Novel approaches to aminoglycoside-induced nephrotoxicity in children

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor in Philosophy

Stephen James McWilliam

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This thesis is the result of my own work and the material contained within the thesis has not been presented, nor is currently being presented, either wholly, or in part, for any other degree or qualification.

Stephen James McWilliam

The research presented in this thesis was carried out in the Wolfson Centre for Personalised Medicine, Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine

This thesis is dedicated to my wife, Liz.

Thank you for your constant love and support, and for always helping me see what's really important in life

Abstract

Background: Aminoglycoside antibiotics are commonly used in paediatric clinical practice, especially for the treatment of neonatal sepsis and pulmonary exacerbations in cystic fibrosis (CF). However, megalin-mediated endocytosis of the aminoglycosides by renal proximal tubule epithelial cells leads to toxicity, and may result in acute kidney injury and chronic kidney disease. Current approaches to identify and prevent toxicity are limited. Several novel biomarkers have shown utility in preclinical studies for the identification of aminoglycoside-induced nephrotoxicity, but clinical data and an understanding of their clinical utility is lacking. The potential of statins to prevent aminoglycoside-induced nephrotoxicity by inhibition of megalin-mediated endocytosis has been previously demonstrated *in vitro* and in a rat model, but its potential in man is unclear.

Aims: Firstly, to investigate the utility of novel urinary biomarkers for the early identification of aminoglycoside-induced nephrotoxicity in children. Secondly, to develop a novel intervention using statins to prevent aminoglycoside-induced nephrotoxicity in children with CF.

Methods and Results: Urine samples were collected from 41 premature neonates at least once per week, and daily during courses of gentamicin. Three urinary biomarkers were measured using Luminex-based (Kidney Injury Molecule-1 (KIM-1) and Neutrophil Gelatinase-associated Lipocalin (NGAL)) and colorimetric assays (N-acetyl-β-D-glucosaminidase (NAG)). All three biomarkers were elevated during treatment with gentamicin, but when adjusted for potential confounders, only the elevation in KIM-1 remained significant (mean difference from not treated, 1.35ng/mg urinary creatinine; 95% CI 0.05-2.65).

Electrochemiluminescent assays for both KIM-1 and NGAL were validated, and were compared to Luminex-based assays by analysing samples from healthy children in the UK (n=120) and the US (n=171). 95% reference intervals for both biomarkers were derived using quantile regression.

Urine samples were collected from a cohort of children with cystic fibrosis (n=158) at outpatient clinic appointments and during exposure to tobramycin. Biomarkers were measured using the validated electrochemiluminescent assays. Elevations in both KIM-1 and NGAL (median peak fold-change was 2.28 (IQR 2.69) and 4.02 (IQR 7.29) respectively) were observed during exposure to tobramycin. In a multiple regression model, baseline KIM-1 was associated with the number of previous courses of IV aminoglycoside ($p<0.0001$; $R^2=0.11$).

An *in vitro* model of aminoglycoside-induced nephrotoxicity was developed using a conditionally immortalized proximal tubule epithelial cell line (ciPTECs). Dose and time-dependent toxicity was demonstrated with neomycin, gentamicin, and tobramycin (from most to least potent).

In rats, the addition of rosuvastatin significantly reduced nephrotoxicity compared to gentamicin alone ($p<0.01$). In guinea pigs, dose-dependent inhibition of gentamicin-induced nephrotoxicity was seen with rosuvastatin (at a minimum concentration of 0.94mg/kg/day, $p<0.0001$), but not with simvastatin. *In vitro* models demonstrated that neither rosuvastatin nor atorvastatin had any effect on the minimum inhibitory concentration of tobramycin for *Pseudomonas aeruginosa*.

Conclusion: Urinary KIM-1 has shown potential as a biomarker of both acute and chronic proximal tubular injury associated with exposure to aminoglycosides in children. Inhibition of aminoglycoside-induced nephrotoxicity by statins was demonstrated in further animal models, allowing the selection of a statin and dose (rosuvastatin 10mg) which have been taken forward into a clinical trial which will test this hypothesis in children with CF, utilising urinary KIM-1 as the primary outcome measure.

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Abbreviations

ADD	Attention Deficit Disorder
ADHD	Attention Deficit and Hyperactivity Disorder
ADR	Adverse drug reaction
AE	Adverse Event
AKI	Acute kidney injury
AKIN	Acute Kidney Injury Network
ALT	Alanine transaminase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AR	Adverse Reaction
AST	Aspartate transaminase
ATP	Adenosine-5'triphosphate
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BCRP	Breast cancer resistance protein
BNFc	British National Formulary for Children
BSA	Bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
BUN	Blood urea nitrogen
CCD	Charge-coupled device
CDSS	Centre for Drug Safety Science
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CI	Confidence interval
CI	Chief Investigator
ciPTEC	Conditionally immortalised human proximal tubule epithelial cell
CKD	Chronic kidney disease
CMFDA	5-chloromethylfluorescein diacetate
CRF	Case report form
CRN	Clinical Research Network
CRP	C-reactive protein
CTA	Cliical trial authorisation
CTIMP	Clinical Trial of an Investigational Medicinal Product
CTRC	Clinical Trials Research Centre
CTU	Clinical Trials Unit
CV	Coefficient of variation
CYP	Cytochrome P450
DERIVE	DEtermining Reference Values of renal biomarkers in healthy children
DIKI	Drug induced kidney injury
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DSUR	Development Safety Update Report
EC	Effective concentration
EGF	Epidermal growth factor
eGFR	Estimated glomeruar filtration rate

EMA	European Medicines Agency
EudraCT	European Union Drug Regulating Authorities Clinical Trials
FBS	Foetal Bovine Serum
FDA	US Food and Drug Administration
FEV1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
GCLP	Good Clinical Laboratory Practice
GCP	Good clinical practice
GEEs	Generalised estimating equations
GFR	Glomeruar filtration rate
GTP	Guanosine-5'-triphosphate
GWAS	Genome wide association study
HDL-c	High-density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
hTERT	Human telomerase reverse transcriptase
ICH	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IDSMC	Independent Data and Safety Monitoring Committee
IFCC	International Federation of Clinical Chemistry
IMP	Investigational Medicinal Product
IP	Intraperitoneal
IQR	Inter-quartile range
ISB	Iso-sensitest broth
ITS	Insulin-Transferrin-Sodium Selenite
IV	Intravenous
IVH	Intraventricular haemorrhage
KIM-1	Kidney injury molecule-1 (human)
Kim-1	Kidney injury molecule-1 (rat)
LB	Lysogeny broth
LDH	Lactate dehydrogenase
LDL-c	Low-density lipoprotein cholesterol
LLC-PK1	Lilly Laboratories Cell-Porcine Kidney 1
LLOQ	Lower limit of quantitation
LOD	Limit of detection
MAGIC	Molecular Genetics of Adverse Drug Reactions in Paediatric Patients
MCRN	Medicines for Children Research Network
MHRA	Medicines and Healthcare products Regulatory Agency
MIC	Minimum inhibitory concentration
MRC	Medical Research Council
MREC	Multi-centre Research Ethics Committee
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
MSD	Meso Scale Discovery
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide
NAG	N-Acetyl-β-D-glucosaminidase

NGAL	Neutrophil gelatinase-associated lipocalin
NHS	National Health Service
NICE	National Institute of Health and Care Excellence
NICU	Neonatal Intensive Care Unit
NIHR	National Institute for Health Research
NRES	National Research Ethics Service
NSAIDs	Non-steroidal anti-inflammatory drugs
OAT	Organic anion transporter
OCT	Organic cation transporter
OK	Opossum kidney
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline plus 0.05% Tween-20
PCR	Polymerase chain reaction
PCRN	Primary Care Research Network
PDA	Patent ductus arteriosus
Pgp	P-glycoprotein
PI	Principal investigator
PMDA	Japanese Pharmaceuticals and Medical Devices Agency
PMS	Phenazine methosulfate
pRIFLE	paediatric-modified Risk, Injury, Failure, Loss, End-Stage Kidney Disease criteria
PROteKT	Phase IIa, randomised, controlled, open-label trial of ROsuvastatin for the prevention of aminoglycoside-induced Kidney Toxicity in children with cystic fibrosis
PSTC	Predictive Safety Testing Consortium
PTEC	Proximal tubule epithelial cell
R&D	Research and development
RAP	Receptor-associated protein
REC	Research Ethics Committee
RI	Reference interval
RN	Research Nurse
RNA	Ribonucleic acid
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
sCr	Serum creatinine
SD	Standard deviation
SmPC	Summary of Product Characteristics
SNP	Single nucleotide polymorphism
SOP	Standard Operating Procedure
SUSAR	Suspected Unexpected Serious Adverse Reaction
SV40T	SV40 large T antigen
TMF	Trial master file
TMG	Trial Management Group
TORPEDO-CF	Trial of Optimal Therapy for Pseudomonas Eradication in Cystic Fibrosis
UAR	Unexpected Adverse Reaction
UCL	University College London
uCr	Urinary creatinine
UKCRC	UK Clinical Research Collaboration
UKCRN	UK Clinical Research Network

ULOQ	Upper limit of quantitation
URBAN CF	URinary Biomarkers of Aminoglycoside-induced Nephrotoxicity in children with Cystic Fibrosis

Publications

McWilliam SJ, Antoine DJ, Sabbisetti V, Turner MA, Farragher T, Bonventre JV, Park BK, Smyth RL, Pirmohamed M. ***Mechanism-Based Urinary Biomarkers to Identify the Potential for Aminoglycoside-Induced Nephrotoxicity in Premature Neonates: A Proof-of-Concept Study.*** PLoS ONE 7(8): e43809, 2012

McWilliam SJ, Antoine DJ, Sabbisetti V, Pearce RE, Jorgensen AL, Lin Y, Leeder JS, Bonventre JV, Smyth RL, Pirmohamed M. ***Reference intervals for urinary renal injury biomarkers KIM-1 and NGAL in healthy children.*** Biomark Med. 8(10):1189-1197, 2014

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Abstracts

McWilliam SJ, Antoine DJ, Turner MA, Sabbisetti V, Bonventre JV, Park BK, Smyth RL, Pirmohamed M. ***Urinary Kim-1 As A Biomarker of Aminoglycoside-Induced Nephrotoxicity in Premature Neonates.*** Pediatr Res 70: 107, 2011
European Society for Paediatric Research, Newcastle, October 2011. Oral Presentation.

McWilliam SJ, Antoine DJ, Smyth RL, Pirmohamed M. ***Reference Intervals for Urinary Kidney Injury Molecule-1 And Neutrophil Gelatinase-Associated Lipocalin In Children.***
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McWilliam SJ, Antoine DJ, Smyth RL, Pirmohamed M. ***Association of Urinary Kidney Injury Molecule-1 with Aminoglycoside Exposure in Children with Cystic Fibrosis.*** Journal of Cystic Fibrosis 13(2): S63, 2014
European Cystic Fibrosis Society Conference, Gothenburg, June 2014. Oral & Poster Presentation.

1 Introduction

1.1 Adverse drug reactions

An adverse drug reaction (ADR) has been defined by the World Health Organization as 'a response to a drug which is noxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function' (World Health Organization, 1972). More recent definitions have sought to improve upon this, with the following definition proposed by Edwards and Aronson widely cited: 'An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product' (Edwards and Aronson, 2000).

1.1.1 ADRs in adults and children

Researchers at the University of Liverpool have undertaken prospective observational studies to determine the prevalence of ADRs. ADRs were related to 6.5% of adult hospital admissions, with aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), diuretics and warfarin the most common causative agents (Pirmohamed et al., 2004). 14.7 % of adult hospital inpatients experienced one or more ADR, with diuretics, opioid analgesics and anticoagulants most commonly implicated (Davies et al., 2009). Exposure to multiple medicines increased the likelihood of an ADR occurring, and ADRs prolonged inpatient stays by 0.25 days per patient admission (Davies et al., 2009).

In a tertiary paediatric hospital, ADRs were associated with 2.9% of admissions, with cytotoxic agents, corticosteroids, NSAIDs, vaccines and immunosuppressants most commonly implicated (Gallagher et al., 2012). 17.7% of paediatric inpatients experience one or more ADR, with opioid analgesics and anaesthetic agents the most common causative agents (Thiesen et al., 2013). Undergoing a general anaesthetic, increasing age,

exposure to multiple medicines, and treatment for an oncological condition all increased the likelihood of experiencing an ADR (Thiesen et al., 2013).

1.2 Drug induced kidney injury (DIKI)

Table 1.1 – Types and causes of drug induced kidney injury (data taken from (Taber and Pasko, 2008))

Type of kidney Injury	Causative Drugs
Haemodynamically mediated	Non-steroidal anti-inflammatory drugs (NSAIDS), Angiotensin II-converting enzyme (ACE) inhibitors, Angiotensin II-receptor blockers, Calcineurin inhibitors, Triamterene
Glomerulonephritis	Nephrotic syndrome: NSAIDs, Ampicillin, Rifampin, Foscarnet, Lithium. Focal segmental glumerulosclerosis: Lithium, heroin, pamidronate. Membranous nephropathy: NSAIDs, gold therapy, mercury, penicillamine. Membranoproliferative glomerulonephritis: Hydralazine
Acute tubular necrosis	Aminoglycosides, Amphotericin B, Radiocontrast dye, Cisplatin
Interstitial nephritis	Antibiotics (penicillins, cephalosporins, sulphonamides, vancomycin), NSAIDs, Proton pump inhibitors, calcineurin inhibitors
Papillary necrosis	Aspirin, Paracetamol (acetaminophen), NSAIDs
Obstructive nephropathy	Aciclovir, Sulfadiazine, Methotrexate, Triamterene, Foscarnet, Indinavir

The kidneys receive a quarter of resting cardiac output. This, combined with their combined roles of filtration and homeostasis, means that the kidneys are exposed to significant concentrations of medications (Choudhury and Ahmed, 2006). There are numerous medications with the potential to cause renal toxicity via a variety of mechanisms (Table 1.1) and sites of action (Figure 1.1). Some are more likely to cause toxicity than others, and some are used more frequently. DIKI is reported to cause between 18 and 27% of all cases of Acute kidney injury (AKI) in US hospitals (Taber and Pasko, 2008).

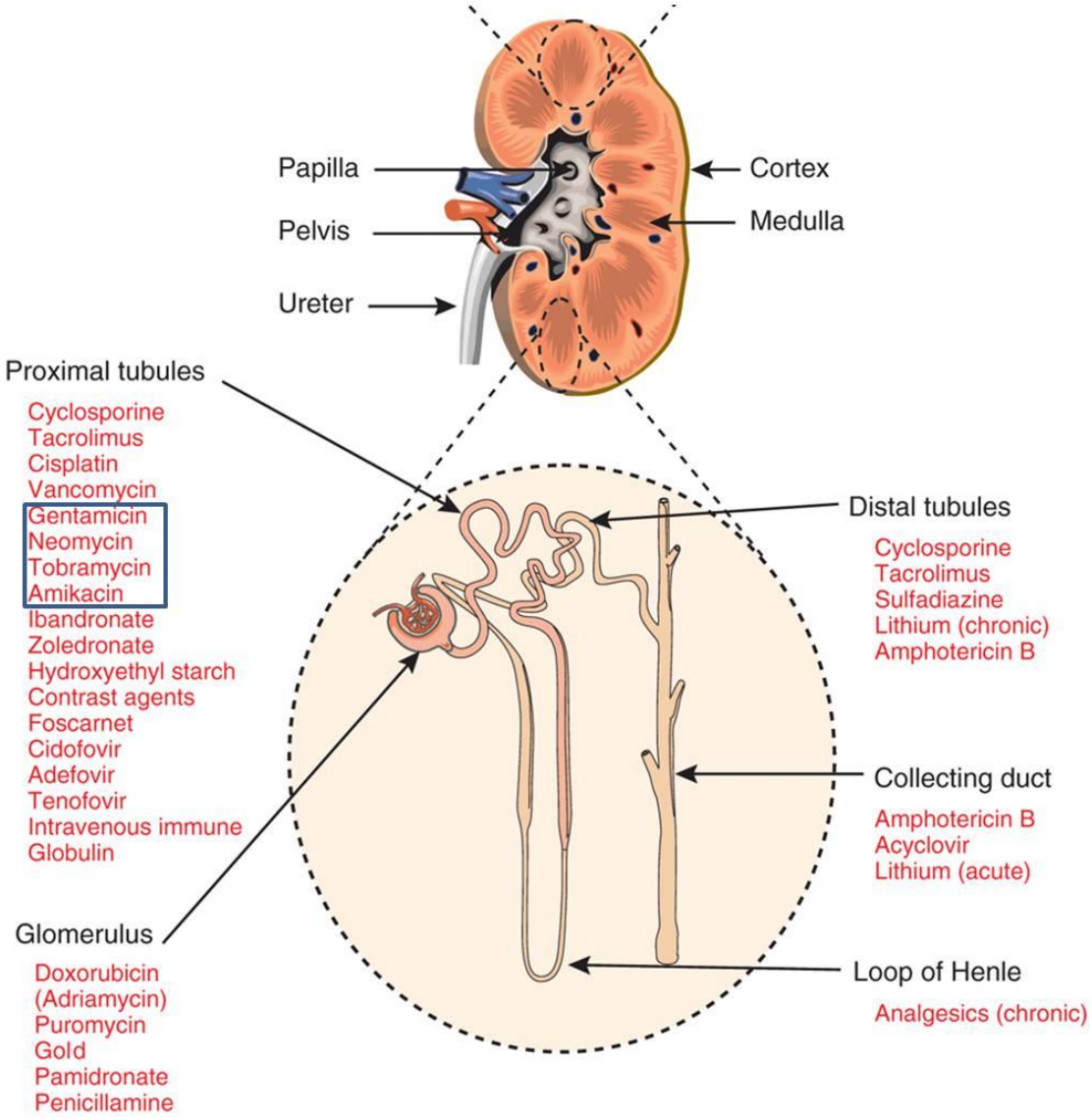


Figure 1.1 – Common nephrotoxins and their sites of action in the kidney (adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology 28;436-440, copyright 2010 (Bonventre et al., 2010)).

1.2.1 DIKI in children

There has not been much work done to quantify the burden of drug-induced kidney injury in children. A retrospective review of episodes of AKI over a three year period at a tertiary paediatric centre in the US identified 248 children who had one or more episodes of AKI (Hui-Stickle et al., 2005). They identified patients who had the diagnosis of AKI listed on their discharge or death summary, and who also had a glomerular filtration rate (GFR) less

than or equal to 75 ml/min/1.73m². From their review of each case they identified a primary cause of the AKI, although they do acknowledge that the causes were frequently multifactorial. Overall the most common cause was ischaemic (in 21% of cases), but the second most common was nephrotoxic drugs (16% of cases). Nephrotoxins were the most common cause of AKI in children aged 6 and above. They were also the most common primary cause in children with an underlying haematology or oncology diagnosis. Interestingly, primary renal diseases only accounted for 7% of cases.

The importance of early diagnosis allowing intervention to prevent AKI is highlighted by survival to discharge of only 70% of children with AKI in this study (those with AKI due to nephrotoxins had 77% survival) (Hui-Stickle et al., 2005). A follow-up study established that survivors of AKI may have significant renal morbidity, and mortality (Askenazi et al., 2006).

There has been little work characterising the incidence of nephrotoxicity in children as a result of individual drug classes. A retrospective case-control study looked at nephrotoxic medication exposure in 561 children with AKI defined using the paediatric-modified Risk, Injury, Failure, Loss, End-Stage Kidney Disease (pRIFLE) criteria (Moffett and Goldstein, 2011). 86% of children who developed AKI had had exposure to one or more potentially nephrotoxic medications (from a list of 36). This was significantly greater than controls (Odds ratio 1.7, 95% confidence interval 1.04-2.09, p=0.03). The percentage of patients developing AKI increased with increased exposure to nephrotoxins.

From these studies it appears that drug-associated AKI is a relatively common occurrence amongst paediatric patients, and that nephrotoxins are the probable cause or a contributory factor in a significant proportion of cases of AKI in children.

1.3 Aminoglycoside-induced nephrotoxicity

1.3.1 Aminoglycoside antibiotics

The first aminoglycoside, streptomycin, was introduced in 1944, and since then the family has grown (Begg and Barclay, 1995). The ones most commonly used in UK clinical practice are gentamicin and tobramycin. The aminoglycosides are particularly active against aerobic Gram negative bacteria, including enterobacteriaceae and pseudomonas species. In clinical practice the aminoglycosides are generally used in combination with an antibiotic providing better activity against Gram positive bacteria (such as a gylcopeptide or beta-lactam antibiotic) to provide broad spectrum coverage for the empirical treatment of suspected serious systemic sepsis. Streptomycin, the first aminoglycoside, has activity against Mycobacterium tuberculosis. They exert their bactericidal effect by binding to the bacterial 30S ribosomal subunit where they prevent protein synthesis by inhibiting binding of transfer ribonucleic acid (RNA) to the ribosome (Begg and Barclay, 1995).

1.3.2 Clinical presentation of aminoglycoside-induced nephrotoxicity

Aminoglycoside-induced nephrotoxicity is described as non-oliguric renal failure with a varying degree of renal tubular dysfunction (Begg and Barclay, 1995). A reduction in GFR is reported to be a late event, occurring between 5 and 10 days after starting treatment (Taber and Pasko, 2008). The renal impairment is reported to be completely reversible in most patients upon withdrawal of the aminoglycoside (Luft, 1984).

1.3.3 Mechanisms of aminoglycoside-induced nephrotoxicity

Aminoglycoside-induced nephrotoxicity is characterized by selective targeting of the proximal tubule cells within the renal cortex. Approximately 5% of the administered dose accumulates within the proximal tubule epithelial cells (PTECs), following glomerular filtration (Mingeot-Leclercq and Tulkens, 1999). This is thought to be the key determining mechanism for the development of toxicity (Mathews and Bailie, 1987). Endocytosis via the multi-ligand receptor, megalin, has been demonstrated to be the principal pathway for this

accumulation (Schmitz et al., 2002). It is the expression of this receptor by proximal tubule epithelial cells which explains the targeted nature of this toxicity. Once inside the cell, aminoglycosides are localised and accumulated within the lysosomes (Taber and Pasko, 2008), as well as the golgi and endoplasmic reticulum (Silverblatt and Kuehn, 1979). Aminoglycosides bind to phospholipids and inhibit phospholipase activity, resulting in lysosomal phospholipidosis (Lopez-Novoa et al., 2011, Taber and Pasko, 2008). At some unknown threshold concentration of aminoglycoside, leakage occurs from the lysosomal structures into the cytoplasm (Regec et al., 1989). Cytoplasmic aminoglycoside acts both directly and indirectly upon the mitochondria, activating the intrinsic pathway of apoptosis via cytochrome c (Servais et al., 2005), leading to disruption of electron transport and ATP production, and the formation of reactive oxygen species (Lopez-Novoa et al., 2011). Lysosomal cathepsins, released into the cytoplasm, also activate the intrinsic apoptotic pathway (Chwieralski et al., 2006), and in higher concentrations may cause necrosis (Golstein and Kroemer, 2007). Furthermore, aminoglycoside in the endoplasmic reticulum inhibits the protein synthesis and associated functions of this organelle, resulting in endoplasmic reticulum stress, and apoptosis via calpain and caspase 12 (Peyrou et al., 2007). The proposed mechanisms of aminoglycoside-induced cytotoxicity in proximal tubule epithelial cells are summarised in Figure 1.2.

