.1 Seated Haemodynamic Characteristics (Table 6-3)

Results of seated haemodynamic characteristics are presented in Table 6-3. The mean peripheral systolic blood pressure (SBP) was 103.8 (SD 11.31) mmHg with the MAP being 76.6 (SD 7.65) mmHg. The seated central SBP (mean 89.0, SD 8.76 mmHg) was lower than seated peripheral SBP. The seated central diastolic blood pressure (DBP, mean 63.6, SD 7.57 mmHg) was similar to peripheral DBP (mean 62, SD 7.6 mmHg).

6.4.4.2 Seated AIx-adjustment for heart rate

Seated aortic AP was adjusted to heart rate 75 bpm (HR75) by Sphygmocor software; the mean value was 3.0 (SD 2.76) mmHg. Seated AIx (%), was adjusted to heart rate 75 bpm by the Sphygmocor software (AIx_HR75); mean seated AIx_HR75 was 11.6 (SD 10.41)%. Unadjusted AIx showed significant correlation with heart rate, (R^2=0.159, P=0.020, β=-0.432). To adjust AIx to HR75 following equation was used (Equation 6-4*)*:

$$\begin{aligned} \boldsymbol{Seated\ AIx\ \ adjusted\ at\ HR75\ (AIx@HR75)} \\ = \boldsymbol{AIx - \beta * (HR - 75)} \\ = \boldsymbol{AIx - (-0.432) * (HR - 75)} \end{aligned}$$

$$\boldsymbol{AIx = augmentation\ index, HR = heart\ rate}$$

Equation 6-4

The seated AIx at HR75 calculated by above equation (AIx@HR75) showed similar results (mean 11.4%, SD 10.37) as by the software (AIx_HR75).

6.4.4.3 Seated AIx-adjustments for confounder

Seated AIx@HR75 showed significant correlation with height (P=0.004, adjusted R^2=0.282). There was no significant association of seated

AIx@HR75 with age (age groups), (R^2=0.196, P=0.148), sex (R^2=0.019, P=0.491) and seated central MAP (R^2=0.054, P=0.232).

6.4.4.4 Seated AIx- correlations

Regression analysis was done using seated AIx@HR75, which was calculated by Equation 6-4, and the results are presented in Table 6-4.

There was no significant association of seated AIx@HR75 with GFR, R^2=0.001, P value=0.901, (Figure 6-4)**.**

There was no significant correlation of AIx@HR75 with percentage predicted values of FEV_1 (R^2=0.008, P=0.649), FVC (R^2=0.011, P=0.600) and PEF (R^2=0.049, P=0.255).

There was no significant association of AIx@HR75 with the level of inflammatory markers, hsCRP (R^2=0.00, P=0.938) and IL-6 (R^2=0.028, P=0.484). There was no significant association of AIx and chronic colonisation with *P. aeruginosa (*R^2=0.003, P=0.765).

Regression analysis following adjustment of AIx@HR75 with confounders individually (age group, sex, height, central MAP and FEV_1%) did not show any significant association of seated AIx@HR75 with any variable.

Table 6-3 Seated Haemodynamic Characteristics				
Haemodynamic Characteristics Seated	**MEAN N=28**	**SD**	**95% CI lower**	**95% CI upper**
Brachial SBP Seated (mmHg)	103.8	11.31	99.44	108.21
Brachial DBP Seated (mmHg)	62	7.6	59.05	64.95
Brachial PP Seated (mmHg)	41.8	7.68	38.84	44.79
Peripheral Mean Pressure seated (mmHg)	76.6	7.65	73.65	79.59
Central SBP Seated (mmHg)	89.0	8.76	85.62	92.41
Central DBP Seated (mmHg)	63.6	7.57	60.6	66.54
Central PP Seated (mmHg)	25.3	4.87	23.45	27.22
Central MAP Seated (mmHg)	76.6	7.66	73.65	79.59
Heart Rate (HR) Seated (bpm)	81.9	11.63	77.38	86.4
Aortic AP Seated[$] (mmHg)	2.3	2.98	1.16	3.47
Aortic AP Seated Adj.[#] (HR_75) (mmHg)	3.0	2.76	1.94	4.12
Aortic AIx Seated (%)[$]	8.4	11.52	3.94	12.87
Aortic AIx Seated Adj.[#] (AIx _HR75) (%)	11.6	10.41	7.58	15.66
AIx @HR75_Seated_Calc.[£]	11.4	10.37	7.36	15.41

([$]): raw values by Sphygmocor; ([#]): values adjusted to HR 75 bpm by Sphygmocor; ([£]): values adjusted to HR 75 bpm calculated by regression equation; CI: confidence interval; SD: standard deviation; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; MAP: mean arterial pressure; AP: augmentation pressure; AIx: augmentation index; Adj: adjusted for; HR: heart rate; bpm: beats/minute; Calc: calculated

Table 6-4 Regression analysis for Seated_AIx@HR75_calc with various exposure variables				
Parameter	**Regression coefficient-B**	**P value**	**Regression coefficient 95% CI**	
			Lower Bound	**Upper Bound**
GFR ml/min/1.73m^2	0.011	0.901	-0.177	0.199
FEV_1 %	-0.044	0.649	-0.242	0.153
FVC %	-0.058	0.600	-0.280	0.165
hsCRP mg/ L	-0.160	0.938	-4.154	4.474
IL-6 pg/ml	0.337	0.484	-0.654	1.327
Chronic *P.aeruginosa* colonization (=No)*	-1.20	0.765	-9.401	6.993

For binary variables (denoted by *), results are presented against the reference group (chronic P.aeruginosa colonization=yes) which was supposed to have a regression coefficient (B)=0; AIx@HR75_calc: augmentation index adjusted to heart rate 75 beats/min, calculated by regression equation; CI: confidence interval; GFR: glomerular filtration rate; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; hsCRP: high sensitivity C-reactive protein; IL-6: interleukin 6; P. aeruginosa: Pseudomonas aeruginosa

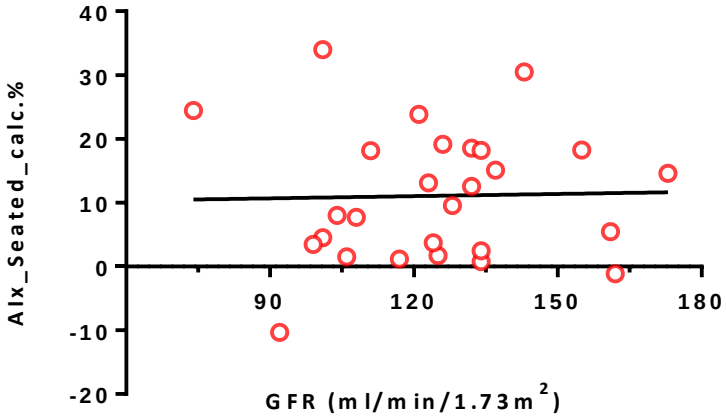


Figure 6-4 Scatter diagram of seated AIx with GFR (ml/min/1.73m^2); (R^2=0.001 P=0.901); AIx_seated_calc%: seated augmentation index (adjusted to heart rate 75 beats/min, calculated by regression equation) (%); GFR: glomerular filtration rate

6.4.5 Supine Haemodynamic Results

6.4.5.1 Supine Haemodynamic Characteristics (Table 6-5)

The results of supine haemodynamic characteristics are presented in Table 6-5.

6.4.5.2 Supine AIx-adjustment for heart rate

Supine aortic AP was adjusted to heart rate 75 bpm by Sphygmocor software; the mean value was 2.0 (SD 2. 32) mmHg. Supine AIx (%), was adjusted to heart rate 75 bpm by the Sphygmocor software (AIx_HR75); mean supine AIx_HR75 was 7.9 (SD 9.25) %. Unadjusted AIx showed significant correlation with heart rate, (R^2=0.144, P=-0.047, β=-0.266). To adjust AIx to HR75 following equation was used (Equation 6-5):

$$AIx\ \ supine\ adjusted\ at\ HR75(AIx@HR75)$$
$$= AIx - \beta * (HR - 75)$$
$$= AIx - (-0.266) * (HR - 75)$$
$$AIx = augmentation\ index, \qquad HR = heart\ rate$$

Equation 6-5

The supine AIx adjusted to HR75 (AIx@HR75) calculated by above equation (6-5) showed similar results (mean 8.1, SD 8.78%) as the mean AIx_HR75 calculated by the software.

6.4.5.3 Supine AIx-adjustments for confounders

The Supine AIx@HR75 had significant negative correlation with height (R^2=0.248, P=0.007). There was no significant variation in supine AIx@HR75 with other factors including age groups (R^2=0.112, P=0.406), sex (R^2=0.016, P=0.524) and supine central MAP (R^2=0.042, P=0.296).