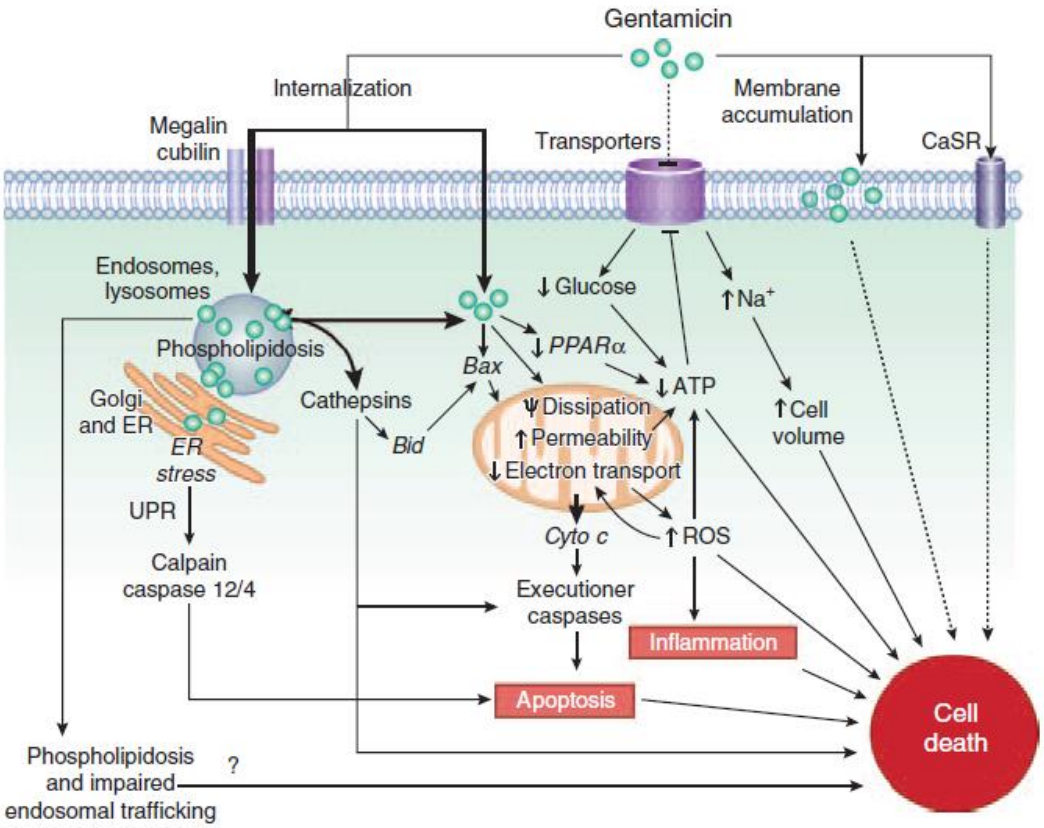


Figure 1.2 – Mechanisms of aminoglycoside-induced cytotoxicity in the renal proximal tubule epithelial cell. ATP, adenosine triphosphate; CaSR, extracellular calcium-sensing receptor; Cyto c, cytochrome c; ER, endoplasmic reticulum; PPARα, peroxisome proliferator-activated receptor-α; ROS, reactive oxygen species; UPR, unfolded protein response (reprinted by permission from Macmillan Publishers Ltd: Kidney International 79;33-45, copyright 2011 (Lopez-Novoa et al., 2011)).

Results of clinical trials and pre-clinical studies has suggested the following order of nephrotoxicity of the aminoglycosides from highest to lowest: neomycin, gentamicin, tobramycin, amikacin, netilmicin, streptomycin (Begg and Barclay, 1995). This rank order of toxicity corresponds to the affinity of each aminoglycoside for the proximal tubule cell brush border membrane.

1.3.4 Defining aminoglycoside-induced nephrotoxicity

Table 1.2 – Paediatric AKI definitions as per the pRIFLE and AKIN criteria (adapted from (Akcan-Arikan et al., 2007), (Mehta et al., 2007) and (Zappitelli et al., 2011))

Paediatric Risk, Injury, Failure, Loss, End-Stage Kidney Disease (pRIFLE)			**Acute Kidney Injury Network (AKIN)**		
AKI severity	**Estimated CCl***	**Urine output**	**AKI severity**	**Serum creatinine**	**Urine output**
'Risk' (R)	Decrease by 25%	<0.5 ml/kg/h for 8 h	**Stage 1**	≥0.3 mg/dL (26.5 µmol/L) rise or rise to 1.5–1.99 × baseline	<0.5 ml/kg/h for >6 h
'Injury' (I)	Decrease by 50%	<0.5 ml/kg/h for 16 h	**Stage 2**	Rise to ≥2–2.99 × baseline	<0.5 ml/kg/h for >12 h
'Failure' (F)	Decrease by 75% or CCl <35 mL/min./1.73 m^2	<0.3 ml/kg/h for 24 h or anuria for 12 h	**Stage 3**	Rise to ≥ 3 × baseline or ≥ 4 mg/dL (354 µmol/L) rise with an acute rise of at least 0.5 mg/dL (44 µmol/L)	<0.3 ml/kg/h for 24 h or anuria for 12 h
'Loss' (L)	Persistent failure >4 weeks				
'End stage' (E)	End-stage renal disease (persistent failure > 3 months				

* CCl, Creatinine Clearance

A key difficulty in establishing the epidemiology of DIKI, including that caused by aminoglycosides, has been the absence of widely agreed criteria for defining episodes. In recent years a number of classification systems have been proposed for the classification of AKI in children. The best validated are the paediatric-modified RIFLE (pRIFLE) criteria (Akcan-Arikan et al., 2007, Moffett and Goldstein, 2011) and the Acute Kidney Injury Network (AKIN) criteria (Mehta et al., 2007). The pRIFLE criteria depend on using estimated creatinine clearance, whereas the AKIN criteria use measured serum creatinine (

Table 1.2). Studies suggest that pRIFLE is more sensitive, but the AKIN criteria have a stronger association with poor outcomes, suggesting they are more specific (Zappitelli et al., 2011, Zappitelli et al., 2008).

1.3.5 General epidemiology

In 1989 Nuyts *et al*. reviewed 29 studies which provided estimates of the incidence of aminoglycoside-induced nephrotoxicity (Nuyts et al., 1989). Within these studies there were wide variations in the populations studied, the criteria used to define nephrotoxicity, and the aminoglycoside of interest, not to mention differences in study design and patient monitoring. There was also variation in whether incidence was reported per patient or per treatment episode. It is therefore unsurprising that within these studies the reported incidence of aminoglycoside-induced nephrotoxicity varied greatly, with a range from 0% to 55% (Nuyts et al., 1989).

Slaughter and Cappelletty reviewed the literature again in 1998 (Slaughter and Cappelletty, 1998). They again found difficulty in comparing the literature due to a large variety of definitions used for nephrotoxicity. They did, however, find a number of studies in adults published in the 1980s and 1990s, which used a reasonably stringent definition of nephrotoxicity: an absolute increase in serum creatinine level of 0.5mg/dl from baseline. In these studies they identified a reduction in incidence of nephrotoxicity from 30% to 8% over time, and suggested that one reason for this may have been increased use of therapeutic drug level monitoring during this period (Slaughter and Cappelletty, 1998).

Table 1.3 – Risk factors for aminoglycoside-induced nephrotoxicity (Adapted and reprinted by permission from Macmillan Publishers Ltd: Kidney International 79;33-45, copyright 2011 (Lopez-Novoa et al., 2011)).

Patient	Treatment	Other drugs
Older age	Longer treatment course	NSAIDs
Reduced renal function	Higher total dosage	Diuretics
Pregnancy	Multiple daily dosing	Amphotericin
Dehydration	Choice of aminoglycoside	Cisplatin
Renal mass reduction	High trough concentration	Cyclosporin
Hypothyroidism		Iodide contrast media
Hepatic dysfunction		Vancomycin
Metabolic acidosis		Cephalosporin
Sodium depletion		
Hypotension		

In one US hospital 16% of episodes of hospital-acquired renal insufficiency were secondary to medications (Nash et al., 2002). Of these, the most common medication indicated was aminoglycosides, which contributed to 29.5% of medication-associated episodes.

1.3.6 Paediatric epidemiology

Aminoglycosides are frequently indicated as a cause of nephrotoxicity in children, but there are little data documenting its incidence, in part due to the lack of an accepted definition for AKI. A recent study at a tertiary children's hospital used the pRIFLE criteria and the AKIN Staging definition (Zappitelli et al., 2011) (

Table 1.2). Of the 557 children who received aminoglycoside treatment for 5 days or longer over the year of the study, the AKI rate was 33% (pRIFLE) and 20% (AKIN) using the two definitions. AKI was associated with longer hospital stay and total hospital costs.

A meta-analysis of studies comparing once-daily with multiple daily doses of aminoglycosides assessed nephrotoxicity related to the different regimens (Contopoulos-Ioannidis et al., 2004). Definitions of toxicity were not consistent across studies. The meta-analysis defined primary nephrotoxicity as 'a rise in serum creatinine or decrease in creatinine clearance with thresholds as defined in each study'. They found that 1.6% of children experienced primary nephrotoxicity in both the once-daily and multiple daily dosing groups (Contopoulos-Ioannidis et al., 2004).

1.3.7 Neonatal epidemiology

Nephrogenesis begins at 4 weeks gestation (Faa et al., 2012) and is completed by 36 weeks gestation (Osathanondh and Potter, 1963) with 60% of nephrons formed in the third trimester (Hinchliffe et al., 1991). Those born at term have a full complement of nephrons, but these nephrons are immature. Glomerular filtration rate (GFR) in a term neonate is 2 to 4 ml/min/1.73m^2 (Kearns et al., 2003). Changes in renal blood flow and maturation of glomerular function, including tubular secretion, occur over the first year of life, with GFR

reaching an adult level (around 120 ml/min/1.73m^2) by 8 to 12 months of age (Kearns et al., 2003). Those born prematurely begin life with a reduced number of immature nephrons. These immature nephrons may then be exposed to a range of insults, including asphyxia, haemodynamic changes, inadequate nutrition, sepsis, and nephrotoxic medications (especially aminoglycosides and non-steroidal anti-inflammatory drugs (NSAIDs)). They are therefore not only at immediate risk of acute kidney injury (AKI) (estimated incidence 6-24% in neonatal intensive care units) (Andreoli, 2004) but there is a growing body of evidence to suggest this in itself is a risk factor for the development of chronic kidney disease (CKD) (Carmody and Charlton, 2013, Askenazi et al., 2009).

Gentamicin is the drug most commonly prescribed to neonates in the UK (Turner et al., 2009). An American study found that 57.5% of all neonates discharged from NICU had received treatment with gentamicin (Clark et al., 2006). The UK National Institute of Health and Care Excellence (NICE) guidelines recommend its use (in combination with a penicillin) for first line therapy in neonates with suspected early-onset sepsis (National Institute of Health and Care Excellence, 2012). The potential for nephrotoxicity is acknowledged, and prevention strategies are employed, including extended-interval dosing and drug trough level monitoring with dose adjustment. A local protocol for the dosing and monitoring of aminoglycoside antibiotics in neonates is included as appendix (Appendix 10.1).

Despite their widespread use, there is a paucity of data quantifying aminoglycoside-induced nephrotoxicity in neonates (Nestaas et al., 2005). A recent review included 10 studies of gentamicin in neonates that reported nephrotoxicity as an outcome measured by plasma creatinine (Kent et al., 2014). Seven of these studies reported no nephrotoxicity. The remaining three reported various rates, with the maximum being 27% (Martínková et al., 2010).

1.3.8 CF epidemiology

Cystic Fibrosis (CF) is a common, autosomal recessive, life-limiting disease which affects around 9000 people in the UK alone (Cystic Fibrosis Trust, 2011). The gene affected, located on chromosome 7, encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (Collins, 1992, Riordan et al., 1989). Mutations in this gene lead to defective chloride transport across the membranes of epithelial cells, which is associated with decreased transport of sodium and water, resulting in dry, viscous secretions (Quinton, 1990). CFTR is widely expressed and therefore CF is a multisystem disease, with major effects on the respiratory, hepatobiliary, pancreatic, gastrointestinal and reproductive systems. Whilst significant problems can be related to other systems, the major morbidity and mortality is associated with the respiratory effects of CF. Here, thick airway secretions lead to reduced activity to the mucociliary escalator, and predispose to secondary bacterial infection and pulmonary colonisation, often by resistant organisms, in particular *Pseudomonas aeruginosa*. The resulting inflammatory response and airway damage results in progressive bronchiectasis and obstructive pulmonary disease (Ramsey, 1996). Recent data have shown 25% of children with CF aged 12-15 years have chronic pulmonary infection with *P. aeruginosa*, and 40% by age 16-19 years (Cystic Fibrosis Trust, 2011).

The aminoglycosides have good efficacy against *P. aeruginosa* and are commonly used to treat pulmonary exacerbations in CF in combination with a beta lactam antibiotic such as Ceftazidime. Treatment courses usually last for 2 weeks and have been shown to improve patient outcomes (Ramsey, 1996). It has been suggested that regular elective courses of intravenous (IV) antibiotics (an aminoglycoside plus a beta-lactam) four times a year may improve survival in patients with CF colonised with *P. Aeruginosa (Szaff et al., 1983)*. However, there is a lack of evidence from randomised trials about whether this elective

approach to treatment of exacerbations improves either lung function or survival (Elborn et al., 2000, Breen and Aswani, 2012).

CF affects multiple systems, and CFTR is expressed in the kidney, but it is not thought to directly cause any renal disease (Morales et al., 2000, Stanton, 1997). Whilst CF patients may be at risk of stone formation, this is felt to be secondary to hyperoxaluria (Hoppe et al., 2005) and dehydration (Perez-Brayfield et al., 2002).

However, some patients with CF do develop AKI and case reports have suggested that nephrotoxicity related to aminoglycosides, either alone or in combination with colistin (Al-Aloul et al., 2005b), ibuprofen (Kovesi et al., 1998, Scott et al., 2001) or cephalosporins (Drew et al., 2003, Kennedy et al., 2005), is an important cause. A UK national survey of Acute Kidney Injury (AKI) in patients with CF found 24 cases between 1997 and 2004 (Bertenshaw et al., 2007). 88% were receiving an aminoglycoside at the time of developing AKI, or within the previous week. They estimated an incidence of AKI of 4.6 to 10.5 cases per 10,000 CF patients per year. Renal biopsy was performed in seven of these individuals, and acute tubular necrosis found in six, again suggesting aminoglycoside-induced nephrotoxicity. In a follow-on case-control study, the same group identified an 80-fold increase in the risk of AKI if CF patients received an aminoglycoside within the preceding week (Smyth et al., 2008). Whilst complete recovery was reported in 92% of those patients who developed AKI, only one actually had GFR measured, and therefore it is not possible to rule out more mild impairment in the others. AKI was associated with significant acute morbidity, with 54% requiring dialysis (Bertenshaw et al., 2007).

In a cohort of adults with CF from Liverpool, chronic renal impairment (reduced creatinine clearance) was reported in 31-42% of adult CF patients (depending on definition), and was associated with cumulative aminoglycoside exposure (P=0.0055) (Al-Aloul et al., 2005a). This effect was exacerbated by the concomitant use of IV colistin, although IV colistin alone

did not have an impact on renal function (Al-Aloul et al., 2005a). A previous, slightly smaller study, in a Danish centre using tobramycin, found no association between previous tobramycin exposure and measured creatinine clearance (Pedersen et al., 1987).

Whilst episodes of AKI do occur, studies which have looked for acute changes in serum creatinine during aminoglycoside exposure have rarely shown a significant change (Pedersen et al., 1987, Halacova et al., 2008). A number of studies have previously quantified other biomarkers to assess changes during exposure to aminoglycosides in patients with CF. Increased urinary excretion of tubular enzymes during aminoglycoside exposure is well documented in CF. Of these, the most widely and consistently reported is elevation of N-Acetyl-β-D-glucosaminidase (NAG) (Reed et al., 1981, Steinkamp et al., 1986, Glass et al., 2005, Etherington et al., 2007). Halacova *et al* demonstrated increases in urinary NAG and serum cystatin C during treatment with amikacin in patients with CF (Halacova et al., 2008). Hypomagnesaemia associated with aminoglycoside exposure in CF is also widely reported (Green et al., 1985, Holben et al., 1995), but is not a consistent finding (Glass et al., 2005).

Patients with CF have been reported to demonstrate altered aminoglycoside pharmacokinetics, with increased volume of distribution and increased clearance (Touw, 1998). However, more recently a population pharmacokinetic meta-analysis in over 700 adults and children with and without CF has demonstrated no effect of CF on tobramycin pharmacokinetics, when the model included age, fat-free mass, sex and renal function (Hennig et al., 2013). Typically aminoglycosides are dosed in mg/kg bodyweight, not on a measure like fat-free mass. Patients with CF in general have less fat, and therefore will need a higher aminoglycoside dose per kg to achieve the same circulating concentration. In another study of aminoglycoside pharmacokinetics in CF, there was no change in clearance associated with cumulative aminoglycoside exposure (Alghanem et al., 2013).

As life expectancy in CF continues to improve (Dodge et al., 2007), there is potential for DIKI to have significant implications for quality of life. Additionally, renal impairment is a common complication following lung transplantation in CF patients, and is predicted by pre-existing renal impairment (Bech et al., 2004, Quattrucci et al., 2005). Therefore, for the long term benefit of these patients it is important to take steps from an early age to prevent DIKI.

1.4 Biomarkers of aminoglycoside-induced nephrotoxicity

1.4.1 Existing approaches

The traditional indicator of AKI is a rise in serum creatinine concentration, but this has a delayed response with levels rising significantly above baseline levels only when 25-50% of renal function has been lost (Askenazi et al., 2009). Furthermore, it is a marker of glomerular filtration and not an indicator of damage at other sites in the nephron. It is an insensitive early marker, and reliance upon it means that AKI is frequently not identified early, and that the degree of damage may be underestimated (Waikar and Bonventre, 2009). However, even a small rise in serum creatinine is a significant risk factor for mortality (Chertow et al., 2005). Interpretation is made more difficult by the variation in the production of creatinine, which depends on age, sex, and weight (in particular muscle mass). Interpretation is made more difficult in the newborn, in whom serum creatinine initially reflects maternal values.

Blood urea nitrogen (BUN) is frequently measured as part of a renal blood profile, but infrequently relied upon in definitions of AKI. It is freely filtered, but reabsorbed by different parts of the nephron to differing degrees. It is therefore possible for it to increase as a result of volume depletion (commonly with dehydration) in the absence of any kidney injury. It is also sensitive to increased urea production, which occurs, for instance, with protein-rich diets.

Oliguria is, at best, a late sign of AKI, and may not be present in many forms of AKI especially those related to toxins (Karlowicz and Adelman, 1995).

1.4.2 Potential of novel biomarkers

Therefore, to identify children who are at increased risk of AKI, there is a need for biomarkers that reflect different regions of the kidney that can be quantified earlier than currently used indicators. This would, in turn, allow for early treatment adjustment, intervention, and the avoidance of further injury.

An ideal biomarker would have the following characteristics (Adapted from (Bonventre et al., 2010)):

- Sensitivity and specificity for kidney injury
- Identify injury early
- Correlates with and tracks the severity of injury and recovery
- Predictive of clinical outcome
- Able to guide clinical decision making (results are available in real time)
- Translatable from pre-clinical to clinical use
- Localise injury to a specific site in the nephron
- Mechanistic basis
- Measurable in the population of interest
- Minimally invasive (easy to obtain necessary sample)
- Measured using robust assays
- Cost-effective

It is unlikely that one biomarker alone will fulfil all of this wish list for all causes of AKI or even of nephrotoxicity. Some may have more specificity for a nephron site making them useful for identifying toxicity at that site, whereas others may have less specificity but

greater sensitivity for AKI. Some biomarkers may increase earlier than others, therefore making them more useful for early diagnosis. Therefore the clinical setting and suspicion is likely to indicate the best biomarker, or panel of markers to use in a given situation.

1.4.2.1 Qualification of Renal Biomarkers

There exist a large number of candidate renal biomarkers (Figure 1.3), with varying degrees of preclinical and clinical evidence to support their use (

Table 1.4).

The Critical Path Institute is an organisation established out of the US Food and Drug Administration (FDA) to facilitate public-private collaboration with the aim of improving and accelerating drug discovery. As part of this the Predictive Safety Testing Consortium (PSTC), a collaboration of academic, industry (both pharmaceutical and biotechnology companies) and regulatory (the FDA, and the European Medicines Agency, EMA) partners, was established to expedite the qualification of renal biomarkers of nephrotoxicity. Much of this work has been published in a special issue of Nature Biotechnology (May 2010, 28(5)). They have focused on rigorously testing a number of renal biomarkers in animals to demonstrate the association between biomarker levels and renal histopathology: work which cannot be so readily achieved in human subjects.

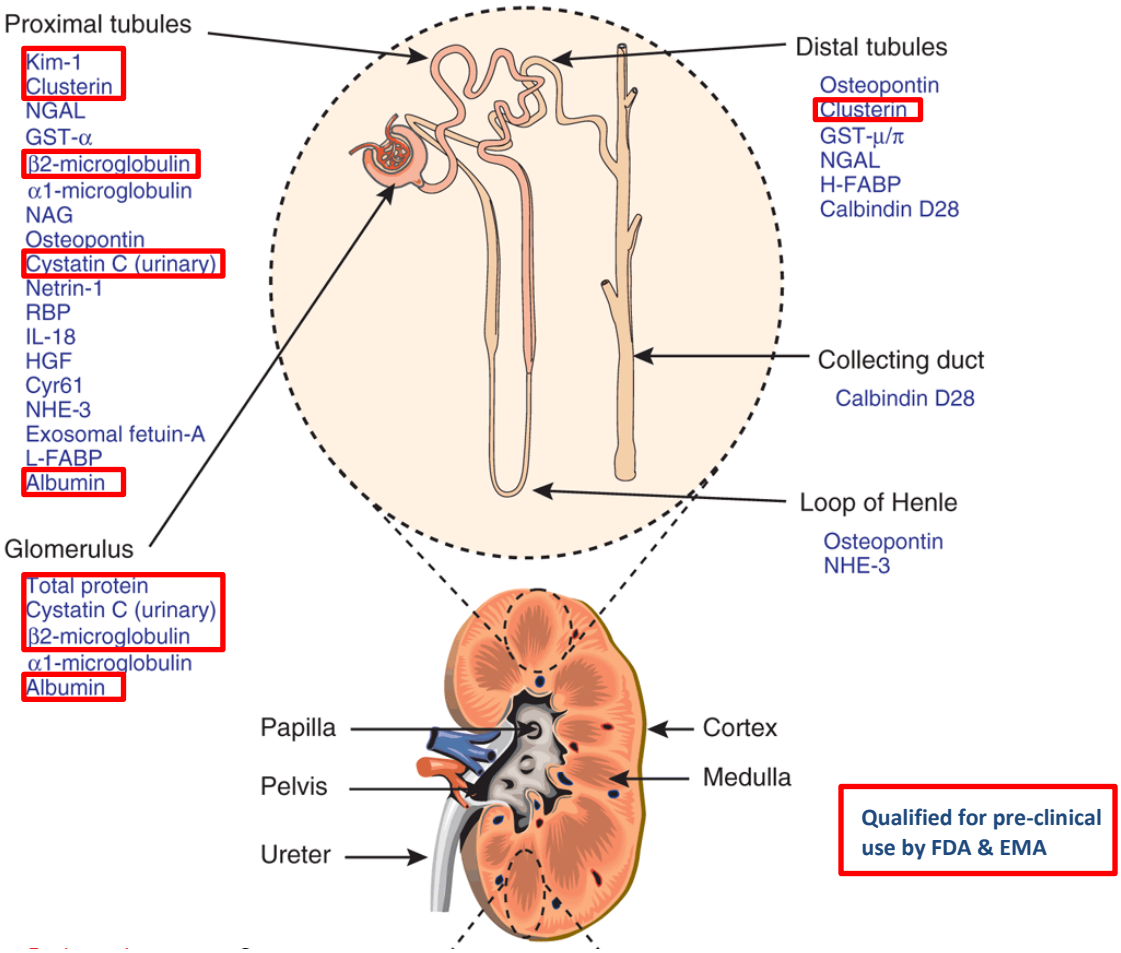


Figure 1.3– Candidate renal biomarkers and their specificity for nephron segments (adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology 28;436-440, copyright 2010 (Bonventre et al., 2010)).

Table 1.4 – Candidate renal biomarkers of nephrotoxicity (adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology 28;436-440, copyright 2010 (Bonventre et al., 2010)).

Biomarker	Preclinical model	Clinical model	Nephron segment	Comments
Albumin	Nephrotoxic AKI or ischemic AKI	Nephrotoxic AKI, ischemic AKI or septic AKI	Glomerulus and proximal tubule	Increased urinary excretion may reflect alterations in glomerular permeability and/or defects in proximal tubular reabsorption; increased urinary levels in the setting of fever, exercise, dehydration, diabetes, hypertension, etc., limit specificity for AKI
α-GST	Nephrotoxic AKI	Nephrotoxic AKI, septic AKI, ischemic AKI or renal transplantation	Proximal tubule	Samples require stabilization buffer for appropriate quantification; clinical data are limited
α_1-microglobulin	Nephrotoxic AKI	Nephrotoxic AKI, ischemic AKI, septic AKI or renal transplantation	Proximal tubule	Clinical applicability limited by lack of standardized reference levels; increased urinary levels in the setting of a number of non-renal disorders may limit specificity; and levels may predict adverse outcome (renal replacement therapy (RRT, dialysis) requirement)
β_2-microglobulin	Nephrotoxic AKI	Nephrotoxic AKI, ischemic AKI, septic AKI or renal transplantation	Proximal tubule	Clinical applicability limited by instability in urine
Clusterin	Nephrotoxic AKI, ischemic AKI, unilateral ureteral obstruction or subtotal nephrectomy	No AKI clinical studies to date	Proximal tubule and distal tubule	Increased urinary levels observed in rat models of tubular proteinuria but *not* glomerular proteinuria
Cysteine-rich protein	Ischemic AKI	Ischemic AKI	Proximal tubule	Urinary levels do not reflect progressive injury; levels assessed via immunoblotting (semiquantitative)
Cystatin-C	Nephrotoxic AKI	Nephrotoxic AKI, ischemic AKI or septic AKI	Glomerulus and proximal tubule	Urinary levels may predict adverse outcome(RRT requirement)
Exosomal fetuin-A	Nephrotoxic AKI or ischemic AKI	Septic AKI or ischemic AKI	Proximal tubule	Levels assessed via immunoblotting (semiquantitative); limited clinical data (n = 3)
Heart-type fattyacid-binding protein	Nephrotoxic AKI or ischemic AKI	Nephrotoxic AKI or renal transplantation	Distal tubule	Increased urinary levels in the setting of heart disease may limit specificity
Hepatocyte growthfactor	Nephrotoxic AKI, ischemic AKI or unilateral nephrectomy	Nephrotoxic AKI, ischemic AKI, septic AKI or renal transplantation	Proximal tubule and distal tubule	Urinary levels may predict adverse outcomes(death or RRT); may play an important role in renal repair and regeneration following AKI
Interleukin-18	Nephrotoxic AKI or ischemic AKI	Nephrotoxic AKI, ischemic AKI, septic AKI or renal transplantation	Proximal tubule	Urinary levels may predict adverse outcomes (death)
Kidney injury molecule-1	Nephrotoxic AKI orischemic AKI	Nephrotoxic AKI, ischemic AKI, septic AKI or	Proximal tubule	Levels may predict adverse outcome (death or RRT)

		renal transplantation		
Liver-type fattyacid-binding protein	Nephrotoxic AKI,ischemic AKI or unilateral ureteral obstruction	Nephrotoxic AKI or ischemic AKI Septic AKI or renaltransplantation	Proximal tubule	Levels may predict adverse outcome (death or RRT); increased urinary levels in acute liver injury may limit specificity
N-Acetyl-β-glucosaminidase	Nephrotoxic AKI or ischemic AKI	Nephrotoxic AKI, ischemic AKI, septic AKI or renal transplantation	Proximal tubule	Levels may predict adverse outcome (death/RRT); decreased activity in the presence of heavy metals may limit sensitivity for AKI; and increased urinary levels in the setting of several non-renal disorders may limit specificity
Netrin-1	Nephrotoxic AKI orischemic AKI	Nephrotoxic AKI, ischemic AKI or septic AKI	Proximal tubule	Levels assessed via immunoblotting (semiquantitative); limited clinical data (*n* = 14)
Neutrophil gelatinase-associated lipocalin	Nephrotoxic AKI orischemic AKI	Nephrotoxic AKI, ischemic AKI or septic AKI	Proximal tubule and distal tubule	Levels may predict severity of AKI and adverse outcome (RRT); increased levels in the setting of urinary tract infections or sepsis may limit specificity
Osteopontin	Nephrotoxic AKI, ischemic AKI or unilateral ureteral obstruction	No AKI clinical studies to date	Proximal tubule, loop of Henle and distal tubule	Increased urinary levels observed in rat models and humans following nephrotoxicity
Retinol-binding protein	Nephrotoxic AKI	Nephrotoxic AKI, septic AKI, ischemic AKI or renal transplantation	Proximal tubule	Decreased sensitivity may be observed in vitamin A–deficient states
Sodium/hydrogen exchanger isoform 3	Nephrotoxic AKI	Nephrotoxic AKI, septic AKI, ischemic AKI or renal transplantation	Proximal tubule and loop of Henle	Levels assessed via immunoblotting (semiquantitative)

The work of the PSTC has led to the qualification of seven renal biomarkers for pre-clinical use by the FDA, EMA and Japanese Pharmaceuticals and Medical Devices Agency (PMDA): KIM-1, Albumin, Total Protein, B2-Microglobulin, Cystatin C, Clusterin and Trefoil factor-3 (Dieterle et al., 2010). This opens the way for use of these biomarkers in pre-clinical drug development work, but also lays the foundation for the translational work envisaged by the consortium in humans. The investigation of these renal biomarkers in clinical studies has been encouraged by the regulatory bodies with the view to producing the validation data required for clinical qualification.

1.4.2.2 Renal biomarkers in children

In paediatric practice there are significant gains to be made from the availability of non-invasive biomarkers, which can be measured on urine samples, potentially reducing the burden of invasive blood tests. However, children are not small adults, and data from adults cannot be directly extrapolated to children. Despite its clear short-comings, this approach has been frequently adopted in many areas of health-related research.

With respect to nephrotoxins a number of factors will affect the potential for toxicity throughout childhood development, and these will be different for each compound. Developmental factors may affect the drug pharmacokinetics by a combination of effects on absorption, distribution, metabolism, and elimination, all of which could combine to leave some children at increased risk of AKI (Kearns et al., 2003). Clearly renal maturation will also play a role in determining the susceptibility to renal toxicity. The glomerular filtration rate and tubular secretion both rise from low levels at birth to reach adult values during the first year of life. Preterm neonates are born with a smaller renal capacity as nephrogenesis is not complete until 36 weeks of gestation (Goldman et al., 2011). This already vulnerable population is therefore most likely to be at risk when exposed to nephrotoxic compounds.

Having considered this renal development, and the variation in normal values of GFR and of serum creatinine during childhood and compared to adults, it becomes obvious that an adult reference range for a novel renal biomarker could not simply be applied to children. Indeed it is important to recognise that the mechanism of toxicity may vary between paediatric and adult patients due to differences in the maturation of the pathways involved. Establishing applicability to paediatric disease, taking account of ontogeny and deriving paediatric reference intervals can clearly best happen through studies carried out in a paediatric population.

Further to these considerations, there are clearly some additional desirable features to those listed above, for a biomarker of renal toxicity to be used in children. Goldman and colleagues identify five characteristics of an ideal paediatric biomarker (Goldman et al., 2011):

- Noninvasive
- Applicable to pediatric-specific diseases
- Results correspond with age-dependent physiologic changes
- Cost effective
- Well-established pediatric normative values

1.4.3 Novel biomarkers

1.4.3.1 N-acetyl-β-D-glucosaminidase (NAG)

NAG is a 130 to 140 kDa lysosomal enzyme specific to proximal tubule epithelial cells (Skálová, 2005). Its size precludes filtration through the glomerular basement membrane, and therefore its urinary concentration reflects its release from proximal tubule cells. NAG has been utilised experimentally for many years for a number of renal diseases, including nephrotoxicity (Price, 1992). It has been used for detecting proximal renal tubular damage including that caused by aminoglycosides (Kos et al., 2003, Ring et al., 1998). It is a predictor of mortality risk following AKI (Coca et al., 2008). Whilst it is generally regarded as a marker of proximal tubule epithelial cell damage, it may also reflect increased lysosomal turnover in renal tubular cells which occurs secondary to proteinuria (Bosomworth et al., 1999).

1.4.3.2 Neutrophil Gelatinase-associated Lipocalin (NGAL)

NGAL is a 25 kDa protein, part of the lipocalin family, expressed by kidney epithelial cells (and other tissues, as well as neutrophils) (Kjeldsen et al., 1993). It appears to have a role in the response to renal injury. NGAL is involved in the delivery of iron to proximal tubule

epithelial cells, which leads to upregulation of heme oxygenase-1 (Mori et al., 2005). This enzyme is thought to play a role in reducing apoptosis and increasing renal tubule proliferation in response to kidney injury (Mishra et al., 2004b). Expression of NGAL messenger RNA (mRNA) and protein are upregulated in response to both renal ischaemia and nephrotoxins in mouse models (Mishra et al., 2003, Mishra et al., 2004a). It has been shown to be a highly sensitive and specific early predictor for the development of AKI in adults (Coca et al., 2008) and children (Wheeler et al., 2008, Zappitelli et al., 2007). However, NGAL is not specific to the kidney. Whilst urinary NGAL does increase as a result of renal injury, it can also increase as a result of systemic inflammation or sepsis (Bagshaw et al., 2010, Friedl et al., 1999)

1.4.3.3 Kidney Injury Molecule-1 (KIM-1)

KIM-1 is a cell membrane glycoprotein. The mRNA and protein are highly upregulated in proximal tubule epithelial cells in response to ischaemia and to nephrotoxins, and the protein ectodomain is cleaved and released into the urine in rats and humans (Han et al., 2002, Ichimura et al., 2004). Rat models of gentamicin-induced nephrotoxicity show that both the mRNA and the Kim-1 protein (the equivalent protein in rats) are upregulated in proximal tubule epithelial cells (Ozaki et al., 2010, Zhang et al., 2008). In rats, urine and kidney Kim-1 upregulation occurs at lower gentamicin doses than upregulation of urinary NAG levels, serum creatinine or blood urea nitrogen, and parallels the degree of nephropathy seen on histopathology (Zhou et al., 2008). A multi-site validation investigation in animal models treated with a number of nephrotoxins, suggests that Kim-1 (KIM-1 in man) outperforms, with respect to sensitivity and specificity, a number of traditional and novel biomarkers of AKI (serum creatinine, blood urea nitrogen, and NAG), as confirmed by histopathology (Vaidya et al., 2010). In clinical trials KIM-1 has been shown to be an early diagnostic marker of AKI (Bonventre, 2007). A systematic review found it to

be one of the best performing biomarkers for the differential diagnosis of established AKI and for the prediction of mortality risk following AKI (Coca et al., 2008).

1.4.3.4 Other biomarkers

As mentioned previously, a number of other biomarkers have been qualified for use in pre-clinical stuidies, and have translational potential (Dieterle et al., 2010). Amongst those approved by the FDA, EMA and PMDA, only KIM-1 is specific purely to the proximal tubule. Albumin, B2-Microglobulin, and Cystatin C may reflect damage to both the proximal tubule and the glomerulus, whereas clusterin may reflect damage to both the proximal and distal tubule (Bonventre et al., 2010). Albumin/creatinine ratio is widely utilised clinically as a marker of renal disease and response to treatment. However, its urinary concentration may be altered by both changes in glomerular permeability and tubular reabsorption. Furthermore, albumin concentration may be increased by additional factors including fever, exercise, dehydration, diabetes and hypertension, limiting its specificity for AKI, and in particular for aminoglycoside-induced nephrotoxicity (Bonventre et al., 2010). Therefore, albumin, and other biomarkers were not assessed.

1.5 Preventing aminoglycoside-induced nephrotoxicity

1.5.1 Existing approaches

Choice of aminoglycoside is important in reducing nephrotoxicity. The following rank order of nephrotoxicity is reported, from most toxic to least: neomycin > amikacin > gentamicin > tobramycin > netilmicin (Begg and Barclay, 1995). A meta-analysis of 43 randomised trials comparing the efficacy and toxicity of aminoglycosides calculated pooled odds ratios for the relative nephrotoxicity of different aminoglycosides (Buring et al., 1988). A weakness of this approach is that studies used different definitions of nephrotoxicity, and the validity of combining these can be argued. However, they reported that tobramycin demonstrated less nephrotoxicity compared to gentamicin (odds ratio 0.64, 95% CI 0.42-0.97). In a case-

control study of patients with cystic fibrosis, gentamicin exposure in the previous year was associated with AKI (19/24 cases v 1/42 controls, p<0.001, odds ratio incalculable), whereas tobramycin was not (9/24 cases v 16/42 controls, p=0.9, odds ratio 1.0, 95% CI 0.3-2.6) (Smyth et al., 2008). Moreover, gentamicin resistance is reported in the majority of *P. aeruginosa* isolates from CF patients (Pitt et al., 2003). Only 13% of isolates were susceptible to gentamicin at a concentration of 1µg/ml, a standard cut-off value recommended by the British Society for Antimicrobial Chemotherapy (BSAC). Mechanisms of resistance include the expression of aminoglycoside-modifying enzymes and efflux mechanisms (Livermore, 2002, Poole, 2005). There has therefore been a shift away from using gentamicin, and towards tobramycin in the management of acute exacerbations of CF.

In theory, extended interval dosing of aminoglycosides may be safer for the kidneys. A higher single dose may result in saturation of megalin-mediated uptake of aminoglycoside in the proximal tubule, resulting in a greater percentage excreted in the urine (Bockenhauer et al., 2009). The TOPIC study was a well powered clinical trial comparing once versus three times daily IV tobramycin for pulmonary exacerbations in CF (Smyth et al., 2005). They demonstrated equivalent efficacy of both regimens using change in forced expiratory volume in 1 second (FEV1) as their primary outcome measure. In children, the study demonstrated a 3.7% increase in serum creatinine with three times daily and a 4.5% decrease with once daily tobramycin. This difference was significant, and suggested a reduction in nephrotoxicity with once daily dosing. This was supported by measurements of NAG pre-treatment and after 14 days of tobramycin. The increase in urinary NAG was 33% less in the once daily compared to the three times daily treatment group (p=0.049 in adults and children, and p=0.02 in children alone). With evidence of equal efficacy, and reduced nephrotoxicity, this study has led to a widespread move from three times daily to once

daily dosing of tobramycin for pulmonary exacerbations in CF. The once daily dose of tobramycin (10mg/kg) used in this study has been widely adopted.

Therapeutic drug monitoring is felt to be helpful for monitoring both efficacy and toxicity. No consistent regime exists for therapeutic drug monitoring, and practice varies widely. For instance, UK CF guidelines recommend that levels taken 18 hours after the previous dose (with a single daily dose) should be <1mg/l (UK CF Trust Antibiotic Working Group, 2009). US CF guidance recommends levels taken between 9-11hours post-dose should be undetectable (Flume et al., 2009). Several CF patients who developed AKI had trough tobramycin levels above 2mg/l (Bertenshaw et al., 2007). With a peak, and another level 6 to 8 hours post-dose, it is possible to use pharmacokinetic principles to adjust dosing to a target area under the curve (AUC) (Coulthard et al., 2007). This approach is used in some centres, but there is little evidence at present for the optimum AUC to give the correct balance of benefit and risk (Coulthard et al., 2007).

Nebulised aminoglycosides (especially tobramycin) have become increasingly widely used in CF. They tend to be used long term in those patients with *P. Aeruginosa.* There are no published data to support their use for acute exacerbations. The perceived benefits are that the antibiotic is delivered directly to the desired site of action, and that it is therefore felt to be possible to deliver high local concentrations, with reduced systemic exposure. Inhaled tobramycin is systemically absorbed, however. One recent study reported a mean serum tobramycin concentration of 1.3mg/l after 56 days of inhaled tobramycin 300mg twice daily (Ratjen et al., 2010), whilst another reports a median concentration of 0.6mg/l using the same dose of inhaled tobramycin (Hennig et al., 2014). A meta-anaysis of randomised trials of long term inhaled antibiotics in CF suggests they result in improvements in FEV1 and reductions in time spent in hospital (Ryan et al., 2003). AKI associated with nebulized tobramycin has been reported (Hoffmann et al., 2002). However,

nephrotoxicity is thought to be lower than with intravenous aminoglycosides, but this has not been investigated in detail.

Therefore current best practice for intravenous aminoglycosides involves the use of less nephrotoxic aminoglycosides, extended interval dosing, and therapeutic drug monitoring with dose adjustment. Avoidance of dehydration and the concomitant use of other nephrotoxic medications should also be observed (Smyth et al., 2008). Current local protocols for the dosing and monitoring of aminoglycoside antibiotics in children (including children with CF) are included as an appendix (Appendix 10.2).

1.5.2 Novel approaches

A recent review of the literature has identified a large number of drugs which have been proposed to have potential to inhibit aminoglycoside-induced nephrotoxicity (Table 1.5) (Balakumar et al., 2010).

Of all the medications proposed, only two have been investigated in humans. One small randomized controlled trial assessed the calcium channel blocker, nifedipine, in patients receiving gentamicin for the treatment of upper urinary tract infection (Vlašiæ-Matas et al., 2000). It was a small study, with a total of 32 participants. Their primary outcome measure was change in creatinine clearance and they demonstrated an improvement in creatinine clearance in the intervention group, with a deterioration demonstrated in the placebo group. Unfortunately the trial had methodological limitations, and it is difficult to draw conclusions as to whether there was any true positive impact of nifedipine. Indeed it is possible that the effect on GFR was a direct effect of the nifedipine itself, secondary to vasodilation of the afferent arteriole leading to increased glomerular perfusion, rather than due to any inhibition of gentamicin-induced nephrotoxicity. There has been no further investigation of calcium channel blockers for this indication in humans, and animal studies

do not demonstrate prevention of aminoglycoside-induced nephrotoxicity consistently with calcium-channel blockers (Balakumar et al., 2010).

Table 1.5 – Drugs reported to have potential to prevent aminoglycoside-induced nephrotoxicity (adapted and reprinted from from Pharmacological Research, 62(3);179-186, Copyright 2010 (Balakumar et al., 2010), with permission from Elsevier)

Pharmacological class	Pharmacological interventions
Antibiotics	Fosfomycin, fleroxacin
Calcium channel blockers	Nifedipine, amlodipine
Beta blocker	Carvedilol
Cytoprotective antianginal	Trimetazine
iNOS inhibitor	L-NIL
NO precursor	L-arginine
Hormones	Melatonin, thyroxine
Antiplatelet	Trapidil
Statin	Atorvastatin
PPAR-γ agonist	Rosiglitazone
TNF-α synthesis inhibitor	Pentoxifylline
Biguanides	Metformin
Antioxidants	Probucol, aminoguanidine, L-carnitine, ebselen, N-acetylcysteine, lycopene, curcumin, thymoquinone, fish oil, vitamin E, vitamin C, sesame oil, halofuginone, resveratrol, quercetin
Free radical scavengers	S-allylcysteine, diallyl sulfide, caffeic acid phenethyl ester, S-allylmercaptocysteine
Antioxidant enzyme	Superoxide dismutase
Superoxide dismutase mimetic	M40403
Herbal extracts	*Rhazya stricta*, garlic, *Cassia auriculata*, soyabean, *Phylanthus amarus*,*Morchella esculenta*, green tea, *Nigella sativa*, *Ligusticum wallichi*, *Viscum articulatum*

A randomized crossover study of fosfomycin, an infrequently used broad-spectrum phosphonic acid antibiotic, in eight patients with CF receiving tobramycin and colistin demonstrated some reduction in proteinuria with fosfomycin treatment but no difference in serum creatinine(Al-Aloul et al., 2004). The size of this study is limiting, and it has only been published in abstract form. Once again, there has been no further study of fosfomycin in humans.

1.5.2.1 Inhibition of megalin-mediated endocytosis of aminoglycosides

Megalin is a 517kDa transmembrane protein which is highly expressed by proximal tubule epithelial cells (Christensen and Birn, 2001). It functions as an endocytic receptor, and is a ligand for numerous low molecular weight proteins, including albumin, vitamin D binding protein, retinol binding protein, α1-microglobulin and β2-microglobulin (Christensen and Birn, 2001, Antoine et al., 2010). It is involved in the clearance of these proteins from the glomerular filtrate, and megalin knock-out mice demonstrate a low molecular weight proteinuria (Leheste et al.).

Endocytosis via the multi-ligand receptor, megalin, has been demonstrated to be the principal pathway for the accumulation of aminoglycosides in proximal tubule epithelial cells. Megalin knock-out mice do not exhibit renal accumulation of aminoglycosides (Schmitz et al., 2002). This suggests a potential pathway for reducing renal accumulation of aminoglycosides by inhibition of megalin-mediated endocytosis. Indeed, known megalin substrates, including cytochrome c and cationic peptide fragments, have demonstrated dose-dependent competitive inhibition of megalin-mediated uptake of gentamicin *in vitro* (Opossum kidney (OK) cells) and have demonstrated inhibition of nephrotoxicity *in vivo* (rat and mouse) (Watanabe et al., 2004).

However, blockade of megalin with endogenous peptides does not provide a viable therapeutic strategy in man as such peptides would be relatively unstable and there would be a risk of immune response developing. However, if it were possible to target the megalin-mediated uptake pathway using an existing medication with a known safety profile, this would potentially be a strategy worth pursuing in man.

Megalin-mediated endocytosis is dependent upon active Guanosine-5'-triphosphate (GTP)-binding proteins which require post-translational modification for trafficking to and anchoring in the cell membrane (Brunskill et al., 1996, Clague, 1998). Two isoprenoid

intermediates derived from mevalonate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, play important roles in this prenylation of GTP-binding proteins (Magee and Marshall, 1999). These isoprenoid intermediates are derived from mevalonate, which itself is the down-stream product of reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), catalysed by HMG-CoA reductase, part of the cholesterol synthesis pathway (Khwaja et al., 2000). Therefore, the process of cholesterol synthesis also results in the formation of important intermediaries for a variety of cell processes mediated by GTP-binding proteins. This leads to the hypothesis that inhibition of the HMG-CoA reductase enzyme by the statin class of drugs, may also result in inhibition of some cellular processes dependent on GTP-binding proteins (Goldstein and Brown, 1990). Indeed this mechanism has been postulated to lie behind a number of the additional effects of statins, such as inhibition of inflammatory processes and augmentation of endothelial function (Cordle et al., 2005). Specifically, in relation to megalin-mediated endocytosis, it has been demonstrated that statins can inhibit this *in vitro*, and that this is associated with both reduced cholesterol synthesis and an increased concentration of unprenylated GTP-binding proteins (Verhulst et al., 2004, Sidaway et al., 2004).

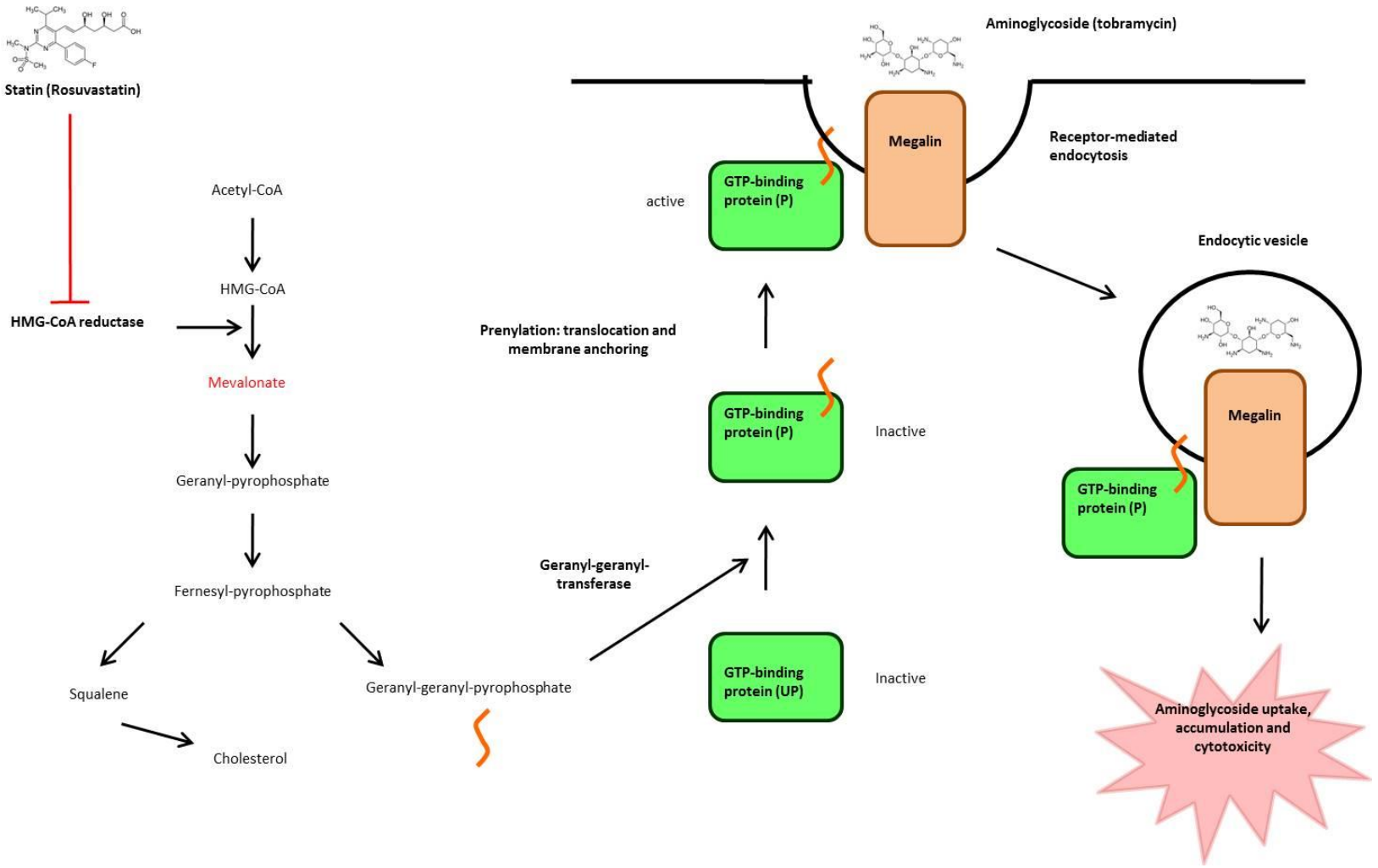
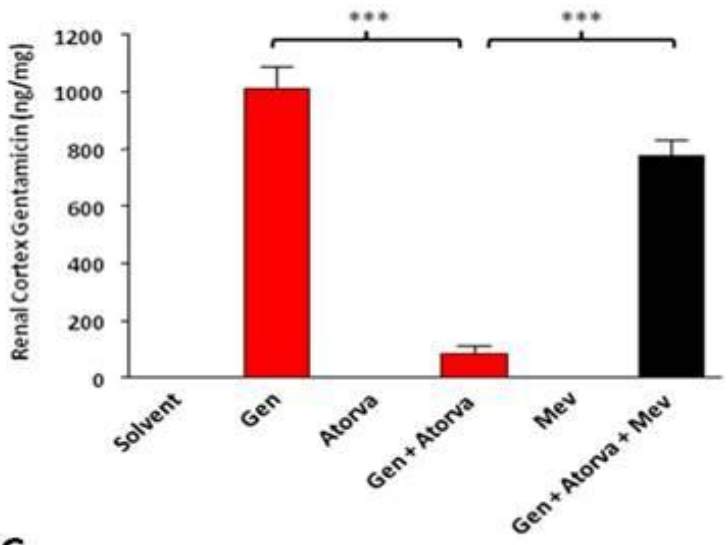


Figure 1.4 - Mechanism of inhibition of aminoglycoside nephrotoxicity by statins

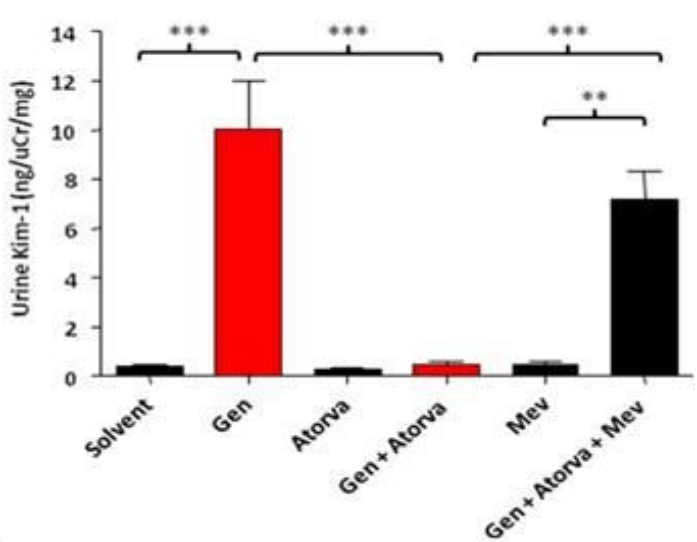
From this develops the hypothesis that statins, by inhibiting megalin-mediated endocytosis, would inhibit uptake of aminoglycosides, and therefore toxicity, in the proximal tubule. This hypothesis was tested in an *in vitro* study using an OK cell model for the proximal tubule (Antoine et al., 2010). Statins (simvastatin, pravastatin and rosuvastatin) were shown to inhibit cholesterol synthesis in OK cells in a dose-dependent manner. They also led to dose-dependent inhibition of gentamicin accumulation and cytotoxicity (measured by lactate dehydrogenase (LDH) leakage), which were related to the degree of GTP-binding protein unprenylation. Addition of mevalonate reversed the protective effect of statins, adding weight to the argument that the inhibition of megalin-mediated uptake is directly related to inhibition of HMG-CoA and not to any off-target effect.