Table 6-5 Supine Haemodynamic Characteristics					
Haemodynamic Characteristics (N=28)	**MEAN (SD)**	**95% CI lower**	**95% CI upper**	**Min.**	**Max.**
Brachial SBP Supine (mmHg)	101.7 (10.99)	97.45	105.98	78	126
Brachial DBP Supine (mmHg)	59.2 (8.18)	56.08	62.42	44	81
Brachial Mean Pressure (mmHg)	72.3 (8.46)	69.64	76.21	59	96
Brachial PP Supine (mmHg)	42.5 (6.41)	39.97	44.94	32.0	60.0
Central SBP Supine (mmHg)	86.4 (8.89)	82.90	89.90	70.0	107.5
Central DBP Supine (mmHg)	60.4 (8.25)	57.17	63.57	45.5	82.5
Central PP Supine (mmHg)	25.9 (4.08)	24.38	27.54	19.0	35.5
MAP Supine (mmHg)	72.9 (8.46)	69.64	76.21	59.0	96.0
Heart Rate Supine (bpm)	74.2 (13.54)	68.92	79.43	52.5	99
Aortic AP Supine (mmHg)[$]	2.2 (2.5)	1.26	3.20	-3.0	8.0
Aortic AP Supine Adj. (HR_75) (mmHg)[#]	2.0 (2.32)	1.15	2.95	-2.0	6.5
Aortic AIx Supine (%) [$]	8.3 (9.49)	4.60	11.96	-17.0	25.0
Aortic AIx Supine Adj (HR_75) (%) [#]	7.9 (9.25)	4.26	11.44	-9.50	25.50
cfPWV Supine (m/s)*	4.5* (1.0)			4.0	6.0
AIx_Supine@ HR75_Calc. [£]	8.1 (8.78)	4.66	11.47	-12.48	25.13

(*) Median (Interquartile range); ([$]): raw values by Sphygmocor; ([#]): values adjusted to HR 75 bpm by Sphygmocor; ([£]): values adjusted to HR 75 bpm calculated by regression equation; SD: standard deviation; CI: confidence interval; Min: minimum; Max: maximum; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; MAP: mean arterial pressure; AP: augmentation pressure; AIx: augmentation index; Adj: adjusted for; cfPWV: carotid-femoral pulse wave velocity; HR: heart rate; bpm: beats/min; Calc: calculated

6.4.5.4 Supine AIx- Correlations

Correlations of supine AIx@HR75 are presented in Table 6-6**.**

There was no significant association of supine AIx@HR75 with mGFR (R^2=0.013, P=0.568), (Figure 6-5). Regression analysis, after adjustment for confounders including age (age group), sex, height and supine central MAP (each variable added individually to the analysis), did not show any significant association between the AIx@HR75 and GFR. There was no significant association of Supine AI@HR75 with FEV_1 percent predicted (R^2=0.001, P=0.854) and FVC percent predicted (R^2=0.004, P=0.757).

There was no significant association of supine AIx with the level of inflammatory markers, hsCRP (R^2=0.001, P=0.912), IL-6 (R^2=0.007, P=0.734) or level of *P.aeruginosa* colonisation status (R^2=0.017, P=0.508).

Table 6-6 Regression analysis for Supine_AIx@HR75_calc				
Parameter	**Regression Coefficient B**	**P value.**	**95% Confidence Interval**	
			Lower Bound	Upper Bound
GFR ml/min/1.73m^2	0.044	0.568	-0.113	0.202
FEV$_1$ %	0.015	0.854	-0.152	0.183
FVC %	0.029	0.757	-0.160	0.218
hsCRP mg/L	0.198	0.912	-3.575	3.971
IL-6 pg/ml	0.154	0.734	-0.785	1.093
Chronic *P.aeruginosa* colonisation (=No)*	-2.250	0.508	-9.139	4.639

For binary variables (denoted by *), results are presented against the reference group (chronic P.aeruginosa colonization=Yes) which was supposed to have a regression coefficient (B)=0; AIx@HR75_calc: augmentation index adjusted to heart rate 75 beats/min, calculated by regression equation; GFR: glomerular filtration rate; FEV$_1$: forced expiratory volume in one second; FVC: forced vital capacity; hsCRP: high sensitivity C-reactive protein; IL-6: interleukin 6; P. aeruginosa: Pseudomonas aeruginosa

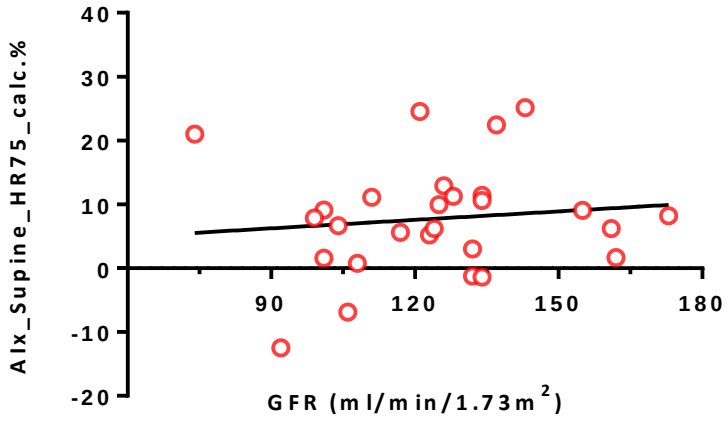


Figure 6-5 Scatter diagram of supine AIx with GFR (ml/min/1.73m^2) (R^2=0.013, P=0.568); AIx_Supine_HR75_calc%: supine augmentation index (adjusted to heart rate 75 beats/min, calculated by regression equation) (%); GFR: glomerular filtration rate

6.4.6 cfPWV

Results for cfPWV and its correlations (Spearman's Rho) are presented in Table 6-5 and Table 6-7 respectively. Median cfPWV was 4.55 (IQR 1.0, range 4.0-6.0) m/sec. There was a significant positive correlation of cfPWV with age (R=0.414, P=0.029) and central MAP (R=0.410, P=0.030). There was no significant correlation of cfPWV with height (R=0.334, P=0.082). There was no difference between cfPWV between male and females (P=0.525, Mann-Whitney U test).

There was no significant correlation of cfPWV with GFR (R=-0.216, P=0.280). There was no correlation of cfPWV with FEV_1 percent predicted (R=0.020, P=0.921) and FVC percent predicted (R=0.129,

P=0.513). There was no significant correlation of cfPWV with hsCRP (R=0.465, P=0.06) and IL-6 (R=0.-0.066, P=0.783). There was no significant association of cfPWV with colonisation status of *P. aeruginosa* (P=0.603, Mann-Whitney U test).

6.4.7 Comparison with historical cohort

The PWV and PWA results from this study were compared to the results from a previously published study (Epicure study) (*799*), where 90 children with mean age 11.27 (SD 0.47) years were recruited as healthy controls to have PWV and PWA measurements performed using the same method as the current study. This control group from Epicure study included 44% males (similar to current study with 46% males). The ethnic origin of the mothers of the children was mainly white British (88% for Epicure cohort and 92.8% for current study). The mean z score for weight and height for Epicure control group were 0.28 (SD 1.12) and 0.26 (SD 0.99) which were higher than the mean z score of weight (-0.47, SD 1.03) and height (-0.59, SD 1.1) for this study. The difference between the mean z scores for weight and height between Epicure and current study cohort was 0.750 and 0.850 respectively.

Supine AIx (8.07%, SD 8.7) in the children with CF was significantly higher than the AIx calculated in the Epicure control group (2.4%, SD 8.7, N=90) with the difference between the means of 5.67%.

Aortic (carotid-femoral) PWV from Epicure cohort (mean 4.72, SD 0.76 m/sec) was similar to cfPWV for current cohort (mean 4.76, SD 0.57 m/sec).

Table 6-7 Spearman's correlations for cfPWV		
Parameter	**Spearman's Rho-Correlation Coefficient**	**Significance**
GFR ml/min/1.73m^2	-0.216	0.280
FEV_1 % predicted	0.020	0.921
FVC % predicted	0.129	0.513
Circulating hsCRP mg/L	0.465	0.06
Circulating IL-6 pg/ml	-0.066	0.783

cfPWV: carotid-femoral pulse wave velocity; GFR: glomerular filtration rate; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; hsCRP: high sensitivity C-reactive protein; IL-6: interleukin 6

6.5 Discussion

This study measured aortic haemodynamic parameters including AIx and aortic PWV in children with CF along with ^{51}Cr-EDTA GFR, lung function and inflammatory markers. The results showed that AIx was significantly greater compared to previously published data from a cohort of healthy control children of similar age using the same device. On the contrary, the aortic PWV was not different. Haemodynamic parameters were not associated with GFR, inflammatory markers and lung function indices.