Following on from this work, an *in vivo* model was developed to further test this hypothesis. Sprague Dawley rats received intraperitoneal dosing with gentamicin (200mg/kg/day), atorvastatin (40mg/kg/day), mevalonate (100mg/kg/day), combined gentamicin and atorvastatin, combined gentamicin, atorvastatin and mevalonate, or saline control for 9 days, and sacrificed 24 hours after the final treatment. Treatment with gentamicin resulted in renal accumulation of gentamicin, and renal toxicity as measured by increased serum creatinine, urinary NAG, Kim-1 and albumin concentrations and confirmed by histopathology (Unpublished). This renal toxicity was inhibited by co-administration of atorvastatin, and this inhibition was reversed by co-administration of mevalonate (Figure 1.5)

A


Cortex Accumulation
Renal Cortex Gentamicin (ng/mg)
1200
1000
800
600
400
200
0
***
***
Solvent
Gen
Atorva
Gen + Atorva
Mev
Gen + Atorva + Mev

B


Urinary Kim-1 (Toxicity)
Urine Kim-1 (ng/uCr/mg)
14
12
10
8
6
4
2
0
***
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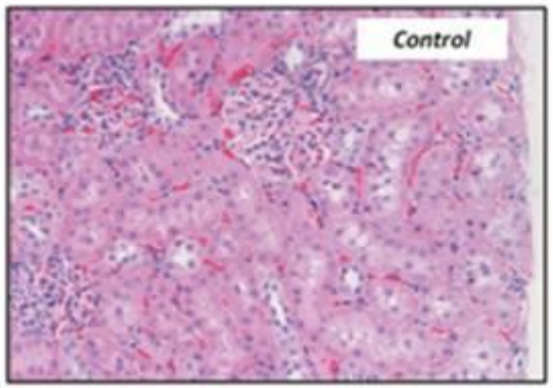
Solvent
Gen
Atorva
Gen + Atorva
Mev
Gen + Atorva + Mev

C


Control

D

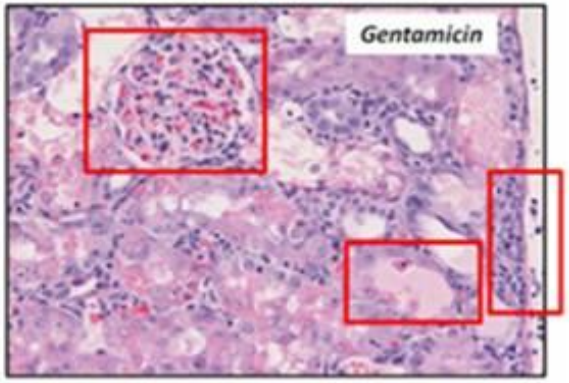

Gentamicin

Figure 1.5 - Gentamicin-induced nephrotoxicity in a rat model, and the impact of atorvastatin. Sprague Dawley (SD) Rats, (n=4 per group) were treated with solvent (control group), gentamicin (200mg/kg/day), Atorvastatin (40mg/kg/day), Gentamicin & Atorvastatin, Mevalonate (100mg/kg/day), and Gentamicin & Atorvastatin & mevalonate for 9 days. Bar charts: Atorvastatin prevents gentamicin accumulation into the renal cortex in SD rats (A) and also the resulting toxicity as measured by urinary Kim-1 (B). Co-treatment with mevalonate, reverses the protective effect of atorvastatin *in vivo*. H&E figures: Gentamicin-induced renal effects in the SD rat. Histological determination reveals proximal tubule cell death by necrosis, luminal protein casts and inflammatory cell infiltration with gentamicin exposure (D) compared to control (C). The degree of proximal tubule cell loss is indicated on the figure and ranges in severity from 0-5. Histology score for gentamicin treated is 2-3, for gentamicin +statin is 0-1.

1.6 Aims, hypotheses and approach

Clinicians considering the use of aminoglycoside antibiotics for the treatment of infections in their patients will be well aware of the potential nephrotoxicity associated with these antibiotics. There is growing evidence that patients, such as those with CF, who are more frequently exposed to treatment with intravenous aminoglycosides are at greater risk. Much is already known about the site and mechanisms of toxicity, and a number of strategies have been established which may lead to a reduction in toxicity. However, traditional markers of renal function are late to indicate damage, non-specific and invasive.

The work described in the following chapters of this thesis seeks to address two key challenges. Firstly, is it possible to develop improved diagnostic tools to identify aminoglycoside-induced nephrotoxicity earlier when it occurs? The potential of novel, non-invasive, urinary biomarkers has already been described. Their utility will be explored in paediatric cohorts exposed to aminoglycosides, and steps taken towards their qualificaton for use in these populations. Secondly, is it possible to prevent aminoglycoside-induced nephrotoxicity from occurring? Improved diagnostic tools must be coupled to the development of further strategies to minimise the nephrotoxic consequences of aminoglycosides. A promising preventive strategy utilising statins has been described, and will be developed and tested through further animal models. The ultimate aim of this thesis

is to combine this two-pronged approach in a first-in-man clinical trial which will investigate the potential of statins to prevent aminoglycoside-induced nephrotoxicity in children with CF, using a novel urinary biomarker as the primary outcome measure.

1.6.1 Aims

1. Develop improved tools for the early identification of aminoglycoside-induced nephrotoxicity in children.
2. Develop a novel intervention to prevent aminoglycoside-induced nephrotoxicity.

1.6.2 Hypotheses

1. KIM-1 represents a novel urinary biomarker with clinical utility as a biomarker of aminoglycoside-induced nephrotoxicity in children.
2. Statins can prevent aminoglycoside-induced nephrotoxicity in children with CF.

1.6.3 Approach

1. Further qualification of KIM-1 and other novel urinary biomarkers by:
    i) Validation of assays
    ii) Establishing reference values in healthy children
    iii) Measurement in children with CF during treatment with tobramycin
2. Test the utility of statins for the prevention of aminoglycoside-induced nephrotoxicity by:
    i) Determining the best statin and dose to take forward into human studies using an animal model that better reflects the human
    ii) Designing and conducting a phase IIa study in children with CF receiving tobramycin.

2 Urinary biomarkers identify the potential for aminoglycoside-induced nephrotoxicity in premature neonates

2.1 Introduction

Nephrogenesis begins at 4 weeks gestation (Faa et al., 2012) and is completed by 36 weeks gestation (Osathanondh and Potter, 1963) with 60% of nephrons formed in the third trimester (Hinchliffe et al., 1991). Those born at term have a full complement of nephrons, but these nephrons are immature. Glomerular filtration rate (GFR) in a term neonate is 2 to 4 ml/min/1.73m^2 (Kearns et al., 2003). Changes in renal blood flow and maturation of glomerular function, including tubular secretion, occur over the first year of life, with GFR reaching an adult level (around 120 ml/min/1.73m^2) by 8 to 12 months of age (Kearns et al., 2003). Those born prematurely begin life with a reduced number of immature nephrons. These immature nephrons may then be exposed to a range of insults, including asphyxia, haemodynamic changes, inadequate nutrition, sepsis, and nephrotoxic medications (especially aminoglycosides and non-steroidal anti-inflammatory drugs (NSAIDs)). They are therefore not only at immediate risk of acute kidney injury (AKI) (estimated incidence 6-24% in neonatal intensive care units) (Andreoli, 2004) but there is a growing body of evidence to suggest this in itself is a risk factor for the development of chronic kidney disease (CKD) (Carmody and Charlton, 2013, Askenazi et al., 2009).

Aminoglycosides are just one of a number of potential renal insults to which preterm neonates may be exposed. Gentamicin is the drug most commonly prescribed to neonates in the UK (Turner et al., 2009). The UK NICE guidelines recommend its use (in combination with a penicillin) for first line therapy in neonates with suspected early-onset sepsis (National Institute of Health and Care Excellence, 2012). The potential for nephrotoxicity is

acknowledged, and prevention strategies are employed, including extended-interval dosing and drug trough level monitoring with dose adjustment. However, despite their widespread use, there is a paucity of data quantifying aminoglycoside-induced nephrotoxicity in neonates (Nestaas et al., 2005).

The traditional indicator of AKI is a rise in serum creatinine concentration, but this has a delayed response with levels rising significantly above baseline levels only when 25-50% of renal function has been lost (Askenazi et al., 2009). Furthermore, it is a marker of glomerular filtration and not an indicator of proximal tubule function. Serum creatinine is difficult to interpret in the newborn: it initially reflects maternal levels, before reducing over the first weeks of life (Gordjani et al., 1988); preterm infants may show an initial rise in serum creatinine, before this decrease becomes apparent (Miall et al., 1999); and in preterm neonates creatinine may be reabsorbed across immature, leaky, tubules, making it a poorer marker of GFR in this population (Matos et al., 1998). Oliguria is, at best, a late sign of AKI, and may not be present in many forms of AKI especially those related to toxins in neonates (Karlowicz and Adelman, 1995). Therefore, to identify infants early who are at increased risk of renal impairment there is a need for biomarkers that reflect different regions of the kidney that can be quantified earlier than currently used indicators. This would, in turn, allow for treatment adjustment and the avoidance of further injury.

There is currently limited data investigating the potential utility of novel renal biomarkers to report nephrotoxicity in the neonatal population. Here we investigate three urinary biomarkers, Kidney Injury Molecule-1 (KIM-1), Neutrophil Gelatinase-associated Lipocalin (NGAL) and N-acetyl-β-D-glucosaminidase (NAG) in preterm infants for the detection of aminoglycoside-associated nephrotoxicity.

The aims of this study were to:

1. Assess the feasibility of collecting serial urine samples from premature neonates in the Neonatal Intensive Care Unit (NICU)setting;
2. Measure a panel of urinary biomarkers in the small volume of samples that could be collected;
3. Determine whether elevations in these biomarkers were associated with the use of nephrotoxic therapeutics in this patient population and relate this to serum creatinine concentrations.

We hypothesized that due to the reported utility of these biomarkers from preclinical data, elevations in all 3 biomarkers would be detected in the absence of serum creatinine changes, and in confirmed cases of AKI, elevations in these biomarkers will precede changes in serum creatinine concentration.

2.2 Methods

2.2.1 Patient recruitment and sample collection

We recruited consecutive neonates with a gestational age less than or equal to 32 weeks admitted to the NICU at Liverpool Women's Hospital, UK, between 1st September 2009 and 24th December 2009. Neonates were excluded if they had a known or suspected renal or chromosomal abnormality, or if they were expected to die within 48 hours of recruitment. The parents or guardians were approached between days 4 to 7 of life of each eligible neonate and provided information about the study. Informed consent was sought over the next 24 hours. The study was conducted with the approval of the Liverpool Paediatric Research Ethics Committee. Informed written consent was obtained from carers or guardians on the behalf of the minors/children involved in our study.

An initial urine sample was collected on day 5 to 8 of life, after consent had been given. Further routine urine samples were collected at weekly intervals for the duration of their stay at the intensive care unit. Urine samples were collected as soon as possible after

beginning treatment with gentamicin (usually the following morning). Samples were collected on a daily basis for the duration of the antibiotic course, and until three days after the last dose of gentamicin. Urine samples were collected using cotton wool balls placed in the perineum inside the nappy. The cotton wool balls were checked after one to four hours. If there was no urine, or the sample was contaminated by faeces, then fresh cotton wool was replaced and checked again later. Once a sample was obtained, a sterile syringe was used to remove the urine from the cotton wool and transfer it to a sterile container. The sample was then centrifuged at 2000g for 4min. Supernatant was then stored at -80°C until analysis.

Serum creatinine concentrations were measured by staff in the clinical biochemistry laboratory at Alder Hey Children's Hospital NHS Foundation Trust, Liverpool, UK, using the compensated kinetic Jaffe reaction (Peake and Whiting, 2006) as part of normal clinical care for each neonate. No additional blood samples were taken, and no extra tests ordered as part of the study.

Neonates who were treated with gentamicin received a dose of 4.5mg/kg every 36 hours in accordance with local protocols and NICE guidance (National Institute of Health and Care Excellence, 2012). Trough serum concentrations were measured just prior to the second dose, and then every 3 to 4 days.

The local guideline for indomethacin recommended its use as prophylaxis for intraventricular haemorrhage (IVH) in neonates with a gestational age of <28 weeks and/or birthweight <1000g: 3 doses of 100µg/kg, given soon after birth, and then every 24 hours. Indomethacin was also recommended for treatment of symptomatic patent ductus arteriosus (PDA) at a dose of 100µg/kg daily for 6 days.

Given that changes in hemodynamics can result in changes in serum creatinine independent of any damage to the nephron, we have defined AKI in this study as a serum

creatinine concentration >132.6micromol/l (1.5mg/dl) which is slightly more than double the median baseline serum creatinine value and/or oliguria (urine output <1ml/kg/hour recorded at any point). This cut-off value of serum creatinine for the definition of AKI in neonates has been commonly used in previous studies (Jetton and Askenazi, 2012). Although newer proposed criteria for the definition of AKI in neonates, such as the modified Acute Kidney Injury Network (AKIN) criteria, may be more sensitive (Koralkar et al., 2011), they had not been validated in preterm neonates at the time of study design. Furthermore, for the purposes of this study, we were most interested in identifying those neonates with the most severe AKI, and felt that choosing a single cut-off value, in line with previous studies, was an appropriate approach.

Neonates were followed-up for the duration of their admission on the unit, or until the end of the study period. Some neonates were discharged home directly from the study unit, whereas others were transferred to satellite neonatal units for further care before discharge.

2.2.2 Determination of urinary biomarkers

All sample analysis for this study was completed at the Bonventre lab, Brigham and Women's Hospital, Harvard University, USA, by Dr D Antoine and Dr V Sabbisetti. Collected urine samples were transported to the USA frozen on dry ice and then stored at -80˚C until analysed. When ready for analysis, samples were thawed, mixed and centrifuged (3000rpm, 5min). Biomarker measurements were performed on the resulting supernatants. Urinary KIM-1 and NGAL measurements were performed using microsphere-based Luminex technology (Figure 2.1), using 25µl of urine per biomarker analysis, and analysed in duplicate (Vaidya et al., 2008). Respective analytes were quantified using a 13-point five parametric logarithmic standard curve for each biomarker. The inter- and intra-assay variability was less than 15% for all assays. Urinary NAG was determined spectrophotometically according to the manufacturers' protocols (Roche Diagnostics),

using 5µl of urine per analysis, and analysed in duplicate. The urinary levels of the respective analytes are expressed as absolute (KIM-1 and NGAL) and enzymic activity (NAG) values and normalized to urinary creatinine concentration. Urinary creatinine (uCr) was determined spectrophotometically as previously described (Waikar et al., 2010), using 25µl of urine per analysis, and analysed in duplicate. Investigators performed all analyte measurements blindly and were unaware of the patients' clinical characteristics.

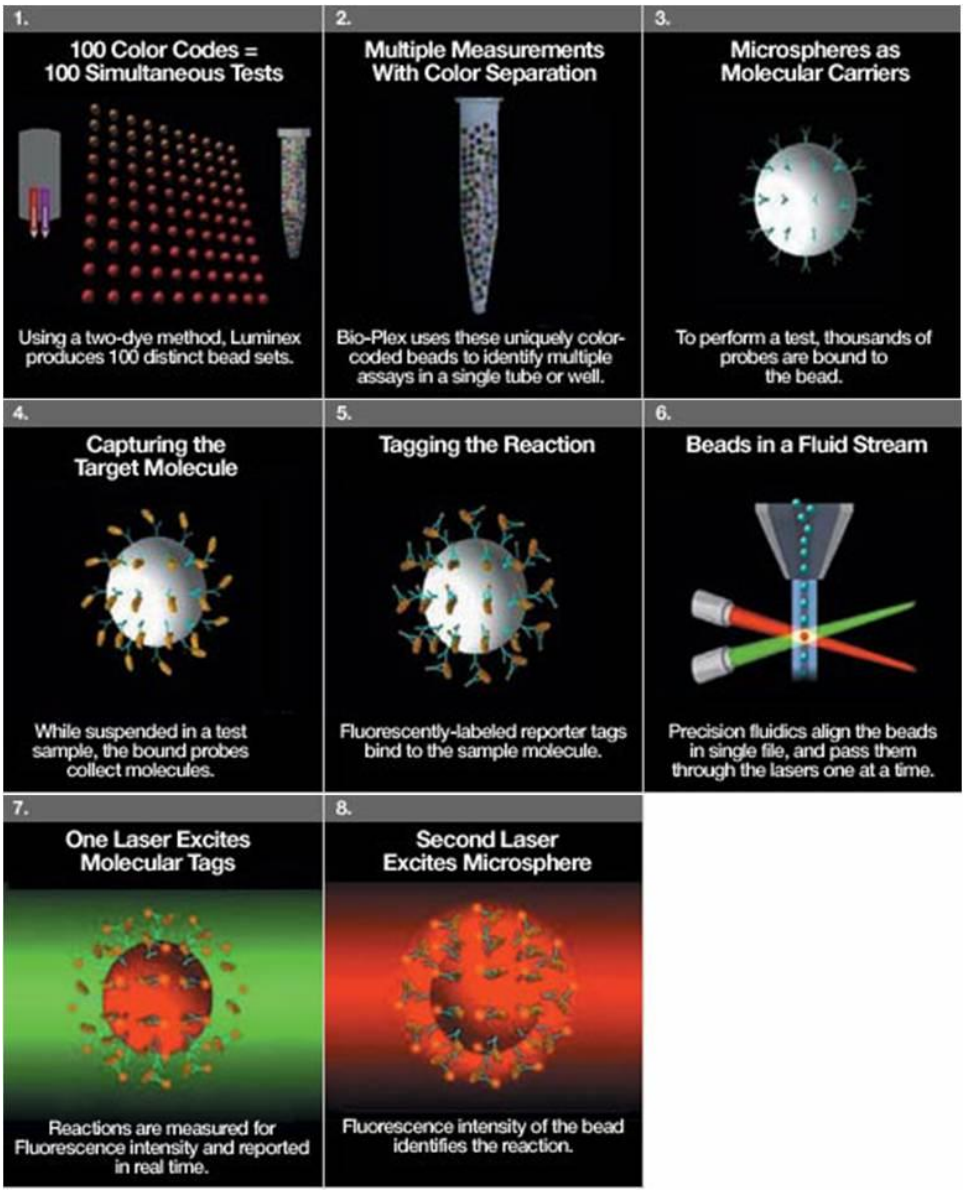


Figure 2.1 – Principle of biomarker analysis using microsphere-based Luminex technology. Adapted with permission from Luminex promotional material (www.luminexcorp.com)

2.2.3 Statistical analysis

Statistical analysis was completed in collaboration with Dr T Farragher, Department of Biostatistics, University of Liverpool.

The study was designed to combine weekly baseline measurements with more frequent, daily, measurements in neonates on gentamicin. This allowed the determination of baseline values taking account of postmenstrual age and assessment of changes in these biomarkers. It was hypothesised that each marker would show an increase related to gentamicin administration due to PTEC damage.

To assess the association between each biomarker and gentamicin treatment, two aspects of the change in each biomarker over the follow-up were first accounted for. The numerous biomarker values available for each neonate over the follow-up are likely to be correlated. To account for this correlation, generalised estimating equations (GEEs) were used with exchangeable correlation (i.e. correlation between biomarker measurements within neonates is constant). GEEs assume linear relationships between each biomarker and time. However, the change in the biomarker values over time is not linear (there are peaks in biomarker values with gentamicin treatment which revert to baseline following cessation of treatment). These changes over time could be accounted for using polynomial equations (i.e. cubic terms) within the GEEs. However, with an increasing number of terms (i.e. knots) multi-colinearity is more likely, affecting the estimate of the association between each of the biomarkers and gentamicin treatment. Therefore, a better solution is to use regression splines, which fit the best-fitting fractional polynominal.

The mfp program in Stata provides a framework for carrying out both tasks simultaneously: selecting (fractional polynomial) functions of the biomarker with time, and the GEE of the biomarker with time (Royston and Sauerbrei, 2005). This approach can account for the change in each of the biomarkers over time and the correlation between measurements.

Accounting for both these aspects allows exploration of the association between treatment and each of the biomarkers.

Postmenstrual age (weeks) was included in the models as it might have an impact on overall differences in biomarkers over the follow-up, and a term was included to assess if this impact changed with time (e.g. impact of gestation diminished/increased over time (later not reported)).

Actual mean difference and percentage difference in each biomarker with gentamicin treatment, as well as baseline values for each biomarker when not on treatment, were calculated. The treatment effects were adjusted for confounders to assess if other factors may explain the association between the biomarker and treatment. Potential confounders were identified from the following factors if they were significantly associated ($p<0.05$) with each of the biomarkers: the episode of treatment received by day of life, gestation (weeks), birth weight (kg), sex, Apgar score at 5 minutes, respiratory distress syndrome, received prophylactic indomethacin in the first week of life, received indomethacin (yes or no, for each day), received furosemide (yes or no, for each day), serum creatinine concentration by day of life, and occurrence of a co-morbidity (yes or no, for each day). Co-morbidities were any of the following: sepsis (blood culture positive), necrotising enterocolitis, cardiopulmonary resuscitation, need for oscillator/high flow oxygen ventilation/nitric oxide, need for inotropes, or surgery.

2.3 Results

2.3.1 Overview of urine collection and the incidence of gentamicin therapy

A total of 41 neonates were recruited to the study. Summary characteristics are presented in Table 2.1. A median of 4 urine samples (Inter-quartile Range (IQR) 2-12) were collected for each neonate. The majority of urine samples measured between 1 and 5ml, with the smallest amount being 200µl in a few patients, but this was still adequate to perform all the

assays. The median length of follow-up per neonate was 30 days (IQR 14-48). The first urine sample was taken at a median of day 7 (IQR 6-7).