These results corroborate the findings of advanced vascular ageing in CF. Hull et al. showed an increased AIx in patients with CF (mean age ± SD 28 ± 8.2years) compared to a healthy control group (*160*). PWV values in people with CF were similar to those in the control populations. The lack of a difference in aortic PWV is perhaps not surprising given PWV changes are more marked in older individuals (>50 years old) (15) whereas AIx is a more sensitive marker of arterial stiffness in people <50 years age. DVP analysis in children with CF, median age 12.2 years (IQR 9.3 to 14.8 years), showed higher SI_{DVP} compared to a control group, median age 10.9 years (IQR 9.7 to 13.4 years); however, these results are not comparable to our study because of a different methodology. DVP is obtained by measuring the transmission of infrared light through the finger-pulp (Plethysmography). The contour of the DVP incorporates a systolic and a diastolic (inflected) component. The stiffness index or SI_{DVP} is defined by subject height divided by the time delay between the systolic and diastolic peaks. The height represents the path-length of the pulse wave from root of subclavian to the sites of reflection and back to subclavian artery. SI_{DVP} provides different information to cfPWV (*800*) as it is influenced by ventricular ejection as well as distensibility of

peripheral muscular arteries (*801*). SI_{DVP} has only a weak correlation with cfPWV (*802*); it is also a less precise measure as it uses height to compute the distance between the arteries (*9*).

This is the first study, to my knowledge, assessing the relationship between arterial stiffness and mGFR in children with CF. There was no significant association of AIx and cfPWV with mGFR. The relationship between arterial stiffness and renal function is complicated, and both the factors are thought to influence each other; there are conflicting results from various studies. People with the advanced renal disease have shown increased PWV compared to controls. However, many, but not all, studies in people with mild to moderately impaired renal function have shown increasing arterial stiffness in relation to progression of renal disease (please refer to Appendix-VI Table 8-4, Table 8-5,Table 8-6,Table 8-7 and Table 8-8). Similarly, there is no consensus yet on the role of arterial stiffness in the progression of renal disease. The evidence is mainly from the studies using creatinine-based formulas to estimate GFR. Creatinine excretion itself can lead to variation in eGFR, as it is dependent on muscle mass and dietary factors. The relation of arterial stiffness with ^{51}Cr-EDTA GFR (in non-CF individuals) has been explored by only one group so far; there was no association of GFR and cfPWV (*761*) and vice versa (*765*). Hull et al. (*160*) showed an inverse association between AIx and GFR in adults with CF (r=-0.55), who also had lower eGFR than the control group. Using this effect size and an $\propto$ value of 0.05, our study had a 90% probability of detecting any association of the same magnitude (*608,803*). This discrepancy in results between these two studies is probably related to inherent differences between the study populations and study methodologies. Apart from the age of the participants, a key difference between our study population and the study by Hull et al., in the context of association with GFR, is that we

had no patients with renal impairment and only one patient with GFR <90 ml/min/1.73m^2. Similarly, a higher proportion of patients in the Hull study had CFRD (one in four) compared to only one patient in our study. A different method to derive GFR (measured versus eGFR using Cockcroft and Gault equation that is creatinine based) might have influenced the results. The reliability of eGFR to predict GFR in patients with CF has also been questioned (*215,429*), (Chapter 2, Section 2.4.3).

This study found no correlation between arterial stiffness measures and the level of circulating inflammatory markers. Previous studies have shown an association between the systemic inflammatory disease and arterial stiffness (*776,804*). Some, but not all, (*779-783*) studies have shown a reduction in arterial stiffness with anti-inflammatory treatments. However, there are differing results for correlation of AIx and PWV with CRP. Some studies have shown an association of CRP with both AIx and PWV (*804*), whereas others have shown association with only one of the two parameters (*771,772,774,775*).

Our results are in agreement with a previous study on children with CF where no association was found between PWV and CRP (*789*). Again, the study on adults with CF (*160*) showed significant association of AIx with CRP which was not replicated in our paediatric study. The difference could have simply been related to different research methods, sample size issues or there could be a real difference. Most of the current literature is cross-sectional in nature. However, the correlation between arterial stiffness (and wave reflection measures) and various cardiovascular risk factors has been shown to vary with age (*770*). On the same principle, it may be possible that the correlation of wave reflection with inflammatory markers is not evident in children with CF. The other explanation may be related to

less advanced lung disease and subsequently, lower level of systemic inflammation in children compared to that in adults with CF. With prolonged disease activity and established chronic infections (*805,806*), adult CF patients are likely to have a higher level of background pulmonary and systemic inflammation compared to children with CF, which could have influenced the correlation with circulating inflammatory markers; The mean (± SD) FEV_1 % predicted was 65.1 (± 21.2) % in the Hull study compared to FEV_1 % predicted of 85.5 (± 21.5) % for the children in our study, which can be a reflection of higher level of pulmonary inflammation in the adult population. Median circulating hsCRP level of our CF population was comparable to the mean CRP level of the control group in the study by Hull et al. IL-6 levels have been shown to be higher in patients with CF (even with stable lung disease) in comparison to healthy controls thus suggesting a background level of ongoing chronic lung inflammation/injury (*791,807*). In our study, >50% of children had hsCRP or IL-6 levels or both levels below detection range (<0.17 mg/dl and <1.5 pg/ml respectively) which reflects low levels of inflammation in this group.

Previous studies on arterial stiffness parameters in patients with CF showed different results for association with lung function values. A study of adult patients with CF (*160*) showed a negative correlation of AIx with percent-predicted FVC. However, it was not predictive of AIx. There was no correlation between AIx and percent-predicted FEV_1. Buehler et al. (*789*) did not show any association between PWV and FEV_1 or LCI in children with CF. A magnetic resonance imaging based study (*788*) revealed that aortic compliance was lower in children with CF compared to controls, but it did not vary between the groups with high (>50% predicted) or low (<40% predicted) FEV_1. The aortic distensibility, however, was significantly lower in the CF patients with

low FEV_1 compared to the CF patients with higher FEV_1 and control subjects. Our study found no significant correlation of AIx or cfPWV with percent predicted values of lung function parameters, which was probably related to good lung function spectrum of our patients or to methodological factors including a small sample size of the study.

Bolton et al. (*808*) showed that the lung function of the middle age men recruited to a population-based study (Caerphilly Prospective Study) were inversely associated with PWV, and lung function in midlife were stronger predictors of the aortic PWV two decades later compared to the contemporaneous lung function. This effect was independent of the effect of various confounding factors e.g. smoking, early life influences like birth weight, socioeconomic factors (evaluated as a common mechanism pathway) or of the effect of inflammation (as a causal mechanism driving the vascular parameters). Authors speculated a possible role of a genetic or developmental process having an effect on both the connective tissue of the alveoli and arterial wall. Though these results cannot be generalised as this study included only males from a particular age group, there may be a possibility that the lung function in childhood predict the PWV in adulthood rather than the concurrent PWV. Such an association will need to be assessed in long-term studies, and indeed cannot be explained by cross-sectional studies.

A recent study suggests the role of CFTR in the regulation of the tone of arterial wall smooth muscles. An augmentation of CFTR function (*809*) by ivacaftor (marketed as Kalydeco®, a CFTR potentiator drug) in people with CF having a CFTR gating defect (mutation G551D) was shown to significantly increase the airway wall distensibility and decrease the PWV and AIx after 48 hour of therapy; there was no change in vascular parameters in control CF patients (with any CF

mutation and not on ivacaftor treatment). A CFTR-related modification of smooth muscle tone in the airway and the vascular wall was speculated. However, it was a small study with a very short duration of treatment, and more studies will be needed to evaluate this association. A study in an experimental mouse model showed that even in the absence of any concomitant lung disease (lung disease is not a feature of mouse model of CF), hearts of the mice with Phe508del mutation showed lower aortic internal diameter, reduced aortic distensibility and increased aortic PWV compared to matched control mice (107). These studies suggest the possibility of a direct influence of CFTR defect on vascular haemodynamics, rather than an indirect one mediated by lung disease.

Our results did not show any association of AIx and PWV with *P. aeruginosa* colonisation status. Although such associations have been shown in previous studies of CF and non-CF children (*789*), any interpretations are difficult in our study due to small numbers involved.

In this study, I measured AIx in seated and supine postures. Both heart rate and central blood pressure can be affected by change in posture (*810,811*); hence, it may be plausible that AIx and PWV are affected by change of posture. There is no specific guidance on which posture to use for AIx. Supine position has been recommended for measurement of PWV (*812*). Due to the need to palpate femoral artery, cfPWV cannot be measured in seated posture. Our study found that seated AIx (mean seated AIx adjusted to HR 75 bpm, 11.4%) was higher than supine AIx (mean seated AIx adjusted to HR 75 bpm, 8.1%). Many studies, using similar methods to this study, have measured and reported both seated and supine values for AIx. Previous studies have shown variable results with some studies showing higher AIx with

supine posture (*813,814*), whereas others showed no effect of posture (*815,816*). More studies are required to explain the effect of posture on vascular haemodynamic parameters (*811*).

Neither seated nor supine AIx showed a positive association with age and MAP; both of these factors have been shown to be independent determinants of AIx in various studies (*770,817-819*). Increasing arterial stiffness (or aortic PWV) with age has been seen in adults and in many studies in children and young adults (*820-822*). A cardiac catheter study in children between 6 months and 20 year age showed decreasing arterial compliance (normalised for body surface area) with increasing age and the decline being fastest during first several years of life (*823*). In the current study, the age of participants was categorised into four age groups in view of the non-normal distribution of the parameter and inability to meet the assumptions of linear regression model when used as a continuous variable for association with supine and seated AIx. Age was used as a continuous variable for its correlation with PWV in a nonparametric test. Lack of association of AIx with the established determinants (i.e. age) may have been related to a very narrow age range for the study participants and with a very narrow range of categories. Besides, a small sample size may have contributed to these results as a type II error.

The main strength of our study was the use of gold standard parameter cfPWV (*9*) and ^{51}Cr-EDTA GFR, which is a more practical and accurate alternative to the inulin clearance method (*824*). Measurement of GFR using a radioisotope method was a unique opportunity to assess the association of arterial stiffness with renal function in CF population, which has several factors predisposing to renal dysfunction. This is the first study, to our knowledge, measuring

both these parameters together in paediatric age group. The study had limitations including small sample size, cross-sectional design and lack of inherent control group. The results show a significant difference between the vascular haemodynamics in children with CF compared with a historical control group and justify a larger study with a control group to investigate the association. The historical control group used for comparison had a similar mean age and ethnic background, and same research methods were used.

Another methodological limitation of this study was the time gap between the measurement of GFR and the vascular parameters in half the study subjects; this ranged from 7 months to 27 months (average 17.5 months). CEFIT CF study was already running and recruiting patients when the current study was added after a major amendment to the study protocol. The time gap was accounted for by the time for approval of amendment by regulatory bodies and time required to organise another study visit. The effect of this time gap on study results, however, is likely to be very limited as changes in GFR or vascular haemodynamic parameters are gradual processes extending over several years (*825*).

Of the whole cohort, 35% and 25% patients had hsCRP and IL-6 levels below detection level (described as left censored data, 0.173 mg/L for hsCRP and <1.5 pg/ml for IL-6) respectively. Left censored data is handled in the literature in a variety of ways (*826*). Advanced statistical tests e.g. maximum likelihood estimation have been described to produce least biased mean and SD, and such tests are recommended widely for use in epidemiological and environmental investigations. Substitution methods replace the left censored data with a value e.g. zero, minimum detection level (*827*) or half detection level (*828*). Various substitution methods, though inferior to higher

order statistical methods, are commonly used in clinical studies as simpler and more practical alternatives to the former (*826,829*); however they are not recommended by many statisticians (*826,830*). As the level of inflammation represented by hsCRP or IL-6 values below detection level would be negligible, we decided to exclude these patients from analysis to avoid any bias introduced by substitution methods. Ignoring the values below detection level can lead to overestimation of mean; however, the influence on our results is likely to be minimal as the overall inflammation (as measured by systemic inflammatory markers) in the whole group was very low with the median hsCRP value (0.6 mg/L) below detection limit of a standard CRP test (<5 mg/L).

Although limited by its cross-sectional nature, this study failed to detect any associations that could explain the mechanism of higher AIx in children with CF. A prospective study in patients with metabolic syndrome showed a higher rate of progression of PWV in patients with more than three cardiovascular risk factors (during a 6-year follow-up period) compared to the groups with a lesser number of risk factors (*831*). Similarly, a population-based study on adult males demonstrated a significant correlation of arterial stiffness with cumulative exposure to CRP and cyclical stress [44]. Likewise, with the constellation of multiple risk factors like longer duration of CFRD and prolonged period of inflammation, there can be a possibility that the difference in AIx between CF and control patients worsens with increasing age. The effect of diabetes could not be studied in our population due to a very small number of children with CFRD (N=1). In addition to the environmental factors, an inherent predisposition to vascular haemodynamic alterations related to the absence of CFTR function (*832*), possibly due to alteration of smooth muscle tone (*809*) has been suggested. This association needs further research.

6.6 Conclusion

This study investigated vascular haemodynamics in children with CF and provides first reference values of AIx and cfPWV in this group. AIx was found to be significantly higher in children with CF compared to healthy children; however, caution needs to be exercised with this interpretation acknowledging the limitation of comparison with controls from a different study and a narrower age range. There was no association of cfPWV and aortic AIx with the level of inflammatory markers, lung function parameters and ^{51}Cr-EDTA GFR.

This pilot study, limited by a small sample size and lack of control group has provided interesting findings, which should be confirmed in another study with larger cohort and a contemporaneous healthy control group. Furthermore, prospective studies will be needed to explore the causative associations, longitudinal progression of altered vascular haemodynamics in people with CF through their adulthood and any associated clinical implications.

Chapter-7 Conclusion

In this chapter, I have summarised the findings from earlier chapters and discussed future perspectives related to this work.

7.1 General Aim

The aim of this thesis was to investigate various comorbidities associated with cystic fibrosis (CF) and to identify ways to reduce the treatment-related adverse effects. The main emphasis of the work was on renal disease in CF and its association with recurrent antibiotic use. The pathophysiology of renal disease in CF was explored using kidneys from pig model of CF.

Renal function in CF was assessed by measurement of ^{51}Chromium-EDTA glomerular filtration rate (^{51}Cr-EDTA GFR) of a group of adults and children with CF in an observational study. Other comorbidities like hearing abnormality and alteration in vascular haemodynamics were assessed. Any correlations of GFR and hearing defect with cumulative antibiotic exposure including aminoglycosides, ceftazidime and colistin were explored. A randomised controlled study was undertaken to compare the pharmacokinetics of intravenous (IV) tobramycin administered in the morning with that in the evening to identify novel and cost-neutral ways to reduce nephrotoxicity of aminoglycosides.

A Cochrane systematic review was conducted to evaluate the evidence for the clinical utility of the practice of antibiotic treatment for pulmonary exacerbations following bronchoalveolar lavage against the standard practice of therapy based on the microbiology of oropharyngeal swabs or other less invasive methods.

Kidneys from pig model of CF were compared to kidneys from CF heterozygotes and wild-type pig kidneys for microscopic appearance and gene expression analysis for cystic fibrosis transmembrane conductance regulator (CFTR) and endocytic receptor proteins. At a protein level, localisation of CFTR and endocytic receptor proteins within pig kidneys was inspected along with measurement of urinary loss of endocytic receptor proteins and the low molecular weight protein transferrin.

7.2 Summary of Findings & Future Directions

Chapter 2 of this thesis describes the findings of renal function measurement and hearing assessment in people with CF. GFR was measured by ^{51}Cr-EDTA method, which was then compared to the GFR estimated by creatinine-based formulas. In our study cohort, the overall prevalence of a low GFR (<90 ml/min/1.73m^2) was 6%. The mean GFR value was 124.8 ml/min/1.73m^2 in paediatric group and 99.6 ml/min/1.73m^2 in the adult patients. There was no association of GFR with cumulative antibiotic exposure. These results are reassuring and indicate that routine GFR measurement may not be required in all the patients. However, due to various predisposing risk factors as discussed in Chapter 2, it will still be important for clinicians to remain mindful of the risk to renal function and to continue to implement measures to reduce any renal damage, e.g. safer prescribing practices for aminoglycosides. With the use of newer disease-modifying treatments, CF patients may experience fewer exacerbations (*79,833*), and the life expectancy of CF patients may improve further. Hence, the prevention of comorbidities becomes even more vital.

The number of adults who had a measurement of GFR was small (Chapter 2). More work is needed in adults with CF in the light of epidemiological evidence which suggests that the prevalence of

chronic kidney disease (CKD) increases with age (*4*). I have demonstrated that the estimated GFR is not reliable when compared to the measured GFR. In the context of recruitment challenges faced by this study, for future studies and for clinical monitoring at any stage, it should be a priority to develop and validate reliable, simple and less invasive methods to measure GFR in the CF population.

An interesting finding from Chapter 2 was that GFR was high in about one in three children. The aetiology cannot be explored fully in an observational study, but there was no evidence of a relationship with increased cumulative antibiotic exposure. This finding has been shown in other cross-sectional studies (*214*) and will need further exploration in longitudinal studies to assess its natural history and implications.

Increased hearing thresholds were observed in one in four people with CF. The hearing abnormality was not related to cumulative exposure to aminoglycosides. Only one of the 10 individuals who had received more than 10 courses of aminoglycosides had raised hearing thresholds. The lack of association between hearing abnormality and cumulative aminoglycoside exposure indicates that there are alternative mechanisms in play, which may be genetic or idiosyncratic in nature or indeed, related to the specific CFTR defect. The same has been speculated in other studies that have found a non-linear relationship between hearing and cumulative aminoglycoside exposure in CF (*449*), or have identified that a proportion of children did not develop any hearing deficit despite receiving high doses of aminoglycosides. This suggests tolerance in some children whereas other children developed a hearing deficit with the same or less aminoglycoside exposure (*506*).