Forty of the 41 patients (97.6%) received treatment with gentamicin during the first week of life. Twenty-one (51.2%) went on to have 2 or more treatment courses of gentamicin during the follow-up period. The median number of days of gentamicin treatment for any particular neonate was 5 (IQR 1-8) and median episodes of treatment was 2 (IQR 1-2). Only one participant did not receive any gentamicin. Eight patients were discharged home, 16 were transferred to other neonatal units, 2 were withdrawn from the study by their parents, 3 died, and 12 remained on the NICU at the end of the study follow-up.

Table 2.1 - Baseline characteristics and clinical signs of neonates treated with gentamicin.

Gestation (weeks)	Number	Mean Birthweight (kg)	Number				Mean biomarker value			
			Males	Respiratory Distress	Indomethacin Prophylaxis	Acute Kidney Injury	Urinary KIM-1 (ng/mg Cr)	Urinary NAG (IU/mg Cr)	Urinary NGAL (ng/mg Cr)	Serum Creatinine (micromol/l)
<26	6	0.758	3	6	6	4	3.64	0.28	1046.72	93.37
26 to <28	9	0.893	6	9	8	1	2.85	0.14	632.68	56.51
28 to <30	11	1.106	6	10	2	0	2.67	0.18	538.49	56.12
30 to 32	15	1.499	10	11	0	0	0.72	0.05	82.76	54.19

2.3.2 Evaluation of novel urinary biomarkers in cases of confirmed AKI

Five patients developed AKI during the course of the study as defined by a sCr concentration >132.6micromol/l (1.5mg/dl) and/or oliguria (urine output <1ml/kg/hour recorded at any point). In this patient group, we investigated the hypothesis that it was feasible to measure a panel of urinary biomarkers, and that in cases when serum creatinine was elevated, the urinary concentration of each of the novel mechanism-based biomarkers would also be increased. During episodes of AKI patients had significantly increased mean values of KIM-1 (mean difference 5.84ng/mg Cr; 95% CI 3.77, 7.92), NGAL (mean difference

2031.7ng/mg Cr; 95% CI 1351.4, 2711.9), and NAG (mean difference 0.53 IU/mg Cr; 95% CI 0.39, 0.68) compared to those without AKI. Representative figures are given for three infants (Figure 2.2), and the data for all five infants with AKI are summarised in Table 2.2.

Table 2.2 - Association between Acute Kidney Injury, Gentamicin treatment and biomarker values.

	Effect on Biomarker		
Predictors of variability of biomarker	**KIM-1 (95% CI) (ng/mg uCr)**	**NAG (95% CI) (IU/mg uCr)**	**NGAL (95% CI) (ng/mg uCr)**
Mean difference in those diagnosed with Acute Kidney Injury (AKI) on a given day: compared to not	5.84 (3.77, 7.92)	0.53 (0.39, 0.68)	2031.7 (1351.4, 2711.9)
Mean difference compared to not diagnosed with AKI and not treated with Gentamicin on any given day:			
Not diagnosed with AKI but treated with Gentamicin on any given day	1.53 (0.64, 2.42)	0.08 (0.01, 0.14)	431.62 (138.14, 725.09)
Diagnosed with AKI and not treated with Gentamicin on any given day	8.11 (5.56, 10.66)	0.64 (0.46, 0.81)	2827.68 (1987.07, 3668.29)
Diagnosed with AKI and treated with Gentamicin on any given day	3.9 (0.97, 6.83)	0.43 (0.23, 0.63)	1226.21 (262.13, 2190.29)

No splines were required in models for KIM-1 and NGAL – i.e. GEEs only.

Interestingly, for KIM-1 and NGAL the models no longer require cubic or spline terms. This means that the diagnosis of AKI explains the peaks, suggesting that these biomarkers are strongly associated with AKI. Within this limited sample of 5 individuals, treatment with gentamicin on a given day explains an increase in each biomarker, but the diagnosis of AKI explains a larger difference. When we look at these factors in combination (i.e. both AKI and treatment with gentamicin on the same day) the mean difference is smaller than for AKI alone. This may be because, if possible, gentamicin would not be given to a neonate with severe renal impairment. However, with such a small number of individuals with AKI, it is likely that these estimates are highly variable, and it would be unwise to draw further conclusions.

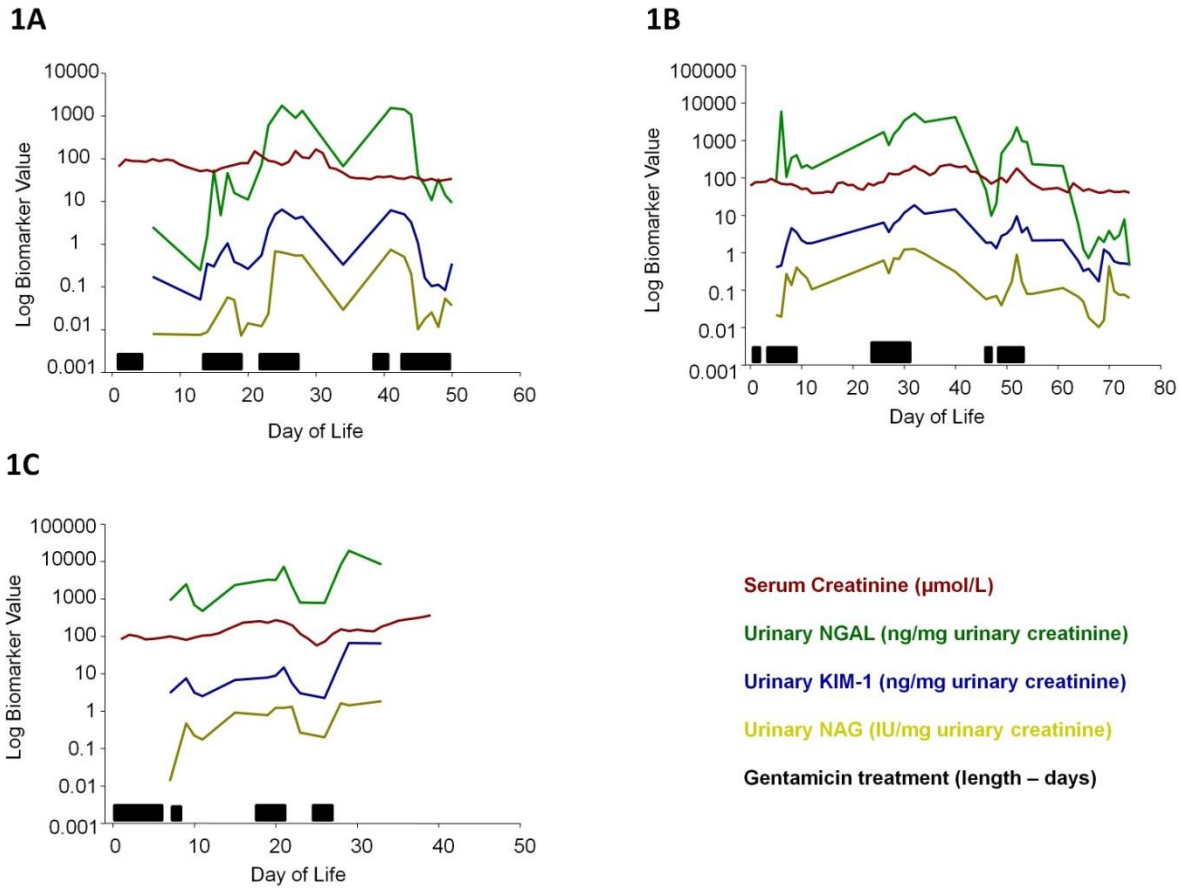


Figure 2.2 - Longitudinal biomarker analysis of infants treated with multiple courses of gentamicin with a change in serum creatinine concentration (AKI). Representative figures demonstrating the longitudinal quantification of the biomarkers KIM-1 (blue; ng/mg. uCr), NGAL (green; ng/mg. uCr), NAG (yellow; IU/mg. uCr) and serum creatinine (red; µmol/L) for three infants treated with gentamicin (A-C). Gentamicin treatment episode and length of treatment (days) are indicated by the black horizontal bar on each figure for that individual patient.

2.3.3 Assessment of the impact of gentamicin treatment on the concentration of urinary biomarkers

After determining that measuring each of these urinary biomarkers was feasible in this patient group in the ICU setting using the method that we employed for urine collection, and that their concentration increased in the presence of confirmed AKI, we next explored the relationship between the urinary biomarker concentration and incidence of gentamicin treatment. Figure 2.3 illustrates representative data from three patients treated with multiple courses of gentamicin. Multiple treatment courses (21/41 patients), in particular, were associated with transient increases in KIM-1, NGAL and NAG. No elevation in any of

the biomarkers investigated was seen in the one patient that was not exposed to gentamicin. Interestingly, in the patients without confirmed AKI that were treated with multiple course of gentamicin (16/21), the urinary concentration increase following gentamicin treatment was observed in the absence of a change in serum creatinine concentration. An analysis of the association between gentamicin treatment and biomarker values is presented in Table 2.3 for all patients recruited to the study.

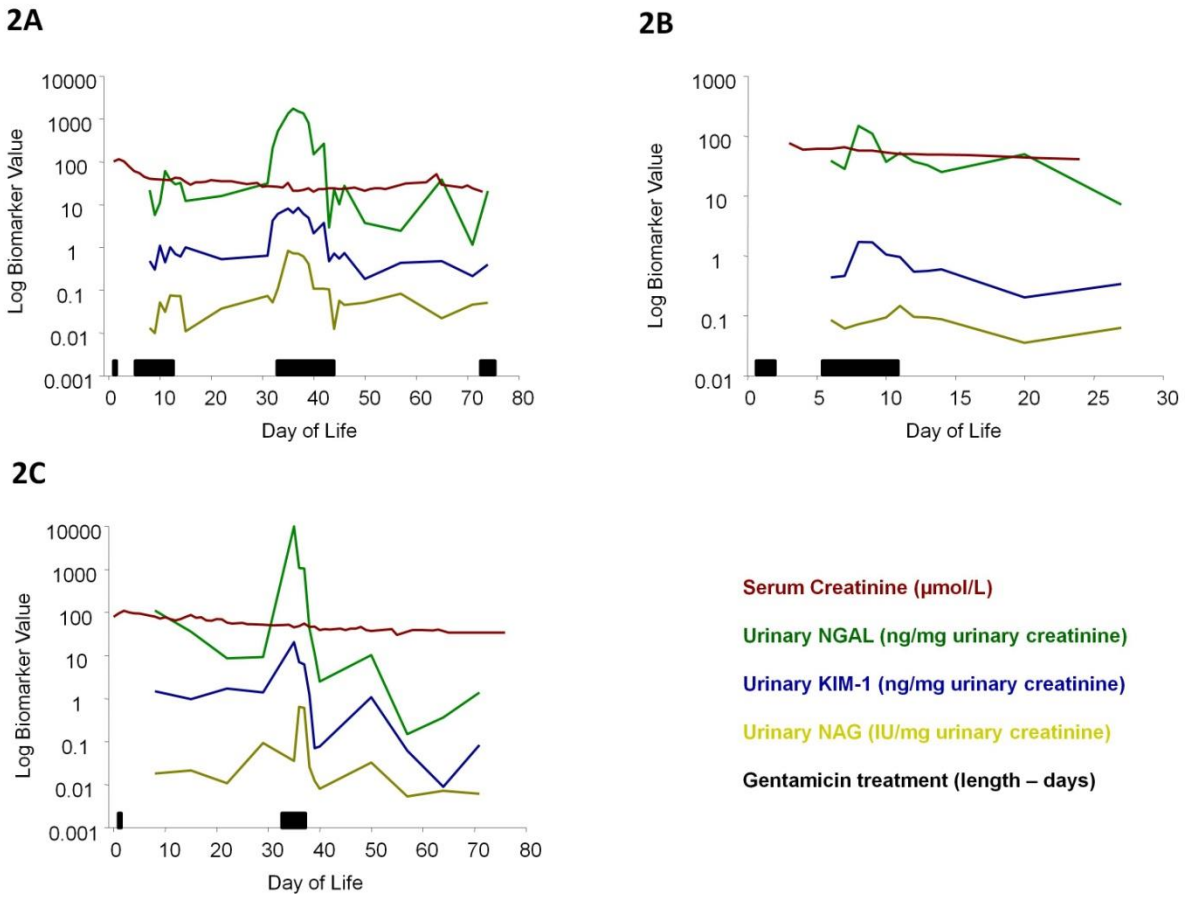


Figure 2.3 - Longitudinal biomarker analysis of infants treated with multiple courses of gentamicin without a change in serum creatinine concentration. Representative figures demonstrating the longitudinal quantification of the biomarkers KIM-1 (blue; ng/mg Cr), NGAL (green; ng/mg Cr), NAG (yellow; IU/mg Cr) and serum creatinine (red; µmol/L) for three infants treated with gentamicin (A-C). Gentamicin treatment episode and length of treatment (days) are indicated by the black horizontal bar on each figure for that individual patient.

Table 2.3 - Association between gentamicin treatment and the change in biomarker values.

Predictors of variability of biomarker	Effect on biomarker KIM-1 (95% CI) (ng/mg Cr)	NAG (95% CI) (IU/mg Cr)	NGAL (95% CI) (ng/mg Cr)	Creatinine (95% CI) (µmol/l)
Mean difference in biomarker value from baseline when receiving gentamicin	1.64 (0.54, 2.75)	0.08 (0.02, 0.15)	453.6 (145.1, 762.2)	-4.64 (-8.64, -0.64)
Predictors of variability of biomarker - adjusted for confounders				
Mean difference in biomarker value from baseline when receiving gentamicin	1.35 (0.05, 2.65)	0.06 (-0.02, 0.13)	298.58 (-56.21, 653.38)	-8.6 (-13.1, -4.2)
Fixed effects: mean difference in biomarker over the follow-up				
Gestation: per week increase	0.22 (-0.44, 0.88)	0.01 (-0.03, 0.05)	33.17 (-145.36, 211.71)	-4.4 (-10.1, 1.2)
Received Indomethacin in the first week of life: compared to not	1.51 (-1.55, 4.57)	0.06 (-0.11, 0.23)	319.2 (-501.9, 1140.2)	-7.1 (-34.4, 20.2)
Time dependent fixed effects: mean difference in biomarker given the predictor				
Episodes of Gentamicin by a given day: per episode increase	-	-0.02 (-0.07, 0.02)	-	2.3 (-0.9, 5.5)
Co-morbidity on a given day: compared to not	1.06 (-0.71, 2.83)	0.12 (0.02, 0.22)	518.3 (30.4, 1006.2)	17.2 (11.3, 23.2)
Creatinine on a given day: per unit 1 increase	0.05 (0.02, 0.07)	0.004 (0.003, 0.005)	15.97 (10.02, 21.91)	-

2.3.4 Determination of baseline urinary biomarker values and the effect of gentamicin treatment

After observing the association between drug treatment and biomarker elevations, we next sought to establish baseline values for each patient to assess the impact and significance of changes in biomarker values. The mean baseline values, in the absence of any gentamicin treatment, were 1.91ng/mg Cr (95% CI 1.07, 2.76) for KIM-1, 0.13 IU/mg Cr (0.07, 0.19) for NAG, 425.4 ng/mg uCr (162.6, 688.3) for NGAL, and 62.39µmol/l (53.1, 71.69) for creatinine. There was a significant difference observed in the value of all three urinary biomarkers between those treated and those not treated with gentamicin on any given day (Table 2.3). These figures take account of the variability and correlation in the biomarker measurements within neonates over the follow-up period, as described in the statistical

analysis section. Between courses of gentamicin, each biomarker returned to a value not significantly different from the value in those patients not receiving gentamicin.

The factors associated with KIM-1 in the univariate analysis, and therefore treated as potential confounders in the multivariate analysis, included gestation, prophylactic indomethacin, co-morbidities and serum creatinine (sCr). The same factors were also associated with NGAL and NAG in the univariate analysis, with the addition of gentamicin episode number for NAG. The association between gentamicin treatment and KIM-1 elevations remained after adjusting for potential confounders (mean difference 1.35ng/mg Cr; 95% CI 0.05, 2.65), but not for NAG and NGAL (Table 2.2).

2.3.5 Evaluation of the impact of other medications on urinary biomarker elevations

The NSAID, indomethacin, is commonly prescribed to neonates in a NICU setting. Its use has been reported to be associated with renal toxicity (Shaffer et al., 2002), although this would not selectively affect the proximal tubule. Indomethacin treatment had no significant effect on the urinary concentration of KIM-1 (-1.31ng/mg Cr; 95% CI -6.06, 3.44) and NGAL (-543.15ng/mg Cr; 95% CI -2119.88, 1033.58), or the urinary enzymatic activity of NAG (-0.09 IU/mg Cr; 95% CI -0.42, 0.24).

2.4 Discussion

Animal models of paediatric gentamicin toxicity have suggested that immature rats (aged 10 days) developed gentamicin-induced nephrotoxicity earlier, and to a greater extent, than more mature rats (Espandiari et al., 2007). The level of renal maturity in 10-day old rats is similar to that found prenatally in humans (Espandiari et al., 2007), suggesting that preterm neonates may be at increased risk of gentamicin-induced nephrotoxicity compared to older children. Thus, it is important to develop methods to investigate renal injury caused by drugs in this vulnerable patient group. Our proof-of-concept investigation shows

that it is feasible to collect serial urine samples, and despite the fact that only small volumes of urine were collected, it was possible to measure a panel of urinary biomarkers in a reproducible manner.

We hypothesized that urinary concentrations of the novel urinary biomarkers, KIM-1, NGAL and NAG would be elevated during AG treatment in the absence of, or before, changes in serum creatinine. Within this study, we found significant elevations in three urinary biomarkers (KIM-1, NGAL and NAG) in premature neonates during courses of treatment with gentamicin, which occurred in the absence of a significant increase in sCr. Once gentamicin was withdrawn, the biomarkers returned to baseline values, similar to that found prior to aminglycoside treatment in the same infant. Those neonates who developed AKI had higher mean values of all 3 urinary biomarkers. Adjustment for potential confounders showed that the increase in biomarker value remained significant only for KIM-1, but the confidence intervals for NGAL and NAG bordered on significance which may reflect our small sample size. It is important to note that this investigation represents the first determination of the urinary abundance of KIM-1 following exposure to nephrotoxic medications in pre-term neonates. Our findings clearly need independent replication, but it is also important to ensure that future studies are adequately powered to determine whether one of the biomarkers has superior predictive ability compared to the others.

NGAL has previously been evaluated in neonates: in a study of 20 patients, although there was marked variability in urinary NGAL levels, it had a sensitivity of 31% for predicting oliguria, and a specificity of 90% (Lavery et al., 2008). Two more recent reports have provided reference values for urinary NGAL in premature neonates (Huynh et al., 2009, Parravicini et al., 2009). A direct comparison with the values reported in our study, however, is not possible as different measurement methods have been used, and our values are corrected for urinary creatinine whereas the other studies have reported only

absolute values. Urinary NGAL serves as a biomarker of sepsis-induced kidney injury (Parravicini et al., 2010); this is consistent with our finding that co-morbidities, including sepsis, were a significant confounder of the gentamicin-associated increase in urinary NGAL. This may indicate that changes in NGAL values are more closely associated with the septic state than with exposure to gentamicin, which would be consistent with its role in the regulation of inflammation (Schmidt-Ott et al., 2006, Schmidt-Ott et al., 2007). As with NGAL, the potential utility of NAG has previously been studied in neonates receiving aminoglycosides. Consistent with our findings, NAG increases during treatment with aminoglycosides and returned to baseline levels following drug withdrawal (Langhendries et al., 1989). Another study, however, found no significant difference in urinary NAG concentrations between neonatal infants treated with gentamicin for 10 days, and a control group (Davidovic-Plavsic et al., 2010). NAG has also been shown to increase following perinatal asphyxia and the related renal ischaemic damage (Willis et al., 1997). Urinary NGAL and KIM-1 were also found to be useful as markers of worsening differential renal function in congenital obstructive nephropathy (Wasilewska et al., 2011).

In preterm neonates, measurement of a panel of urinary biomarkers (which included NGAL and KIM-1) has been reported (Askenazi et al., 2011a). They found that values of these biomarkers were highest in the most premature. Our findings are in agreement with this, our baseline values were broadly similar, and we have accounted for gestational age in our analysis. The same group has also reported that urine biomarkers (again including NGAL and KIM-1) had good predictive value for AKI and mortality in very low birth weight infants (Askenazi et al., 2011b).

We normalised biomarker values to urinary creatinine, but urinary creatinine concentration itself demonstrates age-related changes (Barr et al., 2005, Martin et al., 2008), and creatinine may be reabsorbed in the immature tubules of preterm neonates (Matos et al.,

1998). There is ongoing debate as to the most appropriate standard (Goldstein, 2010, Waikar et al., 2010).

This study was designed to serve as a foundation for evaluation of novel urinary biomarkers in future paediatric studies. It demonstrates the feasibility of measuring a panel of biomarkers in urine samples from premature neonates. Our combination of regular weekly baseline measurements with an increased frequency of sampling during gentamicin treatment enabled us to assess changes in the measured biomarkers during these courses. Despite the relatively small number of neonates who participated in this study, we have been able to show significant changes in our biomarkers related to courses of gentamicin exposure via a longitudinal analysis. In our study, we also noted that biomarker values decreased with increasing postmenstrual age. This suggests a differential sensitivity to renal insults with postmenstrual age, a concept reported pre-clinically (Espandiari et al., 2007) and is similar to a report of baseline levels of hepatic transaminases in preterm neonates (Victor et al., 2010). However, this needs to be confirmed in a larger dataset.

Despite the novel findings of this current investigation, several limitations exist. First, the sample size was small. However, this was a proof-of-concept study, and the longitudinal analysis employed here provides a powerful insight into the dynamics and responses to drug treatment of each of the biomarkers investigated in the clinical setting. Due to the small number of confirmed cases of AKI, the relative sensitivity of each biomarker at reporting renal injury compared to currently used clinical standards could not be assessed with statistical confidence. However, the data provided within this investigation supports the feasibility of such future studies. Second, a number of neonates had only short stays in the NICU, often being transferred to regional units for continuation of their care closer to home. This meant that for some the period of follow-up following their baseline sample was relatively short. Further follow-up studies are planned to determine whether or not

multiple courses of gentamicin exposure in neonates have a lasting effect on renal function. Third, 40 of our 41 participants received gentamicin in the first week of life, and a small number were on gentamicin when the first urine sample was collected. This means we do not have true, pre-gentamicin, baseline measurements of our biomarkers in all patients. Finally, although we have determined urinary biomarker changes associated with gentamicin treatment within this current investigation, we have not correlated this with clinical outcome or clinical decision making.

In conclusion, we have shown that it is possible to collect adequate volumes of serial urine samples to measure a panel of novel urinary biomarkers in premature neonates exposed to gentamicin. The application of the assays, the biomarkers investigated in this patient population in the NICU setting and the statistical analysis used, represent novel outcomes from this research. Of the three urinary biomarkers investigated, KIM-1 appears to be the most promising marker of aminoglycoside-induced nephrotoxicity. We will test this in other paediatric populations.

3 Kidney Injury Molecule-1 (KIM-1) and Neutrophil Gelatinase-Associated Lipocalin (NGAL): Assay validation and reference intervals in healthy children

3.1 Introduction

We have demonstrated the feasibility of measuring urinary KIM-1 and NGAL and their quantitative relationship to aminoglycoside-induced nephrotoxicity as part of a proof of concept pilot study in preterm neonates (Chapter 2) (McWilliam et al., 2012). However, a comprehensive understanding of changes in these biomarkers in urine is critical in order to define reference values in healthy populations to allow appropriate interpretation of novel clinical and translational data, and support their on-going clinical qualification. Historically many paediatric reference intervals have been extrapolated from adult reference data. However, this is not appropriate when one considers the course of renal maturation and other developmental changes during childhood (Goldman et al., 2011).

Any investigation of renal biomarkers designed to produce the desired data for clinical qualification requires the development of validated assays for the biomarker of interest. For the study in preterm neonates, biomarker analysis was undertaken by collaborators at Harvard University using microsphere-based Luminex technology (Section 2.2.2). It was felt that local availability of validated assays would be an important step in developing expertise in these biomarkers. Local availability of a Mesoscale Discovery (MSD) Sector Imager, and of assays for KIM-1 and NGAL from the same company, influenced the decision to select these electrochemiluminescent assays for validation. The existing collaboration with the team at Harvard University afforded an opportunity to compare the MSD assay with the Luminex assay for both biomarkers. The design of the validation experiments were

informed by relevant literature (Lee et al., 2006) and by guidelines produced by the FDA (US Food and Drug Administration, 2001) and EMA (European Medicines Agency, 2011).

The aims of this chapter are:

1. Validate MSD electrochemiluminescent assays for KIM-1 and NGAL according to current guidelines by assessment of:
    a. Calibration curve reproducibility, accuracy and precision;
    b. Intra- and inter-assay precision;
    c. Recovery of spiked samples (accuracy);
    d. Dilutional linearity.
2. Derive reference intervals for KIM-1 and NGAL in healthy children from both the United Kingdom (UK) and United States (US), and to assess:
    a. The impact of sex, age and ethnicity;
    b. Intraindividual and diurnal variability;
    c. Agreement between Luminex and MSD based analytical platforms.

It was hypothesized that:

1. The MSD assays would perform according to the requirements of current guidelines for bioanalytical methods (European Medicines Agency, 2011, US Food and Drug Administration, 2001).
2. Reference intervals for KIM-1 and NGAL would be consistent in both UK and US cohorts.
3. There would be close agreement between Luminex and MSD assays for both biomarkers.

3.2 Methods

3.2.1 Assay Validation

Electrochemiluminescent assays (Meso Scale Discovery (MSD), US), for urinary KIM-1 and NGAL, were selected for this validation (Figure 3.1). The MSD assays are sandwich immunoassays run on 96-well plates. At the base of each well are four spots which can potentially each be coated with a capture antibody allowing the opportunity for multiplex biomarker analysis. The plates validated here, however, used only one spot (precoated with either KIM-1 or NGAL capture antibody by MSD) and the remaining three spots were coated with Bovine Serum Albumin (BSA). When sample is added to the wells, the analyte of interest binds to the capture antibody on the spot. The next phase adds a detection antibody which binds to the bound analyte. The detection antibody is conjugated with an electrochemiluminescent label. A read buffer is added to each well which provides the required environment for electrochemiluminescence, and the plate is read by an MSD SECTOR imager. Within the imager, electrodes built into the base of the plate are energised, initiating the electrochemiluminescent reaction within the well, in which light is emitted by the captured detection antibodies. The imager measures the intensity of light emitted using a charge-coupled device (CCD) camera.

Both assays were run using the same protocol, modified slightly from the manufacturer's protocol. The assay plate was brought to room temperature. MSD Diluent 37, MSD stock calibrator and samples were thawed. 5% MSD Blocker A was prepared by dissolving 1.25g of blocker A powder in 20ml of deionised water, and then adding 5ml of MSD phosphate buffer (5X). Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) was prepared for plate washing. 150µl of Blocker A was added to each well of the plate using a multichannel pipette and reservoir. A reverse pipetting technique was used at all stages in order to avoid

the introduction of bubbles. The plate was sealed using an adhesive plate seal, and incubated on a plate shaker at 300rpm at room temperature for 30minutes.

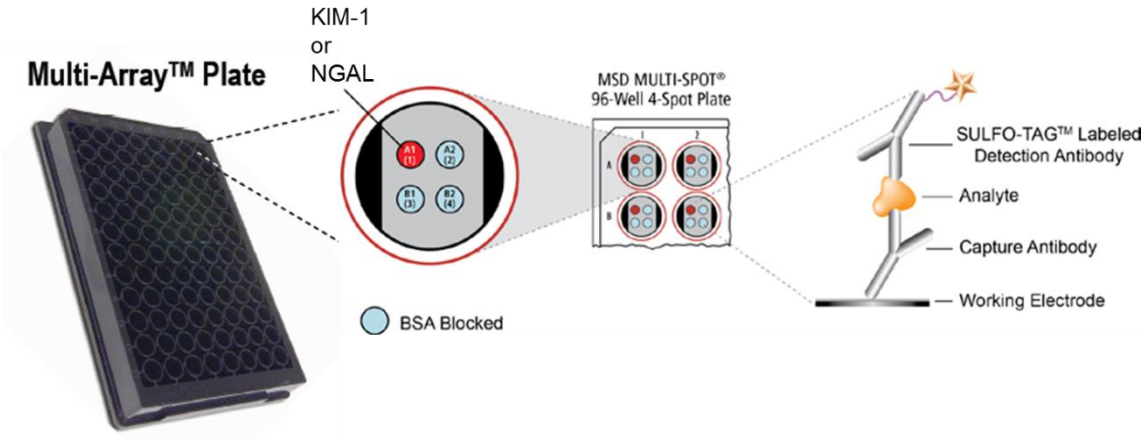


Figure 3.1 - Schematic diagram of Meso Scale Discovery Assay, adapted from diagrams on the manufacturer's website (www.mesoscale.com)

Seven standard concentrations were prepared from the stock calibrator for a standard curve by serial dilutions of the stock calibrator in MSD Diluent 37. Urine samples were thawed, mixed and centrifuged (3000rpm, 5min). The resulting supernatant was prepared for analysis by dilution in MSD Diluent 37.

After 30 minutes, the plate was removed from the plate shaker. The plate was inverted over a sink to remove Blocker A, and washed four times with 150µl of PBS-T per well. Following the fourth wash, excess PBS-T was removed from the wells by inverting the plate and banging sharply onto absorbent paper towel on the bench. 50µl of sample or calibrator was then added to each well of the plate. The plate was sealed and incubated on the plate shaker at 300rpm at room temperature for 2 hours.

After 2 hours the plate was removed and washed as before. 25µl of MSD detection antibody solution was added to each well of the plate. This was prepared within 15 minutes prior to use by dilution of 60µl of the 50X stock detection antibody solution in 2.94ml of

MSD Diluent 37. The plate was sealed and incubated on the plate shaker at 300rpm at room temperature for 2 hours.

After 2 hours the plate was removed and washed as before. MSD read buffer T (2X) was prepared during the previous incubation by dilution of 10ml MSD read buffer T (4X) with 10ml deionised water. 150µl of MSD read buffer T (2X) was added to each well of the plate with a reverse pipetting technique using a multichannel pipette and reservoir. The plate was then immediately read using an MSD SECTOR imager 2400. The plate data was analysed using the MSD DISCOVERY WORKBENCH software (version 3.0), which uses 4-parameter logistic curve fitting to generate the calibration curve.

Healthy volunteer samples were collected as part of the DERIVE study (Determining Reference Values of renal biomarkers in healthy children) (Section 3.2.2.1). Samples with known high concentrations of the biomarkers of interest were taken from our previous study in a neonatal cohort (Chapter 2).

3.2.2 Reference intervals

3.2.2.1 Patient recruitment and sample collection

We recruited healthy children aged from birth to 16 years in Liverpool, UK, between April 2012 and February 2013. Eligible children were recruited at a number of sites, including a nursery, primary school, secondary school, and elective minor surgery admissions wards at Alder Hey Children's Hospital. We also recruited children of staff members at Alder Hey Children's Hospital. Children were excluded if they had a current febrile illness, any history of kidney problems or urinary tract infections, were taking medications known to cause renal problems (especially non-steroidal anti-inflammatory drugs such as ibuprofen), had a diagnosis of CF (by sweat test or genotype), or had a history of exposure to aminoglycoside antibiotics within the last 3 months. We specifically excluded children with CF or a history

of exposure to aminoglycoside antibiotics as we aim to investigate the utility of urinary biomarkers in these patients in another study, and will use this group as a comparison. The DERIVE study received ethical approval from the National Research Ethics Service (NRES) Committee Northwest – Liverpool East, UK. It was registered on the UK Clinical Research Network portfolio (UKCRN 11810) and received support from the Medicines for Children Research Network (MCRN) and the Primary Care Research Network (PCRN). The study was conducted in accordance with the Declaration of Helsinki.

Informed written consent was obtained from carers or guardians on the behalf of the minors/children involved in our study. Those participants aged 16 years were able to consent for themselves. Consent was obtained from all children to provide one urine sample. In schools each participating child produced a urine sample during the school day which was collected by the research team. For children attending the nursery and children of staff at Alder Hey, parents were asked to collect a morning urine sample at home, and bring the sample in to the research team at the site of recruitment on the day of collection. Children recruited on the surgical admissions wards produced a urine sample before their planned surgical procedure.

In addition, some children consented to provide four further urine samples (three morning urine samples at one week intervals following their initial urine sample, and one bedtime urine sample collected the evening before one of the morning urine samples). Morning samples were the first micturition of the day, and the evening samples were collected just before the child went to bed and kept in the home refrigerator overnight. Parents were given sample pots to take home to collect these samples. All samples were brought on the morning of collection to the site of recruitment.

Urine samples were collected from each child by an appropriate method dependent on the age of the child. The normally preferred method was a clean catch urine sample into a

sterile container. Samples were transferred to (if not already collected in) a sterile container and then centrifuged at 2000g for 4min. Supernatant was aliquoted and then stored at -80°C.

Healthy paediatric subjects were recruited at Children's Mercy Hospitals and Clinics (Kansas City, MO, USA) to participate in a longitudinal study investigating changes in cytochrome P450 enzyme drug metabolism activity, specifically CYP2D6 and CYP3A4 activities, through puberty using dextromethorphan as a probe. Dextromethorphan is metabolised to dextrorphan by CYP2D6, and 3-methoxymorphinan by CYP3A4 (Al-Jenoobi et al., 2015). The parent compound and its metabolites are measurable in urine (using high-performance liquid chromatography techniques), allowing quantification of the activity of CYP2D6 and CYP3A4. This study and recruitment was carried out by our collaborators; Dr R Pearce, Dr Y Lin, and Prof JS Leeder and their team. Children of both genders were eligible if they were between the ages of 7 to 16 years; a subset of the study population was diagnosed with Attention Deficit and Hyperactivity Disorder (ADHD) or Attention Deficit Disorder (ADD), and the remainder were non-ADHD/ADD-controls. Exclusion criteria included: i) current therapy with medications metabolized by or known to inhibit CYP2D6; ii) existing asthma or other respiratory diseases associated with hypercapnia; iii) history of metabolic disease, gastro-esophageal reflux disease or gastrointestinal disorders; iv) demonstrated adverse reaction to previous dextromethorphan exposure; v) impaired hepatic activity as determined by routine liver function tests and physical exam; vi) pregnancy; and vii) body mass index less than 5^{th} percentile. Subjects were given a complete medical examination including assessment of Tanner stage and blood samples were taken for liver function testing and DNA collection at the screening visit. The parent study entitled "Exogenous and Endogenous Biomarkers of CYP2D6" and secondary use of the residual urine was approved by the University of Missouri-Kansas City Health Sciences Pediatric Institutional Review

Board. The parent study is also registered as trial NCT01118858 at ClinicalTrials.gov. The study was conducted in accordance with the Declaration of Helsinki.

Subjects were recruited by Children's Mercy Hospital Clinical Pharmacology staff, and parents and children were given verbal and written information about the study. If subjects agreed to participate in the study, a screening questionnaire was completed with the parents; written informed permission was obtained from the subject's parents and assent was obtained from children over 7 years of age.

Paediatric subjects were instructed to fast overnight and report to the Children's Mercy Hospitals and Clinics the following morning for dextromethorphan phenotyping. Subjects were given a single oral dose of dextromethorphan (0.5 mg/kg as Robitussin Cough Syrup, Pfizer, Inc, New York, NY). Urine was collected pre-dose and for 4 hours following dosing by clean catch into sterile containers. At the end of the 4 hour collection period, subjects were instructed to void their bladder. All urine collected during the 4-hour collection interval was kept at 4^{o}C until completion of the collection period, pooled, mixed, dispensed into multiple aliquots of various volumes, frozen and stored at -80^{o}C. Unthawed aliquots of 4-hour urine samples were shipped on dry ice to the University of Liverpool where they were stored at -80^{o}C until analysis. The 171 samples included in this study represent the first of seven study visits at six month intervals for three years.

3.2.2.2 Determination of urinary biomarkers

Collected urine samples were thawed, mixed and centrifuged (3000rpm, 5min). Biomarker measurements were performed on the resulting supernatants. Urinary KIM-1 and NGAL were measured using the validated electrochemiluminescent assays (Meso Scale Discovery, US). Urine samples were run in duplicate at a dilution of 1 in 10 in MSD Diluent 37, and repeated at a dilution of 1 in 100 if they remained too concentrated. Urinary KIM-1 and NGAL measurements were also performed using microsphere-based Luminex technology,

as previously described (see chapter 2), with a turnaround time of 4-6 hours (McWilliam et al., 2012, Vaidya et al., 2008). Biomarker values were normalised to urinary creatinine (uCr) which was determined spectrophotometically as previously described (Waikar et al., 2010). Laboratory analysis was blinded to participants' clinical characteristics. The Luminex-based assay was performed in the Bonventre Lab, Brigham and Women's Hospital, Boston, USA, by Dr V Sabbisetti.

3.2.2.3 Statistical Analysis

Statistical analysis was completed with supervision from Dr A Jorgensen, Department of Biostatistics, University of Liverpool.

Biomarker concentrations from the baseline urine sample produced by each participant were used to calculate the overall reference intervals following approaches recommended for paediatric reference intervals (Daly et al., 2013), using SAS version 9.3 (SAS Institute, Cary, NC) and R (R Development Core Team, 2011). The distributions of KIM-1 and NGAL followed a near Log normal distribution. Therefore a logarithmic transformation was applied to the biomarker values in order to approximate a Gaussian distribution, allowing a parametric approach to analysis. Biomarker levels in the two reference populations were compared using an independent samples t-test. Reference ranges were then calculated in the two cohorts separately. The US cohort included both patients with ADHD/ADD and healthy controls. Although medications licensed for the management of ADHD/ADD do not commonly have nephrotoxic effects, we cannot exclude the possibility of rare adverse effects. An independent samples t-test was used to compare these groups. There were no differences identified between these two groups for KIM-1 or NGAL, and therefore the two groups were analysed together.

Outliers were identified and removed according to Tukey's method (Tukey, 1977). We assessed the need for partitioning of the reference population by the binary factors sex and

ethnicity using independent samples t-tests. In order to assess whether reference ranges varied significantly with age, we used the method of quantile regression (Koenker and Hallock, 2001), with age as the independent variable. Quantile regression was applied either on the complete dataset, or on the dataset partitioned by sex and/or ethnicity depending on which factors were found to be statistically significant in the t-tests. Where quantiles were not found to significantly vary with age (p-value≥0.05 in quantile regression model), the 2.5th and 97.5th quantile were referred to in order to calculate the 95% reference intervals for each biomarker. We also report 90% confidence intervals for the upper limit of the reference interval (Daly et al., 2013, Shaw et al., 2013). Where quantiles were found to significantly vary with age (p-value<0.05), we report the predicted values for the 2.5^{th} and 97.5^{th} quantiles, as obtained from the fitted quantile regression model, for particular age groups, together with 90% confidence intervals for the upper limit.

Differences between morning and evening paired biomarker values were assessed using a paired t-test. Within subject variation over up to four morning urine samples was measured using mean standard deviation (SD). Reference intervals for urinary creatinine were also calculated using the same statistical approach as for KIM-1 and NGAL.

Differences between the MesoScale Discovery (MSD) and Luminex methods of biomarker analysis were assessed using a regression analysis according to the method of Bland and Altman (Bland and Altman, 1999).

3.3 Results

3.3.1 Assay Validation

3.3.1.1 Calibration curve

Some preliminary experiments were done to establish the best calibration curve values to use for the validation and then for sample analysis. We started by using the calibration

values recommended by the manufacturer, and by running urine samples from the neonatal study (chapter 2) with a wide range of known values.

For the calibration curve validation experiments, a calibration curve was run in triplicate on each plate, on three separate plates. The calibration curve consisted of seven serial dilutions of the relevant stock calibrator, and a 'blank' sample which was MSD diluent 37.

The calibration standards should have an accuracy and precision of less than or equal to 20% (≤25% at upper and lower limits) (European Medicines Agency, 2011). Accuracy is calculated by expressing the difference between the expected and the back-calculated concentration of the calibration standard against the calibration curve as a percentage of its expected value. Precision is calculated using the coefficient of variation of the calibration standard from the three replicates on each plate. Providing these criteria were met, the maximum concentration of the calibration curve will be the upper limit of quantitation (ULOQ) and the minimum concentration will be the lower limit of quantitation (LLOQ). The range between these is the dynamic range of the assay. The limit of detection (LOD) of the assay is calculated from the back-calculated concentration of the 'blank' and adding 2 standard deviations. In addition, the reproducibility of the calibration curves was assessed.

3.3.1.1.1 KIM-1 Calibration Curve

For KIM-1 the assay protocol suggests using calibration concentrations of 20000, 5000, 1250, 313, 78.1, 19.5, and 4.88pg/ml. The preliminary experiments demonstrated good precision at the upper concentration of 20000pg/ml (coefficient of variation (CV), 1.46%), but accuracy was 26.4% which is outside the cut-off of 25% for the upper limit. With the range of samples that we ran, we also felt that we were unlikely to have samples with such high KIM-1 concentrations as to require this higher value. We therefore decided to use an

upper limit of 5000pg/ml and to add an additional lower limit of 1.22pg/ml as some of our urine samples were falling below the existing lower limit. Our preliminary work suggested good precision (CV, 3.77%) and accuracy (5.7%) at this new lower limit.

For the validation experiments, the KIM-1 calibration concentrations were 5000, 1250, 313, 78.1, 19.5, 4.88 and 1.22pg/ml. For each plate, 15µl of the stock calibrator (400000pg/ml) was diluted 20-fold in 285µl MSD Diluent 37, to give a solution of 20000pg/ml. Serial 4-fold dilutions using 60µl of the previous concentration in 180µl Diluent 37 were then performed in 0.5ml eppendorfs to give the seven calibrator concentrations.

KIM-1 Calibration curves were run in triplicate on three plates. The three calibration curves are shown in Figure 3.2, and demonstrate good reproducibility between occasions. Accuracy and precision for the three runs are given in

Table 3.1. Accuracy was less than 20% at all concentrations on all three runs. Precision was less than 20% at all concentrations on all runs, except for the 1.2pg/ml concentration on Run 3. However, this value of 21.3% is still below the 25% cut-off value at the lower limit, and is therefore acceptable. A precision profile showing the mean precision at each concentration across the three runs is shown in Figure 3.3. With these results we are able to assign the ULOQ of the assay to 5000pg/ml and the LLOQ to 1.22pg/ml. The mean LOD across the three runs was 0.185pg/ml.

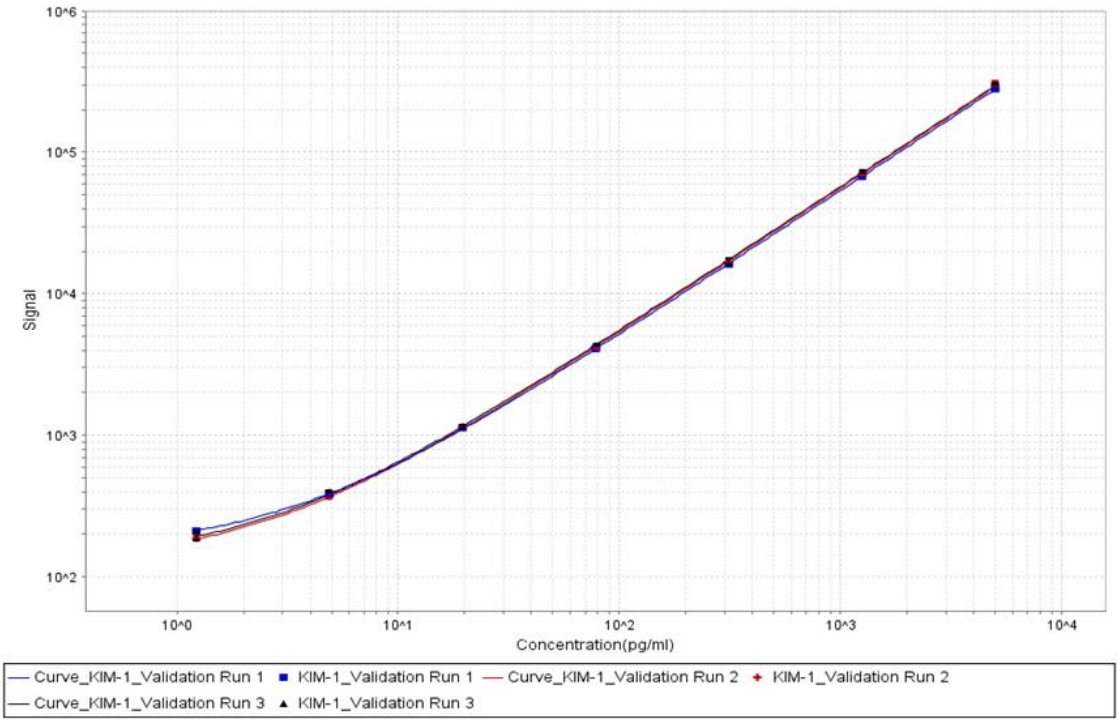


Figure 3.2 - KIM-1 Calibration Curves. KIM-1 calibration concentrations were 5000, 1250, 313, 78.1, 19.5, 4.88 and 1.22pg/ml. Calibration curves were run in triplicate on three plates on different days. Calibration curves were generated using 4-parameter logistic curve fitting in MSD DISCOVERY WORKBENCH software (version 3.0). Results are mean signal (plus or minus standard error).

Table 3.1 - KIM-1 Calibration Curve Accuracy and Precision.

Expected Concentration (pg/ml)	Calculated Concentration (pg/ml)			Accuracy (%)			Precision (CV%)		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
5000.00	5028.66	5210.71	5090.73	0.57	4.21	1.81	2.21	3.59	1.22
1250.00	1246.86	1221.92	1268.92	0.25	2.25	1.51	0.81	0.93	2.45
312.50	308.33	304.48	300.12	1.34	2.57	3.96	1.69	0.55	4.36
78.13	78.26	76.73	77.72	0.18	1.79	0.52	1.10	2.75	1.63
19.53	19.97	19.99	19.30	2.25	2.35	1.18	5.04	1.49	2.80
4.88	4.82	4.98	5.20	1.21	2.01	6.46	3.35	8.44	1.96
1.22	1.17	1.26	1.21	4.27	2.81	0.56	5.21	8.48	21.33

Calibration curves were run in triplicate on three plates on different days.
Results are mean accuracy and precision for each calibrator concentration on each plate.

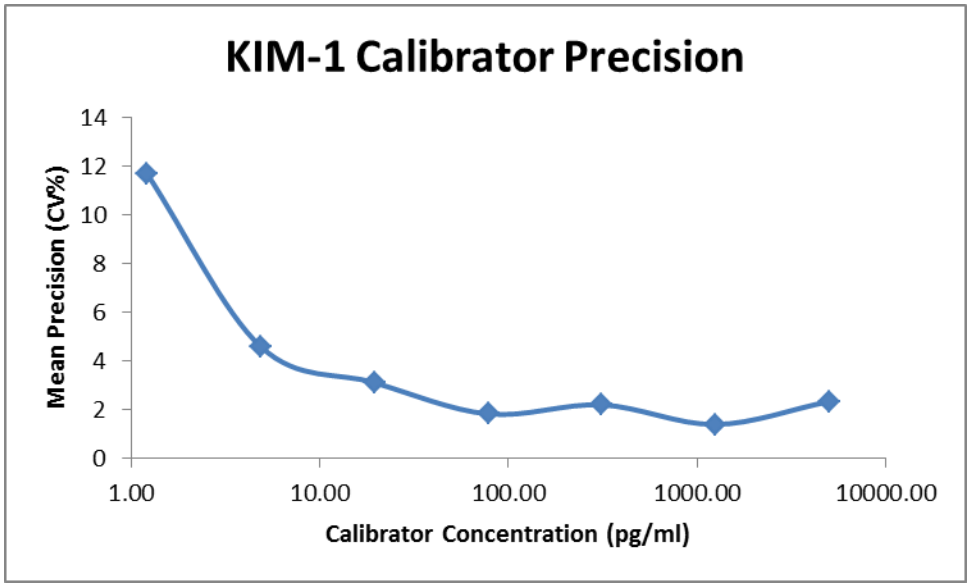


Figure 3.3 - KIM-1 Calibrator Precision Profile. KIM-1 calibration concentrations were 5000, 1250, 313, 78.1, 19.5, 4.88 and 1.22pg/ml. Calibration curves were run in triplicate on three plates on different days. Results are mean precision for each calibrator concentration across the three plates.

3.3.1.1.2 NGAL Calibration Curve

For NGAL, the assay protocol suggests using calibration concentrations of 10000, 2500, 625, 156, 39.1, 9.77 and 2.44pg/ml. The preliminary experiments demonstrated accuracy and precision were both regularly above 25% for the 9.77 and 2.44pg/ml calibrator concentrations. The urine samples analysed as part of the preliminary work suggested that concentrations higher than 10000pg/ml were common, and we therefore investigated use of a higher maximum concentration (up to 40000pg/ml). However, it was found that at concentrations above 10000pg/ml, precision was poor (above 25%) and the assay appeared to start to saturate.

Therefore, for the validation experiments, the NGAL calibration concentrations chosen were 10000, 4000, 1600, 640, 256, 102.4 and 40.96pg/ml. For each plate, 15µl of the stock calibrator (200000pg/ml) was diluted 20-fold in 285µl MSD Diluent 37, to give a solution of

10000pg/ml. Serial 2.5-fold dilutions using 120µl of the previous concentration in 180µl Diluent 37 were then performed in 0.5ml eppendorfs to give the other six calibrator concentrations.