The experiments on kidneys from pig model of CF (Chapter 3) have shown, for the first time that CFTR is expressed in pig kidneys. A

previous study to establish CFTR expression in pigs did not find the CFTR gene or protein within the pig kidneys. The difference in results from our studies may have been related to the difference in techniques. CFTR relative gene expression in the CFTR knockout and wild-type pigs will need further exploration, using primers from a different site. This study established that the gene expression of various endocytic receptor proteins: megalin, cubilin and AMN are equivalent in kidneys from CFTR knockout mice and control (CFTR heterozygotes and wild-type) pig kidneys. Any differences in the amount of endocytic receptor proteins (e.g. cubilin as shown in mouse model of CF (*232*)) are likely to have been driven at the protein or cellular levels. The experiments for detection of various proteins including immunohistochemistry (in pig kidneys) and enzyme-linked immunosorbent assay (ELISA) (in pig urine) were unsuccessful, which appear to have been related to lack of porcine specific antibodies and presence of possible inhibitors in the urine. Further studies will be required with other antibodies to establish the immunocytolocalisation of various endocytic receptor proteins. There were no morphological differences between the kidneys from newborn CFTR knockout pigs and wild-type and CFTR heterozygote pigs. This result suggests that CF kidneys are structurally normal at birth and various histological changes observed in autopsy studies (*219*) (including infants or young children) are probably related to environmental influences. Such influences might include repeated infections; persistent inflammation and comorbidities (e.g. diabetes). Due to the longer lifespan of pigs compared to mice, the pig model of CF provides an opportunity to study the effect of various environmental factors on the kidneys. In a future collaborative study, histological examination of kidneys from pig model of CF who have had several episodes of lung infection and repeated courses of

antibiotics may be possible to help identify any renal morphological changes at a later age.

Chapter 4 and 5 describe two other studies which aimed to evaluate evidence for better management strategies for treatment of exacerbations in CF. In Chapter 4, a randomised controlled study (CRITIC) showed no difference in IV tobramycin pharmacokinetics between the groups receiving the dose in the morning and in the evening. However, the CRITIC study also measured a biomarker of nephrotoxicity (not a part of this thesis), which suggested less nephrotoxicity with tobramycin administration in the morning (*834*). There is also a large body of evidence from animal studies in favour of reduced clearance and increased nephrotoxicity of aminoglycosides when administered during the rest period (*575,576*). Further investigations are required firstly, to compare pharmacokinetics after a longer course of antibiotics, and additionally, to assess other parameters, which have a circadian pattern and can potentially influence nephrotoxicity of aminoglycosides e.g. effect of food intake or urinary pH.

An abnormal circadian rhythm was exhibited in a large proportion of children with CF during hospital admission for a pulmonary exacerbation. This new observation will need confirmation in a larger group of people with CF, preferably during both, stable phase and during pulmonary exacerbation or hospital admission to evaluate or exclude the contribution of illness or hospitalisation.

Chapter 5 describes a systematic review of the outcomes following the treatment of pulmonary exacerbations after a microbiological diagnosis from bronchoalveolar lavage compared to standard treatment practices. Only one randomised controlled study (*685*) was included, and hence, no meta-analysis could be conducted. There was

no difference in the clinical outcomes and the economic analysis between the two groups; routine surveillance with bronchoscopy cannot be recommended at this stage. More evidence will need to be collected from other trials with more sensitive outcome measures like LCI, and the role of other less invasive methods like induced sputum should be assessed. Furthermore, the practice of bronchoscopy at other time points in life, e.g. after initial diagnosis of CF, after diagnosis or eradication of *Pseudomonas aeruginosa*, will need to be evaluated.

Chapter 6 presents findings of the assessment of vascular haemodynamic parameters including augmentation index (AIx) and carotid-femoral pulse wave velocity (cfPWV). Children with CF, age 8-14 years showed increased AIx, which is a surrogate marker of increased arterial stiffness in the younger population. There was no association of AIx or PWV with GFR or any measure of inflammation (C-reactive protein or interleukin 6). These findings illustrate that the advanced vascular ageing noted in adult patients with CF (*160*) may have its origin much earlier in life. There may be other mechanisms driving this phenomenon apart from increased inflammation, the level of GFR or presence of CFRD. The primary role of CFTR-related defect has been suggested and needs further exploration. A larger study with a control group, ideally with a longitudinal design will be required for investigation of aetiological factors and any progression over time.

7.3 Final Remarks

This thesis describes studies of renal function in individuals with CF and has demonstrated a low prevalence of reduced GFR, which did not correlate with cumulative antibiotic exposure. In addition, other comorbidities of CF like hearing abnormality and altered vascular haemodynamics were illustrated. The aetiology and any progression of

these abnormalities need to be assessed in future studies. This study has established that CFTR is expressed in pig kidneys and that pig model of CF may be useful to investigate the renal phenotype in CF.

Chapter-8 Appendix

8.1 Appendix I

8.1.1 Publications from this thesis

Jain K, Smyth AR. Current dilemmas in antimicrobial therapy in cystic fibrosis. Expert Rev Respir Med 2012;6(4):407-22.

Jain K, Wainwright C, Smyth AR. Bronchoscopy-guided antimicrobial therapy for cystic fibrosis. Cochrane Database Syst Rev 2013;12:CD009530.

Prayle AP, Jain K, Touw DJ, Koch BC, Knox AJ, Watson A, et al. The pharmacokinetics and toxicity of morning vs evening tobramycin dosing for pulmonary exacerbations of cystic fibrosis: A randomised comparison. J Cyst Fibros 2016;15(4):510-7

8.1.2 Presentations from this thesis

Jain K, Prayle A, Lewis S, Watson A, Knox A, Dewar J, et al. Assessment of renal function in cystic fibrosis patients by estimated and measured glomerular filtration rate −a cross-sectional study. Journal of Cystic Fibrosis 2012;11(Supplement 1):S52. Oral Presentation at the 35th European CF Society Meeting 2012

Jain K, Prayle A, Symonds M, Hull JH, Bolton CE, Smyth A. Premature vascular ageing in children with cystic fibrosis (CF). Journal of Cystic Fibrosis 2013;12(2):S126, Poster presentation at the 36th European CF Society Meeting, 2013

Jain K, Prayle A, McCulloch TA, Pope M, Symonds M, Smyth AR. Gene Expression of CFTR and Renal Endocytic Receptor Proteins is not altered in the kidneys of young piglets with cystic fibrosis. Pediatric Pulmonology 2013;48(S36):271. Poster presentation at the 27th North American CF Conference 2013

8.2 Appendix Chapter II

8.2.1 Dose of ^{51}Cr-EDTA used in CEFIT CF Study

Scaling of adult administered activity for children or young persons by body weight for Glomerular Filtration Rate (GFR) measurement

Adult dose: 2.8 MBq $Cr^{51}EDTA$

Scaling factor: weight (kg) / 70

Minimum child dose: 0.28 MBq (10% of the adult dose)

Table 8-1-A Dose of ^{51}Cr-EDTA used in CEFIT CF Study

Weight (kg)	Dose (MBq)	Weight (kg)	Dose (MBq)
3	0.28	37	1.48
4	0.28	38	1.52
5	0.28	39	1.56
6	0.28	40	1.60
7	0.28	41	1.64
8	0.32	42	1.68
9	0.36	43	1.72
10	0.40	44	1.76
11	0.44	45	1.80
12	0.48	46	1.84
13	0.52	47	1.88
14	0.56	48	1.92
15	0.60	49	1.96
16	0.64	50	2.00
17	0.68	51	2.04
18	0.72	52	2.08

^{51}Cr-EDTA: ^{51}chromium-ethylenediamine tetraacetic acid; MBq: megabecquerel; CEFIT CF: Cumulative Effects of Intravenous Treatment in Cystic Fibrosis

Table 8-1-B Dose of ^{51}Cr-EDTA used in CEFIT CF Study			
Weight (kg)	Dose (MBq)	Weight (kg)	Dose (MBq)
20	0.80	54	2.16
21	0.84	55	2.20
22	0.88	56	2.24
23	0.92	57	2.28
24	0.96	58	2.32
25	1.00	59	2.36
26	1.04	60	2.40
27	1.08	61	2.44
28	1.12	62	2.48
29	1.16	63	2.52
30	1.20	64	2.56
31	1.24	65	2.60
32	1.28	66	2.64
33	1.32	67	2.68
34	1.36	68	2.72
35	1.40	69	2.76
36	1.44	70	2.80

^{51}Cr-EDTA: ^{51}chromium-ethylenediamine tetraacetic acid; MBq: megabecquerel; CEFIT CF: Cumulative Effects of Intravenous Treatment in Cystic Fibrosis

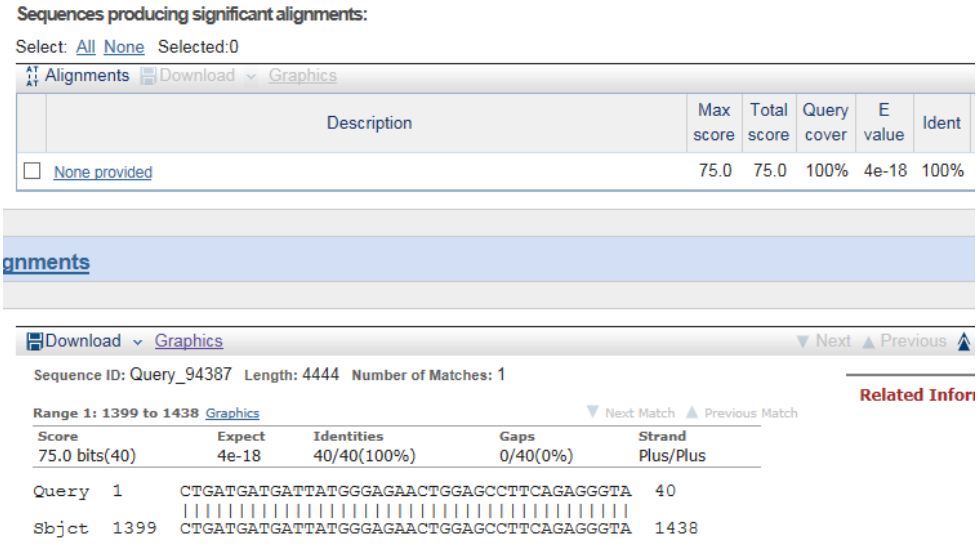
8.3 Appendix Chapter III

8.3.1 DNA sequencing results

8.3.1.1 CFTR2 forward sequence result

CTGATGATGA TTATGGGAGA ACTGGAGCCT TCAGAGGGTA

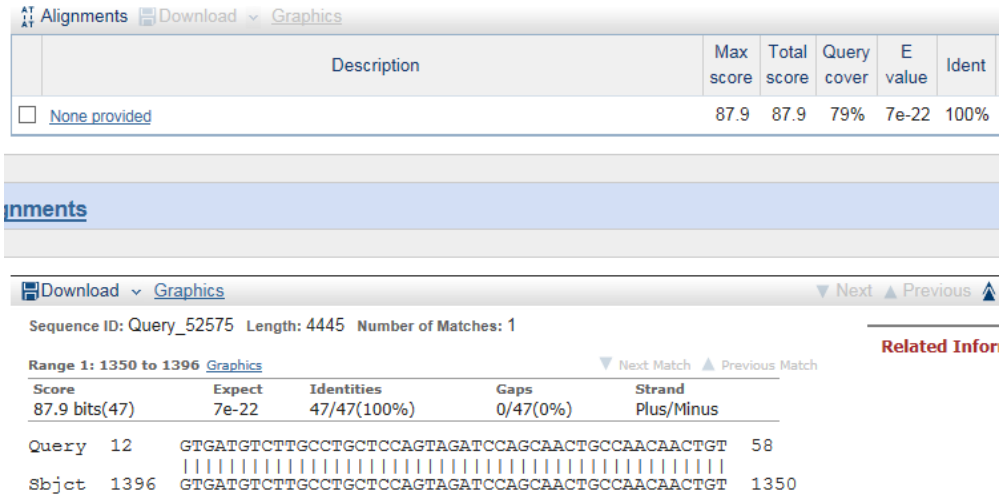
nBLAST (*560*) results:



8.3.1.2 CFTR2 Reverse sequence results

GAACCAGCGCCGTGATGTCTTGCCTGCTCCAGTAGATCCAGCAACTGCCA ACAACTGTA

nBLAST (*560*) results:



8.4 Appendix Chapter IV

8.4.1 CRITIC Paediatric Sleep Questionnaire

8.4.1.1 Sleep questionnaire for children <12 years old

Visual Analogue scale for children 12 year and younger	Version 1.1 Date: 10-05-2011	Patient number	T ☐☐☐☐

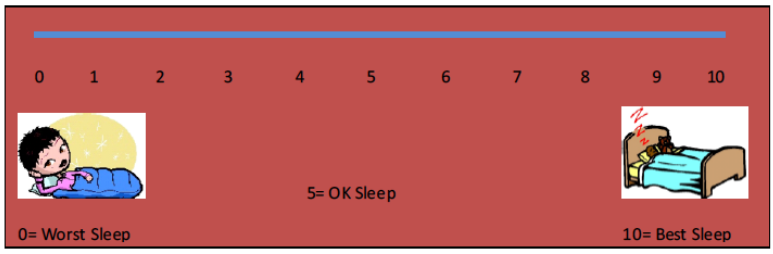
Sleep questionnaire for Children 12 year and Younger

We would like you to answer few questions. These questions will tell us how well did you sleep in the hospital.

1. Today's date: ----------

2. Date you are answering for (like yesterday's date): ----------

3. How well did you sleep on this date? Please circle one number below showing how well you think you slept on a scale on 0-10.



4. How do you sleep when you are at hospital compared to how you sleep at home?

Better ☐ Same ☐ Worse ☐ not sure ☐

Thank you for answering the questions.

1

8.4.1.2 Sleep questionnaire for older children

Sleep Questionnaire for children13 year & older	Version 1.1 Date: 10-05-2011	Patient number	T ☐☐☐☐

Sleep questionnaire for Children 13 year and older

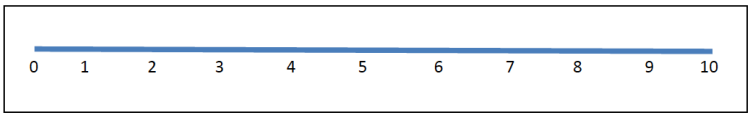
Please take a few minutes to fill out few questions about your sleeping pattern in the hospital.

This will give us idea about the quality of sleep you have had and will give us information about your sleeping pattern. We will use your answers to relate to the information obtained from measuring levels of melatonin (a hormone that regulates sleep pattern).

Your answers will be kept confidential.

1. Date questionnaire filled: ----------
2. Date the responses refer to: ----------
3. How do you rate the quality of your sleep on a scale of 0-10? Please circle one number below.

0= poorest quality of sleep, 10=best quality sleep



To answer the questions 4 & 5, if you are not sure of the times, please write what you guess the time was.

4. The time you went to bed: ----------
5. The time you woke up next morning: ----------
6. Did you wake up during the night? Yes ☐ No ☐

If No: Please go to Question 8, If Yes: Please continue Question 7.

a. How often did you wake up in the night? ----------
b. How long was the duration of night waking? ----------
(Please write the total awake time for all the awake periods during the night)

7. Any associated complaints noticed in the night:

Cough ☐ Breathing difficulty ☐ other ☐ (please specify)

---

8. Compared to your sleep at home, how do you rate your sleep at hospital?

   Better ☐ Same ☐ Worse ☐ Variable ☐ (please explain)

   _____

9. Any other comments about your sleeping behaviour that you would like to make:

   Thanks for your cooperation and your time for answering the questions.

8.4.2 PK analysis equations

The PK analysis was done using a software package: MW/Pharm, version 3.7 (Mediware a.s., Prague, Czech Republic) where a single compartment model was used. Bayesian analysis method was applied which involves the use of predefined population parameters for the initial calculation and subsequently, adjusts these to the individual patient based on their serum levels of tobramycin while taking into account the variability of the population derived parameters and the variability of the drug assay procedure (*835*). Various outputs from the software are described in the methods section of Chapter 4 (Section 4.3.3.6). Details of the analysis methods including various steps and equations used to calculate the clearance values are described here. These were developed with support from the research collaborator Prof. DJ Touw, Professor of Bioanalysis, Therapeutic Drug Monitoring and Clinical Toxicology, University Medical Center Groningen, The Netherlands.

The program estimated the creatinine clearance based on Schwartz formula

$$CLcr = 0.55 * \frac{Height}{Creatinine} * 88.5$$

Equation 8-1

CLcr=creatinine clearance ml/min/1.73m2

PK parameters derived from a population PK model for children with CF “tobramycin (kind, cf) %vinks” were used initially to generate various PK parameters for this study population. These values were then used to create a new population model in following steps:

1. Equation 8-2 was used to adjust Kelm to a value that will result in average half-life:

$$Kelm = \frac{0.693}{T_{1/2}}$$

Equation 8-2

(Kelm=metabolic elimination rate constant, $T_{1/2}$ =The average half-life ($t_{1/2}$) of tobramycin for our cohort), 0.693 equals the natural logarithm of two. (Since drug elimination is an exponential process, the time required for a twofold decrease is proportional to ln (2).

2. Population SD of Kelm was derived from Equation 8-3 using the Kelm values obtained from Equation 8-2.

$$SD = CoV * Kelm$$

Equation 8-3

(CoV coefficient of variation)=SD/mean)

3. The volume of distribution (Vd) value was entered from the initial PK calculations.
4. The PK analysis was repeated with new population parameters and using the following equation to calculate total elimination rate constant (Kel):

$$Kel = Kelm + Kelr * Clcr$$

Equation 8-4

Kelr=renal elimination rate constant, CLcr=creatinine clearance (L/hr)

5. Kelr value was set to zero to eliminate the bias associated with its dependence on eGFR, thus total body elimination rate constant (Kel) was set to be equal to Kelm.
6. Total clearance was calculated as a multiplication product of elimination rate constant and volume of distribution.