NGAL Calibration curves were run in triplicate on three plates. The three calibration curves are shown in Figure 3.4, and demonstrate good reproducibility between occasions. Accuracy and precision for the three runs are given in Table 3.2. Accuracy was less than 20% at all concentrations on all three runs. Precision was less than 20% at all concentrations on all runs. A precision profile showing the mean precision at each concentration across the three runs is shown in Figure 3.5. With these results we are able to assign the ULOQ of the assay to 10000pg/ml and the LLOQ to 40.96pg/ml. The mean LOD across the three runs was 18.6pg/ml.

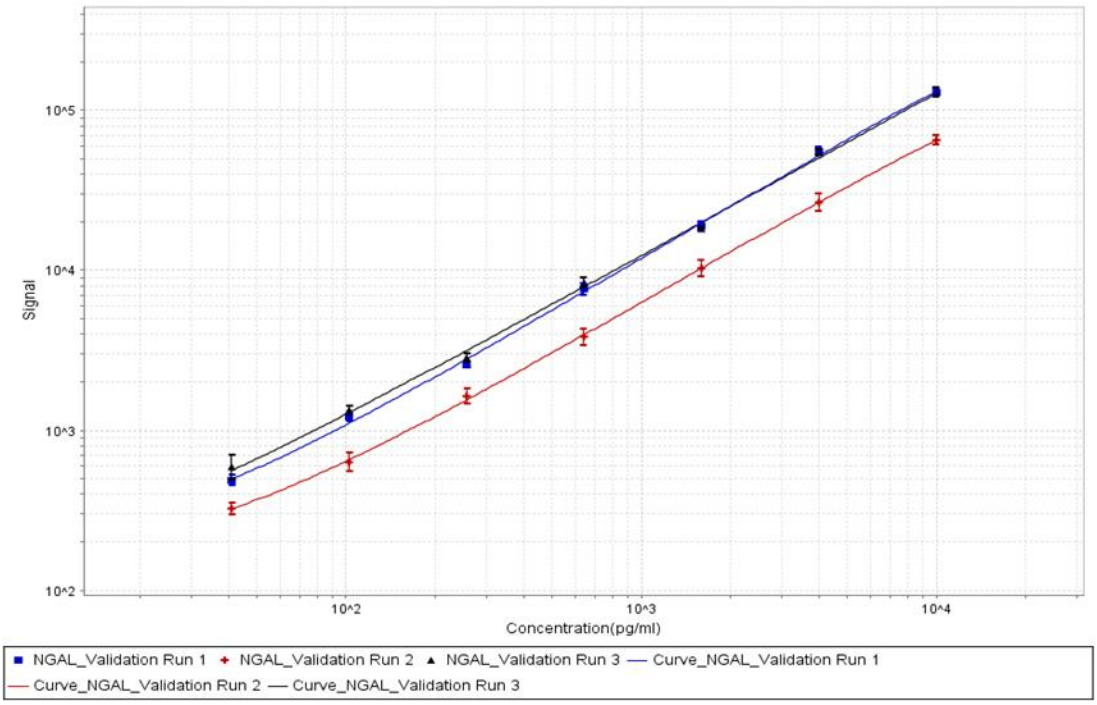


Figure 3.4 - NGAL Calibration Curves. NGAL calibration concentrations were 10000, 4000, 1600, 640, 256, 102.4 and 40.96pg/ml. Calibration curves were run in triplicate on three plates on different days. Calibration curves were generated using 4-parameter logistic curve fitting in MSD DISCOVERY WORKBENCH software (version 3.0). Results are mean plus or minus standard error.

Table 3.2 - NGAL Calibration Curve Accuracy and Precision.

Expected Concentration (pg/ml)	Calculated Concentration (pg/ml)			Accuracy (%)			Precision (CV%)		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
10000.00	9851.41	9999.94	10193.54	1.49	0.00	1.94	4.44	7.39	6.67
4000.00	4225.85	4011.67	4299.57	5.65	0.29	7.49	5.71	12.02	3.68
1600.00	1538.69	1598.69	1463.15	3.83	0.08	8.55	6.06	11.57	1.21
640.00	664.10	625.24	681.37	3.77	2.31	6.46	7.76	11.45	6.07
256.00	236.58	270.32	230.90	7.59	5.60	9.81	3.69	10.82	6.06
102.40	112.40	98.44	106.51	9.77	3.86	4.01	5.72	15.37	6.05
40.96	40.18	41.46	43.75	1.89	1.21	6.80	9.76	11.98	19.66

Calibration curves were run in triplicate on three plates on different days.
Results are mean accuracy and precision for each calibrator concentration on each plate.

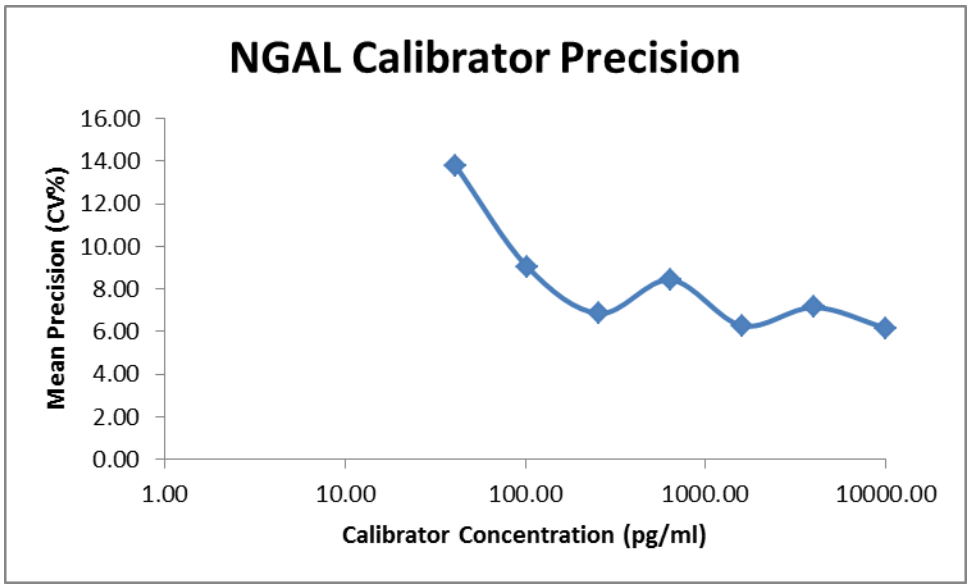


Figure 3.5 - NGAL Calibrator Precision Profile. NGAL calibration concentrations were 10000, 4000, 1600, 640, 256, 102.4 and 40.96pg/ml. Calibration curves were run in triplicate on three plates on different days. Results are mean precision for each calibrator concentration across the three plates.

3.3.1.2 Precision

Precision of the assays was assessed using validation samples. First, stock calibrator was diluted using MSD diluent 37 to give 3 different concentration solutions. Each of these was then used to dilute a urine sample from a healthy volunteer by a factor of 10 to give three

validation samples with different concentrations to cover the dynamic range of the assay. This was done for both KIM-1 and NGAL. With NGAL, a further 3 validation samples were prepared by diluting the urine sample by a factor of 100. The preparation of the validation samples was designed to closely reflect the preparation of study samples to be analysed on the assays, at the dilution factors to be used in practice. With this in mind, validation samples were not prepared fresh, but were prepared in advance, aliquoted and stored at -80°C, before being thawed, mixed and centrifuged on the day of analysis, as would be the case for study samples.

Precision was measured using six replicates per plate of each validation sample, run on three different plates on different days. Intra-assay precision was calculated using the coefficient of variation (CV) for each validation sample on each plate. Inter-assay precision was calculated using the CV of the mean value for each validation sample from each of the three plates. The regulations state that intra- and inter-assay precision should not exceed 20% (25% at the ULOQ and LLOQ) (European Medicines Agency, 2011).

Three standard concentrations of KIM-1 were prepared: 2500, 100 and 1pg/ml. These were used to dilute a healthy volunteer urine sample to give the high, mid and low validation samples. These samples were then run on three different plates on different days with six replicates each. The mean measured concentrations were 2417, 115.4 and 12.29pg/ml respectively for the high, mid and low validation samples. Intra- and inter-assay precision are given in Table 3.3. Intra-assay precision was less than 6% for all validation samples on all runs, and inter-assay precision was less than 7% for all validation samples.

Three standard concentrations of NGAL were prepared: 5000, 500 and 10pg/ml. These were used to dilute a healthy volunteer urine sample to give the high, mid and low validation samples at both 10- and 100-fold dilutions. These samples were then run on three different plates on different days with six replicates each. The mean measured

concentrations were 4884, 841 and 363pg/ml at 10-fold dilution, and 4728, 492 and 43.9pg/ml at 100-fold dilution for the high, mid and low validation samples, respectively. Intra- and inter-assay precision are given in Table 4. Intra-assay precision was less than 15% for all validation samples on all runs, and inter-assay precision was less than 13% for all validation samples.

Table 3.3 - KIM-1 intra- and inter-assay precision.

Validation Sample	Intra-assay Precision (CV%)			Inter-assay Precision (CV%)
	Run 1	Run 2	Run 3	
High	5.11	2.75	5.00	1.31
Mid	1.93	2.35	3.09	5.65
Low	3.57	3.36	4.40	6.01

Table 3.4 - NGAL intra- and inter-assay precision.

Validation Sample	Intra-assay Precision (CV%)			Inter-assay Precision (CV%)
	Run 1	Run 2	Run 3	
High (1in10)	3.65	10.70	7.16	0.72
Mid (1in10)	1.69	4.60	4.32	7.29
Low (1in10)	14.01	5.57	6.31	7.98
High (1in100)	3.94	6.42	8.76	3.50
Mid (1in100)	3.99	3.95	5.96	8.61
Low (1in100)	10.55	5.36	8.94	12.14

3.3.1.3 Accuracy

Accuracy of the assay was measured using recovery. Stock calibrator was diluted using MSD diluent 37 to give a spike sample with a concentration near the middle of the assay dynamic range. Spiked urine samples were prepared by diluting four different urine

samples from healthy volunteers by a factor of 10 with the spike. The urine samples, spike, and spiked urine samples were then run in triplicate on the same plate. Recovery was calculated by expressing the observed value of the spiked sample as a percentage of the expected result (calculated from the measured concentrations of the spike sample and the healthy volunteer samples). Recovery should be within 20% of the expected value (European Medicines Agency, 2011).

For KIM-1, a spike sample with measured concentration of 204.3pg/ml was prepared. Mean recovery was 105.3% (95% CI, 104.6-106.1%) (Figure 3.6). For NGAL, a spike sample with measured concentration of 1841pg/ml was prepared. Mean recovery was 91.8% (95% CI, 87.2-96.4%) (Figure 3.7).

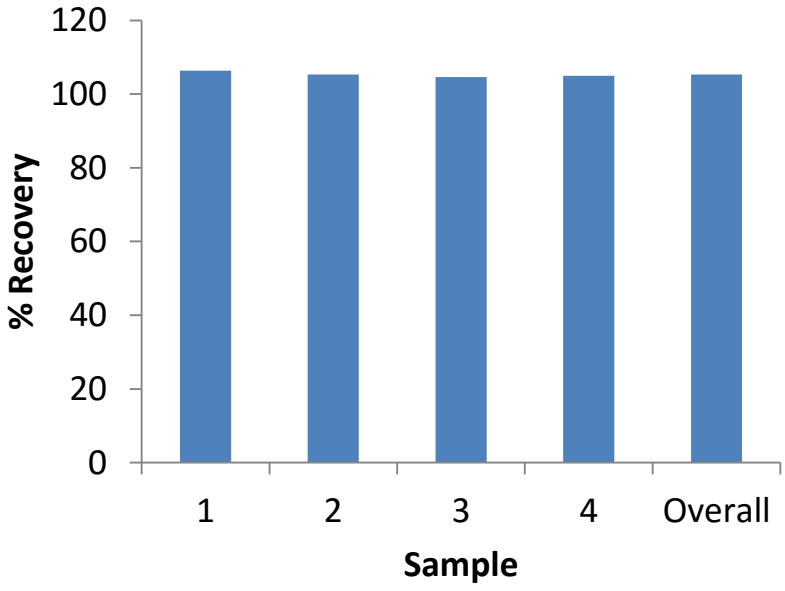


Figure 3.6 - KIM-1 Recovery. A spike sample with measured KIM-1 concentration of 204.3pg/ml was used to spike four urine samples. Unspiked urine samples, spike, and spiked urine samples were run in triplicate on the same plate. Recovery is the mean observed value of the spiked sample expressed as a percentage of the expected result.

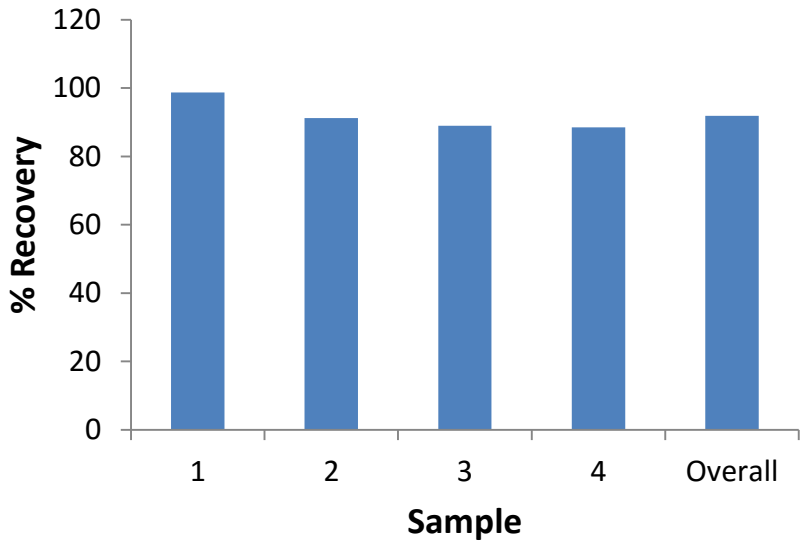


Figure 3.7 - NGAL Recovery. A spike sample with measured NGAL concentration of 1841pg/ml was used to spike four urine samples. Unspiked urine samples, spike, and spiked urine samples were run in triplicate on the same plate. Recovery is the mean observed value of the spiked sample expressed as a percentage of the expected result.

3.3.1.4 Dilutional Linearity

It was important to assess the dilutional linearity of urine samples on the assays in order for sample concentrations to lie within the dynamic range of the assays. Dilutional linearity assesses whether there is any interference caused by the diluents at different degrees of sample dilution. Eight urine samples were chosen to assess dilutional linearity; four samples from neonates with high known concentrations of the analyte, and four samples from healthy volunteers. These samples were diluted by factors of 10, 100 and 1000 using MSD diluent 37. Each sample at each dilution was run in triplicate on a single plate. Back-calculated concentrations for each dilution should be within 20% of the nominal concentration, and precision (measured using CV) should be less than 20% at each dilution (European Medicines Agency, 2011).

The results of the dilutional linearity for KIM-1 are presented in Figure 3.8. The back-calculated concentrations for each sample were within 20% of the nominal value for all

samples at all dilutions, except for samples 1, 2, 3 and 6 at a 1000-fold dilution. At this dilution these samples had concentrations below the LLOQ. At 10-and 100-fold dilutions, these samples were within the dynamic range of the assay. The CV was less than 20% for all samples at 10- and 100-fold dilutions. At 1000-fold dilution the CV was greater than 20% for samples 1 and 3, suggesting some loss of precision at the 1000-fold dilution.

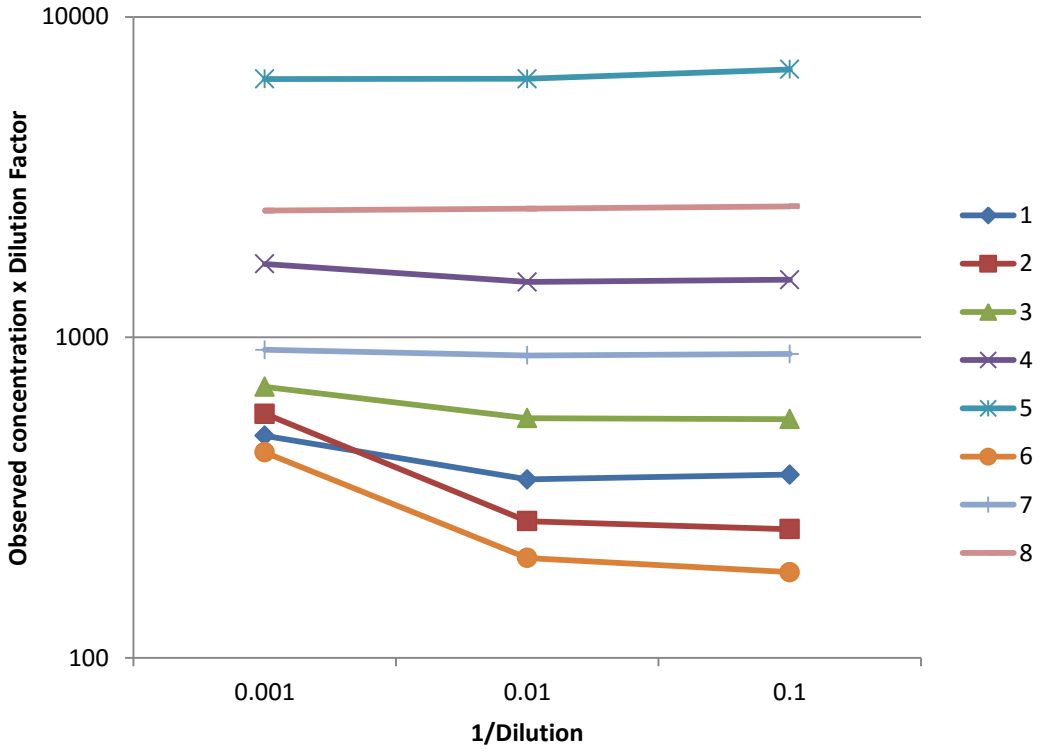


Figure 3.8 - KIM-1 Dilutional linearity. Eight urine samples were diluted by factors of 10, 100 and 1000 using MSD diluent 37. Each sample at each dilution was run in triplicate on a single plate. Results are mean observed concentration multiplied by the dilution factor.

The results of the dilutional linearity for NGAL are presented in Figure 3.9. The back-calculated concentrations for each sample were within 20% of the nominal value for all samples at all dilutions, except for samples 7 and 8 at a 10-fold dilution. At this dilution these samples had concentrations above the ULOQ. Dilution to 100-and 1000-fold brought these samples into the dynamic range of the assay. The CV was less than 20% for all

samples at 10- and 100-fold dilutions. At 1000-fold dilution, the CV was greater than 20% for samples 4 and 5, suggesting some loss of precision at the 1000-fold dilution.

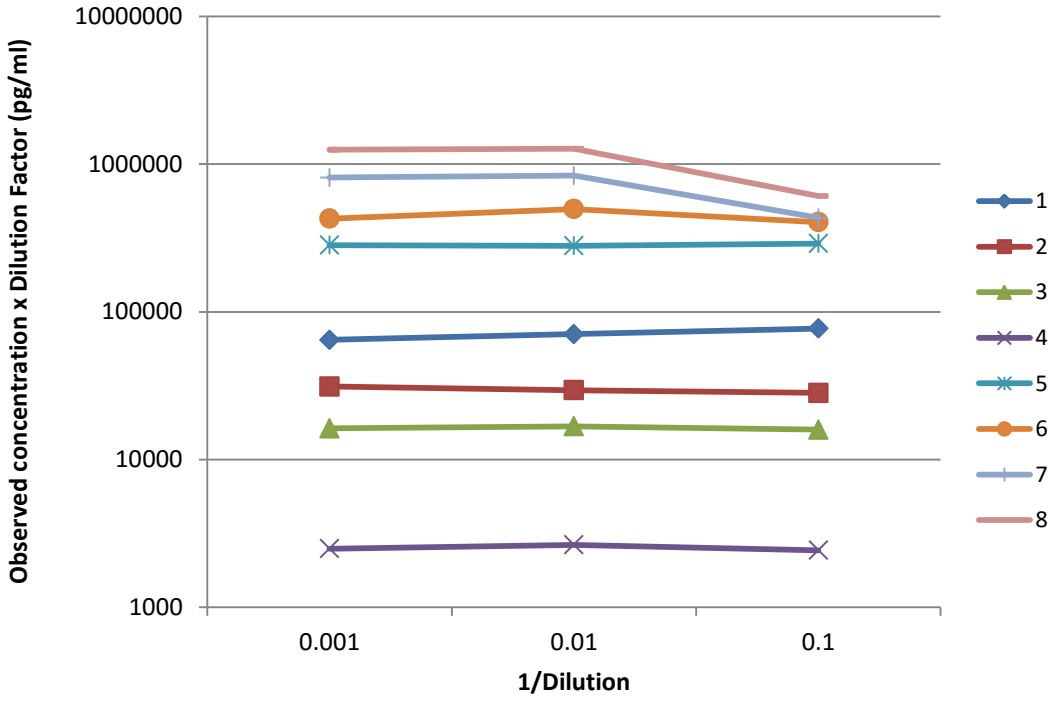


Figure 3.9 – NGAL dilutional linearity. Eight urine samples were diluted by factors of 10, 100 and 1000 using MSD diluent 37. Each sample at each dilution was run in triplicate on a single plate. Results are mean observed concentration multiplied by the dilution factor.

3.3.1.5 Summary

For KIM-1, the values for ULOQ and LLOQ on a 7-point calibration curve were 5000pg/ml and 1.22pg/ml respectively, with a LOD of 0.185pg/ml. Intra- and inter-assay precision were less than 6% and less than 7%, respectively. Mean recovery of spiked samples was 105.3% (95% CI, 104.6-106.1%), and dilutional linearity was shown at 1 in 10, 1 in 100 and 1 in 1000 dilutions in the assay diluent (except for four samples which fell below the LLOQ at a 1000-fold dilution).

For NGAL, the values for the ULOQ and LLOQ on a 7-point calibration curve were 10000pg/ml and 40.96pg/ml, respectively, with a LOD of 18.6pg/ml. Intra- and inter-assay precision were <15% and <13% respectively. Mean recovery of spiked samples was 91.8% (95% CI, 87.2-96.4%), and dilutional linearity was shown at 1 in 10, 1 in 100 and 1 in 1000 dilutions in the assay diluent (except for two samples which lay above the ULOQ at a 10-fold dilution).

3.3.2 Reference intervals

120 healthy children (64 male and 56 female) recruited in the UK provided at least one urine sample as part of the study. The mean age was 9.05 years (standard deviation (SD), 4.41), and 39 participants provided more than one sample. In parallel, one urine sample was collected from 171 healthy children (108 male and 63 female) recruited in the US. This cohort had a narrower age range (mean age 11.20 years, SD 2.49) than the UK cohort and was more ethnically diverse. Whilst we set out to recruit from birth to 16 years of age in the UK cohort, in practice only one child under one year of age was successfully recruited, and we have therefore presented reference intervals for children aged 1 year and above. In the US cohort 65 of 171 (38%) were patients with ADHD/ADD, and the remainder were controls. There were no differences identified between these two groups for KIM-1 or NGAL, suggesting there is not a significant effect of the diagnosis or of medication exposure on the urinary concentrations of these biomarkers. Table 3.5 summarises volunteer demographics for the two cohorts separately.

Table 3.5 - Demographic characteristics of UK and US Cohorts

Cohort Demographics	UK Cohort	US Cohort
Number in cohort	**120**	**171**
Males:Females	64:56	108:63
Age 0-4 years	27	-
Age 5-8 years	32	38*
Age 9-12 years	32	85
Age 13-16 years	29	48
Caucasian	108	79
African American	-	77
Other Ethnicity	12	15

* The US Cohort contained children age 7 to 16 years only.

Reference intervals for each biomarker were determined in both cohorts. For the UK cohort, participants with non-Caucasian ethnicity (n=12) were excluded from the calculation of reference ranges, and we were therefore unable to consider the effect of ethnicity in this cohort. For the US cohort, participants with ethnicity other than Caucasian or African-American (n=15) were excluded. The reference intervals are presented in Table 3.6, stratified by ethnicity and/or sex as appropriate, and then by age. The predicted quantile values reported reflect the value for the middle age within each age group.

Table 3.6 - Biomarker Reference Intervals by quantile regression.