8.5 Appendix Chapter V

8.5.1 Copy of permission letter to use previously published review in thesis

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8.5.2 Study Selection, Quality Assessment & Data Extraction Form

First author	Journal/Conference Proceedings etc	Year

Study eligibility

RCT/Quasi/CCT (delete as appropriate)	Relevant participants	Relevant interventions	Relevant outcomes
Yes / No / Unclear	Yes / No / Unclear	Yes / No / Unclear	Yes / No* / Unclear

*** Iissue relates to selective reporting – when authors may have taken measurements for particular outcomes, but not reported these within the paper(s). Reviewers should contact trialists for information on possible non-reported outcomes & reasons for exclusion from publication. Study should be listed in 'Studies awaiting assessment' until clarified. If no clarification is received after three attempts, study should then be excluded.**

Do not proceed if any of the above answers are 'No'. If study to be included in 'Excluded studies' section of the review, record below the information to be inserted into 'Table of excluded studies'.

References to trial

Check other references identified in searches. If there are further references to this trial link the papers now & list below. All references to a trial should be linked under one *Study ID* in RevMan.

Code each paper	Author(s)	Journal/Conference Proceedings etc	Year
A	*The paper listed above*		
B	*Further papers*		

Participants and trial characteristics

Participant characteristics	
	Further details
Age (mean, median, range, etc)	
Sex of participants (numbers / %, etc)	
Disease status / type, etc (if applicable)	
Other	

Trial characteristics
see Appendix 1, usually just completed by one reviewer

Risk of bias

We recommend you refer to and use the method described in the Cochrane Reviewers' Handbook, Version 5.0.1*

Allocation of intervention	
State here method used to generate allocation and reasons for grading	***Risk of bias*** → ***(circle)***
	Low (Random)
	High (e.g. alternate)
	Unclear

Concealment of allocation **Process used to prevent foreknowledge of group assignment in a RCT, which should be seen as distinct from blinding**	
State here method used to conceal allocation and reasons for grading	***Risk of bias*** → ***(circle)***
	Low
	High
	Unclear

Blinding	
Person responsible for participants care	Yes / No
Participant	Yes / No
Outcome assessor	Yes / No
Other (please specify)	Yes / No
Intention-to-treat An intention-to-treat analysis is one in which all the participants in a trial are analysed according to the intervention to which they were allocated, whether they received it or not.	
All participants entering trial	
15% or fewer excluded	
More than 15% excluded	
Not analysed as 'intention-to-treat'	
Unclear	

Selective outcome reporting	
Have you been able to access the trial protocol?	
Are all outcomes listed in protocol reported in the full trial paper?	

Data extraction

Outcomes relevant to your review Copy and paste from 'Types of outcome measures'	
	Reported in paper (circle)
Outcome 1	Yes / No
Outcome 2	Yes / No
Outcome 3	Yes / No
Outcome 4	Yes / No
Outcome 5	Yes / No
Outcome 6	Yes / No
Outcome 7	Yes / No
Outcome 8	Yes / No

For Continuous data							
Code of paper	Outcomes (rename)	Unit of measurement	Intervention group		Control group		Details if outcome only described in text
			n	Mean (SD)	n	Mean (SD)	
A etc	Outcome A						
	Outcome B						
	Outcome C						
	Outcome D						
	Outcome E						
	Outcome F						

For Dichotomous data			
Code of paper	Outcomes (rename)	Intervention group (n) n=number of participants, not number of events	Control group (n) n=number of participants, not number of events
A	Outcome G		
	Outcome H		
	Outcome I		
	Outcome J		
	Outcome K		
	Outcome L		

Other information which you feel is relevant to the results
Indicate if: any data were obtained from the primary author; if results were estimated from graphs etc; or calculated by you using a formula (this should be stated and the formula given). In general if results not reported in paper(s) are obtained this should be made clear here to be cited in review.

References to other trials

Did this report include any references to **published reports** of potentially eligible trials not already identified for this review?		
First author	Journal / Conference	Year of publication
Did this report include any references to **unpublished data** from potentially eligible trials not already identified for this review? If yes, give list contact name and details		

Appendix 1

Trial characteristics	
	Further details
Single centre / multicentre	
Country / Countries	
How was participant eligibility defined?	
How many people were randomised?	
Number of participants in each intervention group	
Number of participants who received intended treatment	

Number of participants who were analysed	
Drug treatment(s) used	
Dose / frequency of administration	
Duration of treatment ***(State weeks / months, etc, if cross-over trial give length of time in each arm)***	
Median (range) length of follow-up reported in this paper ***(state weeks, months or years or if not stated)***	
Time-points when measurements were taken during the study	
Time-points reported in the study	
Time-points you are using in Meta-View	
Trial design ***(e.g. parallel / cross-over*)***	
Other	

* If cross-over design, please refer to the Cochrane Editorial Office for further advice on how to analyse these data

References

Cochrane Reviewers' Handbook, Version 5.0.1
Higgins JPT, Green S (editors). *Cochrane Handbook for Systematic Reviews of Interventions* Version 5.0.1 [updated September 2008]. The Cochrane Collaboration, 2008. Available from www.cochrane-handbook.org.

8.6 Appendix Chapter VI

8.6.1 Definitions and equations for arterial stiffness

8.6.1.1 Equations for Pulse Wave Velocity

Table 8-2 Definitions and units of indices and terminologies for arterial stiffness		
Index/ Term (unit)	**Definition**	**Formula**
Pulse Wave Velocity, PWV (cm/s)	(1) Speed of travel of the pulse along an arterial segment (2) Proportional to inverse of cross-sectional distensibility coefficient (Bramwell-Hill equation) 3. Proportional to elastic modulus (E_{inc}) and wall thickness/lumen ratio (Moens-Kortweg equation)	1. $\frac{Distance}{\Delta t}$ 2. $\sqrt{\frac{A.\Delta P}{\rho.\Delta A}} = \sqrt{\frac{1}{\rho Dcoeff}}$ 3. $\sqrt{\frac{h.Einc}{D.\rho}}$

P: pressure; D: diameter; V: volume; h: wall thickness; A: area; ρ: blood density; t: time; adapted from references: Laurent et al., Chirinos et al., O'Rourke et al. (9,836,837).

8.6.1.2 Other indices and terminologies for arterial stiffness

Table 8-3 Definitions and units of indices and terminologies for arterial stiffness

Index/ Term (unit)	Definition	Formula
Arterial Distensibility ($mmHg^{-1}$)	Relative change in diameter for a change in arterial pressure (Inverse of Elastic Modulus)	$\frac{\Delta D}{\Delta P . D}$
Elastance ($mmHg/cmH_2O$)	Change in the arterial pressure relative to the change in volume	$\frac{\Delta P}{\Delta V}$
Arterial Compliance (cm/mmHg)	Absolute change in diameter relative to the change in pressure at a fixed vessel length (inverse of elastance)	$\frac{\Delta V}{\Delta P}$
Cross-Sectional Compliance Coefficient (m^2/kPa)	Absolute change in cross-sectional lumen area relative to the change in arterial pressure (local pulse pressure)	$\frac{\Delta A}{\Delta P}$
Cross-Sectional Distensibility coefficient(kPa^{-1})	Relative change in cross-sectional lumen area relative to the change in arterial pressure (local pulse pressure)	$\frac{\Delta A}{A . \Delta P}$
(Peterson's) Elastic Modulus , E_p(mmHg)	The pressure change required for (theoretical) 100% increase in diameter from resting length	$\frac{\Delta P . D}{\Delta D}$
Incremental (Young's) Elastic Modulus, E_{inc} (mmHg/cm)	Elastic Modulus per unit area; the pressure change per cm^2 required for (theoretical) 100% increase in diameter from resting length	$\frac{\Delta P . D}{\Delta D . h}$
β Stiffness Index (β)	Logarithm of the ratio of systolic/diastolic pressure over fractional diameter change	$\frac{Ln\left(\frac{Ps}{Pd}\right)}{[\frac{(Ds - Dd)}{Dd}]}$
Characteristic Impedance (Zc) (mmHg/cm/sec)	Relationship between pressure change & flow velocity in the absence of wave reflections; Assuming constant blood density (ρ), Zc is linearly related to PWV & cross-sectional lumen area (A).	$\frac{\Delta P}{\Delta Q}$ $Zc \approx \rho . \frac{PWV}{A}$

P: pressure; D: diameter; V: volume; h: wall thickness; A: area; s: systolic; d: diastolic; Q: flow velocity; ρ: blood density; t: time; Ln: log normal; adapted from references: Laurent et al., Chirinos et al., O'Rourke et al.(9,836,837).

8.6.2 Studies assessing arterial stiffness in renal impairment

8.6.2.1 Arterial Stiffness in advanced renal impairment

Table 8-4 Table of studies investigating arterial stiffness measures with advanced renal impairment

Reference	Type of study	Number of patients (Mean age, yr.)	Measure of arterial stiffness	Measure of renal function	CKD staging / degree of renal impairment	Results
London 1990 (*754*)	Cross-Sectional	182 (50)	Aortic, femoral & brachial PWV	-	Haemodialysis patients	Aortic PWV significantly higher in haemodialysis patients compared to controls (essential hypertension and no ESRD)
Mourad 1997 (*756*)	Cross-sectional	128 (54)	Radial Incremental Elastic Modulus (E_{inc})	-	End-stage renal disease	Radial artery E_{inc} was significantly elevated in patients with ESRD compared to normotensive subjects or patients with essential hypertension (no ESRD).
Shinohara 2004 (*755*)	Cross-sectional	71 (61)	cfPWV	-	End-stage renal disease, predialysis	Predialysis patients had higher aortic PWV compared to patients on maintenance haemodialysis and healthy controls.
Taal 2007 (*730*)	Prospective	35	Aortic PWV, AIx	eGFR	Stage 4-5	Aortic PWV and AIx were independent risk factors for progression to ESRD.

CKD: chronic kidney disease; PWV: pulse wave velocity; cfPWV: carotid-femoral PWV; ESRD: end-stage renal disease; AIx: augmentation index

8.6.2.2 Arterial Stiffness with mild to moderate renal impairment-1

Table 8-5 Table of studies investigating arterial stiffness measures with mild to moderate renal impairment						
Reference	**Type of study**	**Number (mean age year)**	**Measure of arterial stiffness**	**Measure of renal function**	**CKD stage/ renal impairment**	**Results**
Mourad 2001(*838*)	Cross-Sectional	1290 (47)	cfPWV	eGFR	Mild to moderate	Subjects in the lowest tertile of eGFR showed a significant negative association of eGFR with cfPWV.
Wang 2005(*731*)	Cross sectional	121 (53-65 yrs)	cfPWV	eGFR	Stages 1-5	Stepwise increase in PWV corresponding to advancing CKD staging
Briet 2006(*761*)	Cross-sectional	95 (58)	cfPWV, Carotid E_{inc}	^{51}Cr-EDTA	Stages3-4	Independent negative relationship between GFR and E_{inc}; higher cfPWV in patients with CKD compared to normotensive and hypertensive subjects
Shillaci 2006	Cross-sectional	305 (48)	cfPWV, crPWV	eGFR	Stage 1	Independent inverse association of eGFR with both central and peripheral PWV
Lacy 2006 (*839*)	Cross sectional	55 (55.6)	cfPWV, AIx	I-hexol clearance	Stage 3-5	Patients with reducing GFR have increased large artery stiffness.
Hermans 2007(*760*)	Cross-sectional	806 (68)	cftransit time, AIx	eGFR	Stages 1-3	Lower eGFR associated with higher peripheral arterial stiffness but not with central measures

CKD: chronic kidney disease; PWV: pulse wave velocity; cfPWV: carotid-femoral PWV; E_{inc}: Young's Elastic modulus; cftransit time: carotid-femoral transit time; eGFR: estimated glomerular filtration rate; AIx: augmentation index;; crPWV: carotid-radial PWV; ^{51}CR-EDTA: ^{51}chromium-ethylenediamine tetraacetic acid

8.6.2.3 Arterial Stiffness with mild to moderate renal impairment-2

Table 8-6 Table of studies investigating arterial stiffness measures with mild to moderate renal impairment							
Reference	**Type of study**	**Number**	**Mean Age (year)**	**Measure of arterial stiffness**	**Measure of renal function**	**CKD stage/ renal impairment**	**Results**
Yoshida 2007 (*840*)	Cross sectional	1873	42	baPWV	eGFR	Stage 1-2	Weak association between degrees of eGFR loss and baPWV
Ohya 2007 (*841*)	Cross-sectional	3387	52	baPWV	eGFR	Normal to mild	baPWV is increased in a stepwise fashion according to a decrease in eGFR.
Kawamoto 2008 (*762*)	Cross sectional	310	68	caPWV & brachial PWV	eGFR	Stages 1 to (>3)	Significant contribution of eGFR to PWV and stepwise increase with decreasing eGFR
Upadhyay 2009 (*759*)	Cross-sectional	181	70	cfPWV, AIx	eGFR	Mild to moderate	No association of central arterial stiffness with CKD positive association with elevated urinary albumin.
Makita 2010 (*763*)	Cross sectional	3406	58	Carotid stiffness index (β)**	eGFR	Stages 1 to (>3)	Higher β values in patients with CKD vs no CKD, eGFR associated with β in stepwise manner
Townsend 2010 (*764*)	Cross sectional	2564	60	cfPWV	eGFR	Stages 3-4	Significant negative association of cfPWV with level of kidney function

Carotid stiffness index (β): a measure of carotid arterial elasticity that incorporates end diastolic and peak systolic luminal diameters; CKD: chronic kidney disease; PWV: pulse wave velocity; baPWV: brachio-ankle PWV; caPWV: carotid-ankle PWV; cfPWV: carotid-femoral PWV; eGFR: estimated glomerular filtration rate; AIx: augmentation index

8.6.2.4 Arterial Stiffness with mild to moderate renal impairment-3

Table 8-7 Table of studies investigating association of arterial stiffness measures with mild to moderate renal impairment							
Reference	**Type of study**	**Number**	**Mean Age (year)**	**Arterial stiffness measure**	**Measure of renal function**	**CKD stage/ renal impairment**	**Results**
Ford 2010(*766*)	Prospective	133	69	cfPWV	eGFR	Stages 3-4	Aortic stiffness associated with rate of decline of renal function.
Tomiyama 2010 (*767*)	Prospective	2053	38	Brachial-ankle PWV (baPWV)	eGFR	None to mild	Higher baseline baPWV associated with lower follow-up eGFR (5-6yr) and higher annual rate of decline in GFR; the converse was not true.
Chue 2011 (*842*)	Prospective	255		Aortic PWV, AIx	eGFR	Stage 2-4	Serum phosphate but not aortic PWV or AIx were independent predictors of decline in eGFR.
Briet 2011 (*765*)	Longitudinal	180)	59	cfPWV, Carotid E_{inc}	^{51}Cr-EDTA	Stages 3-5	Aortic and carotid stiffness not associated with CKD progression
Hashimoto 2011 (*843*)	Cross sectional	133	56	cfPWV, crPWV &fdPWV	RI	Mean GFR 72ml/min/1.73 m^2	Renal RI strongly correlates with aortic PP and cfPWV but not with peripheral PWV. Renal RI linked with albuminuria.

CKD: chronic kidney disease; PWV: pulse wave velocity; cf PWV: carotid-femoral PWV; baPWV: brachio-ankle PWV; AIx: augmentation index; crPWV: Carotid-radial PWV; fdPWV: femoral dorsalis pedis PWV; RI:Renal resistive index (1-enddiastolic velocity)/peak systolic velocity; E_{inc}: Young's Elastic Modulus; eGFR: estimated glomerular filtration rate; $^{51}C^{R}$-EDTA: ^{51}chromium-ethylenediamine tetra acetic acid; PP: pulse pressure

8.6.2.5 Arterial Stiffness with mild to moderate renal impairment-4

Table 8-8 Table of studies investigating association of arterial stiffness measures with mild to moderate renal impairment							
Reference	**Type of study**	**Number**	**Mean Age (year)**	**Arterial stiffness measure**	**Renal function measure**	**CKD stage/ renal impairment**	**Results**
Bian 2012 (*757*)	Cross sectional	1131	55	cfPWV, crPWV, AIx	eGFR	None to mild	Decreased eGFR inversely correlated with increments in cfPWV but not with carotid radial PWV
Peralta 2012 (*844*)	Prospective	4853	60	SAE, LAE, PP	eGFR (creatinine and cystatin C)	eGFR>60	Increased brachial artery PP and lower small and large artery elasticity (i.e. higher stiffness) linearly associated with faster decline in eGFR.
Madero 2013 (*845*)	Prospective	2129	70-79	cfPWV	$eGFR_{cystatinC}$	Mild to moderate	cfPWV was associated with incident CKD but not with rapid decline in eGFR (>3m/min/1.73m^2)
McIntyre 2013 (*758*)	Cross sectional	1717	73	cfPWV	eGFR	Stage 3	eGFR was not a determinant of cfPWV, Albuminuria had a weak effect.
Suzuki 2014 (*846*)	Prospective	110	58	cfPWV, baPWV	eGFR	Stage 3-5	Significant positive correlation between baPWV and decline in eGFR over 10 years, baPWV strongest predictor of cardiovascular disease.
Kim 2014 (*847*)	Prospective	913	63	cfPWV, baPWV, PP	eGFR	Mild to moderate	Brachial PP but not baPWV or cfPWV were independent predictors of rapid decline in kidney function (>3ml/min/1.73m^2 per year)

CKD: chronic kidney disease; PWV: pulse wave velocity; cfPWV: carotid-femoral PWV; crPWV: carotid-radial PWV; SAE: small artery elasticity; LAE: large artery elasticity; baPWV: brachial-ankle PWV; PP: pulse pressure; eGFR: estimated glomerular filtration rate; AIx: augmentation index

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