Cohort & Biomarker	Number	2.5th Quantile (90% CI), ng/mg Cr	50th Quantile (90% CI), ng/mg Cr	97.5th Quantile (90% CI), ng/mg Cr	CI:RI ratio
UK KIM-1 (MSD) Caucasians, Male & Female	107				
- Age 1-4 years		0.08 (0.03, 0.12)	0.46 (0.38, 0.57)	2.39 (1.96, 2.91)	0.41
- Age 5-8 years		0.08 (0.03, 0.12)	0.46 (0.38, 0.57)	1.84 (1.62, 2.10)	0.27
- Age 9-12 years		0.08 (0.03, 0.12)	0.46 (0.38, 0.57)	1.42 (1.22, 1.65)	0.32
- Age 13-16 years		0.08 (0.03, 0.12)	0.46 (0.38, 0.57)	1.10 (0.87, 1.39)	0.51
UK NGAL (MSD) Caucasians, Females	48				
- Age 1-4 years		8.34 (3.14, 22.15)	10.49 (8.02, 13.72)	128.84 (54.03, 307.25)	2.10
- Age 5-8 years		3.84 (2.64, 5.60)	15.30 (12.78, 18.32)	143.95 (81.17, 255.29)	1.24
- Age 9-12 years		1.77 (0.95, 3.31)	22.31 (16.79, 29.66)	160.83 (104.23, 248.19)	0.91
- Age 13-16 years		0.82 (0.22, 3.01)	32.55 (20.41, 51.90)	179.70 (100.73, 320.58)	1.23
UK NGAL (MSD) Caucasians, Males	58				
- Age 1-16 years		1.33 (0, 1.89)	5.21 (4.66, 6.49)	64.59 (29.14, Inf)**	Inf
UK KIM-1 (Luminex) Caucasians, Male & Female	108				
- Age 1-16 years		0.03 (0.002, 0.04)	0.17 (0.15, 0.18)	0.63 (0.49, Inf)**	Inf
UK NGAL (Luminex) Caucasians, Females	50				
- Age 1-4 years		1.25 (0.11, 14.06)	63.43 (38.09, 83.93)	706.72 (247.84, 2015.28)	2.51
- Age 5-8 years		2.72 (0.92, 8.06)	63.43 (38.09, 83.93)	581.69 (297.53, 1137.25)	1.45
- Age 9-12 years		5.95 (2.41, 14.70)	63.43 (38.09, 83.93)	478.78 (327.30, 700.38)	0.79
- Age 13-16 years		13.00 (1.46, 116.09)	63.43 (38.09, 83.93)	394.08 (256.72, 604.94)	0.91
UK NGAL (Luminex) Caucasians, Males	58				
- Age 1-4 years		0.36 (0, 0.58)	6.30 (4.53-10.18)	229.90 (118.14, 447.38)	1.43

- Age 5-8 years		0.36 (0, 0.58)	6.30 (4.53-10.18)	180.51 (114.13, 285.51)	0.95
- Age 9-12 years		0.36 (0, 0.58)	6.30 (4.53-10.18)	141.73 (103.94, 193.27)	0.63
- Age 13-16 years		0.36 (0, 0.58)	6.30 (4.53-10.18)	111.28 (80.96, 152.95)	0.65
US KIM-1 (MSD) Caucasians, Male & Female	73				
- Age 7-8 years*		0.014 (0.007, 0.032)	0.23 (0.19-0.29)	0.79 (0.62, Inf)**	Inf
- Age 9-12 years		0.031 (0.022, 0.044)	0.23 (0.19-0.29)	0.79 (0.62, Inf)**	Inf
- Age 13-16 years		0.088 (0.051, 0.151)	0.23 (0.19-0.29)	0.79 (0.62, Inf)**	Inf
US KIM-1 (MSD) African American, Male & Female	65				
- Age 7-8 years*		0.038 (0.018, 0.077)	0.17 (0.13-0.19)	0.42 (0.34, Inf)**	Inf
- Age 9-12 years		0.048 (0.035, 0.066)	0.17 (0.13-0.19)	0.42 (0.34, Inf)**	Inf
- Age 13-16 years		0.067 (0.043, 0.106)	0.17 (0.13-0.19)	0.42 (0.34, Inf)**	Inf
US NGAL (MSD), Females	55				
- Age 7-8 years*		1.30 (0, 4.07)	19.30 (12.36, 30.14)	58.26 (42.06, 80.70)	0.68
- Age 9-12 years		1.30 (0, 4.07)	35.38 (28.40, 44.09)	96.95 (82.72, 113.64)	0.32
- Age 13-16 years		1.30 (0, 4.07)	79.40 (49.91, 126.32)	191.21 (137.58, 265.74)	0.67
US NGAL (MSD), Males	90				
- Age 7-8 years*		1.34 (0, 1.50)	3.10 (2.55, 3.77)	8.28 (6.34, 10.81)	0.64
- Age 9-12 years		1.34 (0, 1.50)	4.23 (3.63, 4.95)	10.38 (8.83, 12.20)	0.37
- Age 13-16 years		1.34 (0, 1.50)	6.42 (4.56, 9.03)	14.02 (10.67, 18.42)	0.61

95% Reference Intervals (2.5th and 97.5th quantiles) along with median values (50th quantile) and their 90% confidence intervals (CI) were estimated using quantile regression methodology. Reference intervals are reported by cohort (UK or US), biomarker (KIM-1 or NGAL) and analytical method (MSD or Luminex). Where biomarker concentrations were significantly associated with sex or ethnicity ($p<0.05$) reference intervals are presented by these partitions. We report quantile values for the middle age within each age group. A ratio of the 90% confidence interval for the 97.5th quantile to the 95% reference interval (CI:RI ratio) is given. * The US Cohort contained children age 7 to 16 years only. **For reference intervals where there was no significant association with age, it was not possible to calculate upper limits for the confidence intervals due to small sample size and the extreme percentile.

Using MSD, there was a significant difference ($p<0.001$) in the mean urinary KIM-1 concentration between the two cohorts (UK (MSD); 0.43ng/mg Cr, 95% Confidence Interval (CI), 0.37-0.50ng/mg Cr. US (MSD); 0.18ng/mg Cr, 95% CI, 0.16-0.20ng/mg Cr). However, there was no difference between the mean NGAL concentrations (UK (MSD); 10.71ng/mg Cr, 95% CI, 8.76-13.11ng/mg Cr. US (MSD); 8.19ng/mg Cr, 95%CI, 6.80-9.86ng/mg Cr).

Within both cohorts, urinary KIM-1 concentrations were not significantly associated with sex. However, we consistently identified a significant difference ($p<0.0001$) in mean NGAL concentration between males and females in both cohorts (UK males (MSD); 6.55ng/mg Cr, 95% CI 5.16-8.32ng/mg Cr. UK females (MSD); 19.18ng/mg Cr, 95% CI, 14.63-25.14ng/mg Cr. US males (MSD); 3.95ng/mg Cr, 95% CI, 3.53-4.42ng/mg Cr. US females (MSD); 28.56ng/mg Cr, 95% CI, 22.09-36.93ng/mg Cr).

The US cohort was more ethnically diverse, with 77 subjects of African American descent, 79 Caucasians, and 15 of dual heritage (although these 15 were excluded from the analysis). Mean NGAL concentrations were not significantly associated with ethnicity. However, for KIM-1 a significant difference was found between Caucasian and African American ethnic groups (Caucasians (MSD); 0.21ng/mg Cr, 95% CI, 0.17-0.26ng/mg Cr. African Americans (MSD); 0.16ng/mg Cr, 95% CI, 0.13-0.18ng/mg Cr; $p=0.02$).

Using MSD, we assessed intra-individual variability in the UK cohort in 39 individuals who provided one morning sample each week for up to four weeks. KIM-1 was less variable within individuals than NGAL (KIM-1 Intra-individual S.D; 0.16ng/mg Cr, 95% CI, 0.12-0.22ng/mg Cr. NGAL Intra-individual S.D; 3.84ng/mg Cr, 95% CI, 2.57-5.74ng/mg Cr). Diurnal effects were assessed using paired urine samples collected in the evening and the following morning in the UK cohort. For KIM-1 ($n=35$) there was a significant diurnal

variation (p<0.001) with lower mean concentration in the evening (0.47ng/mg Cr, 95% CI 0.36-0.62ng/mg Cr) compared to the morning (0.77ng/mg Cr, 95% CI, 0.61-0.97ng/mg Cr). No diurnal effect was seen for NGAL (n=36, Mean evening concentration; 7.35ng/mg Cr, 95% CI, 4.96-10.89ng/mg Cr. Mean morning concentration; 6.77ng/mg Cr, 95% CI, 4.85-9.46ng/mg Cr), p=0.36.

Reference intervals for urinary creatinine were calculated as above utilising a quantile regression approach, and are given in Table 3.7. Both the 2.5th and 97.5th quantiles show a significant increase with age.

Table 3.7 - Reference Intervals of Urinary Creatinine.

Reference Interval	Number	2.5th Quantile (90% CI), ng/mg Cr	50th Quantile (90% CI), ng/mg Cr	97.5th Quantile (90% CI), ng/mg Cr	CI:RI ratio
Urinary Creatinine (mg/ml)	106				
- Age 1-4 years		0.47 (0.19, 0.74)	0.71 (0.54, 0.93)	1.59 (1.21, 2.09)	0.78
- Age 5-8 years		0.69 (0.53, 0.86)	0.97 (0.81, 1.16)	2.00 (1.70, 2.36)	0.51
- Age 9-12 years		0.92 (0.80, 1.04)	1.32 (1.12, 1.56)	2.52 (2.24, 2.84)	0.38
- Age 13-16 years		1.15 (0.97, 1.34)	1.80 (1.41, 2.31)	3.17 (2.63, 3.81)	0.59

95% Reference Intervals (RI) with 90% Confidence Intervals (CI) of the Upper Limit for urinary creatinine in the UK cohort were derived using quantile regression methodology.

Differences between the MesoScale Discovery (MSD) and Luminex methods of biomarker analysis were assessed. For both markers, there was an increase in the difference in biomarker value as the magnitude of the measurement increased. A regression model was used, first to regress the difference between the methods on the average of the two methods, and then to regress the values of the residuals (Figure 3.10). Using this approach the difference (D[KIM-1], pg/ml) between the two methods for KIM-1 (Luminex minus MSD) is described by the equation D[KIM-1] = -105.23 + 1.3124*A[KIM-1], where A[KIM-1] is the mean of the 2 methods (in pg/ml). 95% Limits of Agreement are given by the

equation D[KIM-1] $\pm$ 1.96*216.7. The difference (D[NGAL], pg/ml)) between the two methods for NGAL (Luminex minus MSD) is described by the equation D[NGAL] = -12.894 + 1.2006*A[NGAL], where A[NGAL] is the mean of the 2 methods(in pg/ml). 95% Limits of Agreement are given by the equation D[NGAL] $\pm$ 1.96*65.44.

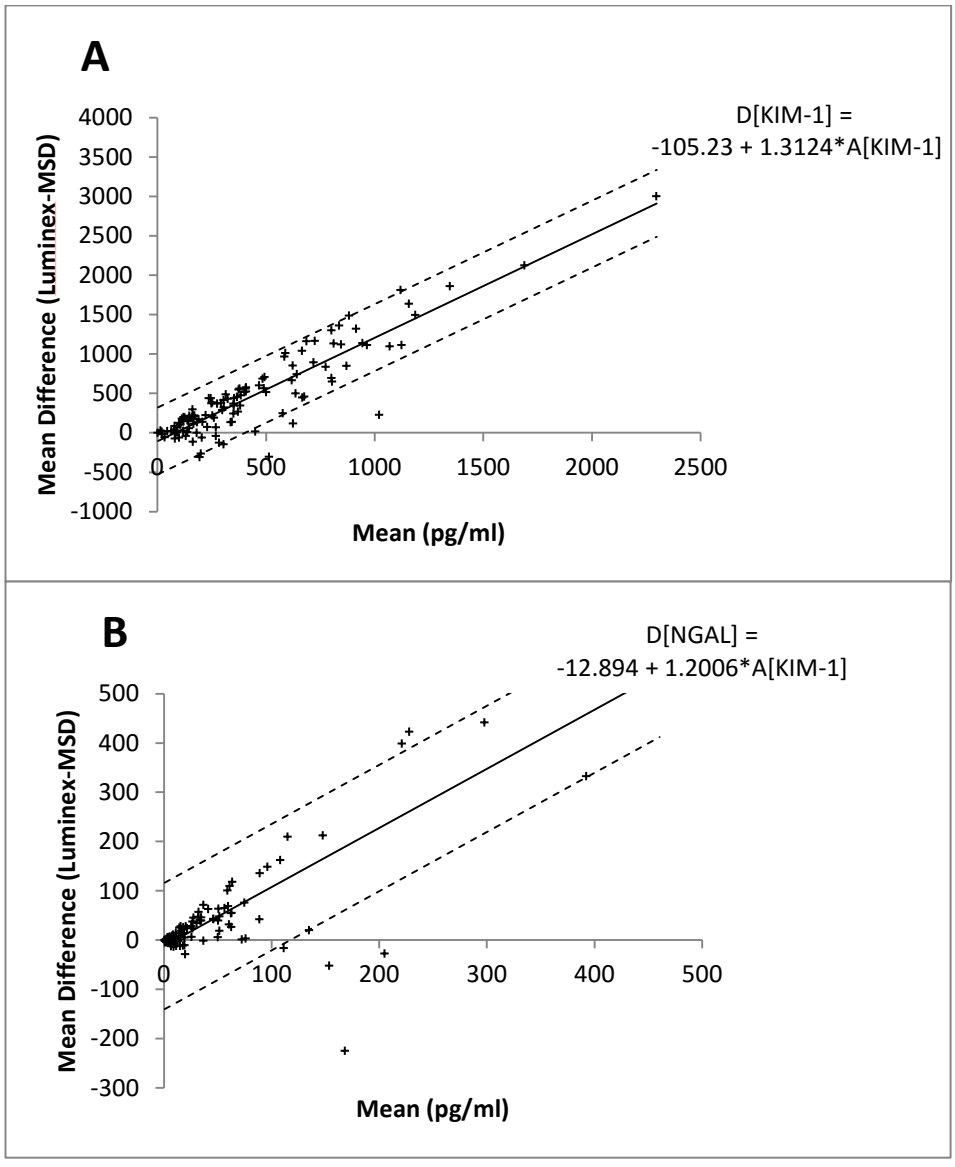


Figure 3.10 - Agreement of MSD and Luminex methods for analysis of urinary KIM-1 (A) and NGAL (B). Regression models describing the differences between the Luminex and MesoScale Discovery (MSD) methods of biomarker analysis. The regression line is given using a solid line, and the upper and lower 95% limits of agreement with dashed lines.

3.4 Discussion

An important aspect for the rolling qualification of novel urinary biomarkers and for their eventual use in man is that independent and robust methodologies are developed for their quantification. Here we present validation of electrochemiluminescent assays for KIM-1 and NGAL using sandwich immunoassays from MSD. When run using the protocol and calibration concentrations described, both assays met the standards set by both the FDA (US Food and Drug Administration, 2001) and EMA (European Medicines Agency, 2011) and can be considered to be fit-for-purpose (Lee et al., 2006).

One published study has reported validation of a multiplex MSD assay which included KIM-1 and NGAL alongside cystatin c and IL-18, and measured these biomarkers in urine samples from adults (Zhang et al., 2013). Despite the differences between this multiplex assay and the two separate assays validated here, there is similarity in performance including inter- and intra-assay precision, accuracy of spike-recovery, and the LLOQ and LOD (Zhang et al., 2013).

We have not looked at sample pH and its effect on the performance of the MSD assay. This has been demonstrated to be important for another commercially available KIM-1 assay (human tim-1/kim-1/Havcr Elisa kit, R&D systems, UK), and adjustment of all samples to neutral pH has been suggested after thawing and before analysis using this assay (Pennemans et al., 2010). We have also not assessed the stability of KIM-1 or NGAL under different storage conditions. For KIM-1, an analysis of storage conditions suggested that adding protease inhibitors or centrifuging prior to storage was unnecessary, and that samples are most stable when frozen at -80˚C within 3 hours of collection and thawed within an hour of analysis (Pennemans et al., 2012). KIM-1 appears to be reasonably stable

at other storage temperatures for short periods of time (Pennemans et al., 2012, Chaturvedi et al., 2009). There is a difference of opinion as to whether KIM-1 may resist degradation with repeated freeze-thaw cycles (Chaturvedi et al., 2009, Pennemans et al., 2012). Furthermore, we have not assessed potential assay interference from medications or endogenous substances which may be present in the urine, although in a murine KIM-1 assay interference was not demonstrated with a range of substances (Sabbisetti et al., 2013). Further validation would seek to address these limitations.

Further validation of the MSD assays was provided by a comparison with quantification of KIM-1 and NGAL via Luminex-based technologies. Urine samples were analysed from the UK cohort of healthy children using assays for both KIM-1 and NGAL. Although absolute differences in biomarker values were observed, there remained strong agreement between both independent analytical platforms. The reason for the absolute differences in biomarker values between the two assays is not completely clear, but may be due to differences in the antibodies used and their affinities for the biomarker of interest, or possibly to the differences in the analytical detection method.

The International Federation of Clinical Chemistry (IFCC) (Hyltoft Petersen and Rustad, 2004) recommends that samples from 120 or more individuals are required for the development of reference intervals for analytes. During the course of this investigation, sufficient healthy volunteers were recruited to surpass these guidelines in both independent cohorts; however, larger numbers would improve the confidence of the reference intervals (RI) we have derived (recommended CI:RI ratio is less than 0.2) (Daly et al., 2013).

An understanding of paediatric renal development suggests that a single reference interval is unlikely to be appropriate for the entire paediatric age span. We therefore felt it was

important to present reference intervals by partitions, where they are significant, in order to give a true picture of our data. However, partitioning the group by sex or ethnicity leads to a loss of confidence, and, in some cases, to very wide confidence intervals. This is a limitation of the study design, but was a necessary balance against the difficulty, length of time and investment that would be required for a much larger study.

Whilst the initial strategy was designed to recruit from birth to 16 years of age in the UK cohort, in practice only one child under one year of age was successfully recruited, and therefore, data are presented regarding reference intervals for children aged 1 year and above. Using the Luminex assay, the mean baseline values in the cohort of preterm neonates were 1.91ng/mg Cr (95% CI 1.07, 2.76) for KIM-1 and 425.4 ng/mg Cr (162.6, 688.3) for NGAL. The baseline figure for KIM-1 lies significantly above the upper limit of the Luminex reference interval (0.63ng/mg Cr), whereas that for NGAL is comparable (upper limit for females is 706.72ng/mg Cr and for males is 229.90ng/mg Cr at 1-4 years). The data and experiences from this current investigation could therefore be used to appropriately design a further investigation to develop useful reference intervals for neonates/infants which take into account the rapid maturation and changes in renal function that occur during this period.

The exclusion criteria for both cohorts were minimal. This was deliberate in order to maximize recruitment to a cohort that would most accurately represent the wider paediatric population. For instance, obesity may be a risk factor for CKD in children (Gunta and Mak, 2013). However, not all children with obesity have CKD. Therefore obese children were not excluded, unless they had known renal impairment, as it was felt that because obesity is common, the resulting cohort would not reflect the wider population.

We normalised biomarker values to urinary creatinine, but urinary creatinine concentration

itself demonstrates age-related changes (Table 3.7) (Barr et al., 2005, Martin et al., 2008), and there is ongoing debate as to the most appropriate standard (Goldstein, 2010, Waikar et al., 2010).

In both cohorts, there was a significantly higher mean NGAL concentration in females compared to males. This gender difference has also been observed in adults (Cullen et al., 2012, Zhang et al., 2013), children (Pennemans et al., 2013), and in very low birthweight infants (Huynh et al., 2009, Askenazi et al., 2011a), although the reasons for the difference remain unclear. One recent study in children, using a different NGAL assay, did not demonstrate this gender difference, although the NGAL values they report are similar to those we found with the MSD assay (Cangemi et al., 2013).

The statistical analysis of both biomarkers showed that urinary concentration was related to age, as has been previously reported (Pennemans et al., 2013). However, age-related changes were not consistent for each biomarker across both cohorts and analytical platforms, and these results must be interpreted with caution.

Significant differences in mean values of KIM-1 were observed between the two cohorts. Factors contributing to this difference may be ethnicity and the time of day of sample collection. In the UK cohort reference intervals were only calculated in Caucasians, whereas the US cohort included both Caucasians and African Americans. KIM-1 concentration was lower in African Americans than Caucasians. Ethnic differences in expression of other renal proteins have been reported, and this is likely related to genetics (Joseph et al., 2015). We have shown, for the first time, diurnal variation in KIM-1 concentration with lower values in the evening. Diurnal variation in expression of other proteins in the kidney, under the control of circadian clock genes, has been previously reported (Rohman et al., 2005). The reference intervals for the UK cohort are based on samples collected in the morning,

whereas the US samples were collected at varying times of day. Furthermore, urine samples for the UK study were spot collections, whereas in the US cohort they were 4 hour collections, although it is unclear whether this would make a difference.

In conclusion, we have reported reference intervals for two urinary renal injury biomarkers, KIM-1 and NGAL, for the first time in two independent healthy paediatric populations, and have shown that they can be robustly quantified on two independent analytical platforms with a high degree of agreement. This represents a key step in the qualification of these two biomarkers for use in children.

4 Urinary biomarkers of aminoglycoside-induced nephrotoxicity in children with cystic fibrosis

4.1 Introduction

Children with cystic fibrosis (CF) are predisposed to secondary bacterial lung infections and pulmonary colonisation, often by resistant organisms, in particular *Pseudomonas aeruginosa*. The aminoglycosides have good efficacy against *P. aeruginosa* and are commonly used to treat pulmonary exacerbations in CF, usually in combination with a beta lactam antibiotic (Ramsey, 1996). Whilst this approach leads to improved patient outcomes, the use of aminoglycosides does introduce the possibility of nephrotoxicity. Episodes of AKI are described in CF related to acute aminoglycoside exposure (Bertenshaw et al., 2007, Smyth et al., 2008). Chronic renal impairment related to cumulative aminoglycoside exposure has also been reported in 31-42% of adult CF patients (Al-Aloul et al., 2005a) (see Chapter 1 for a full discussion).

Measures exist to minimise the potential for aminoglycoside-induced nephrotoxicity, including extended-interval dosing (once daily), drug level monitoring, and monitoring of renal function using serum creatinine and blood urea nitrogen. However, these traditional markers of renal function require invasive blood tests, and, as has previously been discussed (Chapter 1), the information they provide is limited. Therefore, to identify children who are at increased risk of renal impairment, there is a need for improved biomarkers that reflect proximal tubule toxicity at an early stage. This would, in turn, allow for treatment adjustment and the avoidance of further injury.

Children with CF represent a patient group with much to gain from the development of improved biomarkers of aminoglycoside-induced nephrotoxicity. There is potential to

improve both short and long-term renal outcomes in this group of patients. There are currently limited data investigating the utility of novel renal biomarkers in assessing nephrotoxicity in children with CF. We undertook this study to address this issue, with the following questions:

1. In children with CF, is there evidence of a reduction in estimated glomerular filtration rate (eGFR) or chronic elevation in urinary biomarkers associated with cumulative lifetime aminoglycoside exposure?
2. What is the relationship between biomarker values and age, sex and eGFR?
3. In children with CF, is there evidence of acute elevations in urinary biomarkers during treatment with tobramycin?

We hypothesised that, due to the reported utility of these biomarkers from preclinical data, and our findings in a neonatal cohort (Chapter 2), elevations in urinary KIM-1 and NGAL would be detected in the absence of serum creatinine changes during exposure to tobramycin. We also hypothesised that eGFR would show a reduction associated with cumulative aminoglycoside exposure, but that there would be no association between baseline urinary biomarker concentration and previous exposure.

4.2 Methods

4.2.1 Patient recruitment and sample collection

Eligible children were recruited between May 2012 and May 2013 through paediatric CF clinics at ten hospital sites in the north west of England and Wales. Individuals were eligible if they were aged between 0 and 20 years of age (some patients with CF will continue to be seen in a paediatric CF clinic beyond the age of 16) and had a confirmed diagnosis of CF (established by sweat test or genotype). There were no exclusion criteria. The URBAN CF

study (URinary Biomarkers of Aminoglycoside-induced Nephrotoxicity in children with Cystic Fibrosis) received ethical approval from the National Research Ethics Service (NRES) Committee Northwest – Liverpool East, UK. It was registered on the UK Clinical Research Network portfolio (UKCRN 11815) and received support from the Medicines for Children Research Network (MCRN). The study was conducted in accordance with the Declaration of Helsinki. Children with cystic fibrosis and their parents/guardian were approached about the study in the CF outpatient clinics by a member of the research team. Informed written consent was obtained from carers or guardians on the behalf of the minors/children involved in our study. Those participants aged 16 years or above were able to consent for themselves.

Each subject was asked to provide a urine sample at the CF clinic on the day of recruitment, and then on each subsequent clinic visit for the duration of the study. Follow-up was for a minimum of 12 months and a maximum of 24 months. If subjects received one or more courses of treatment with IV tobramycin during this period, urine samples were collected regularly during the treatment course. CF patients can be offered IV treatment as an inpatient or at home, because practice varies widely between sites, and criteria are not consistent. However, it is unlikely that a patient would be treated at home if they were very unwell. The study procedures are outlined below.

In subjects receiving a course of tobramycin treatment as an inpatient or at home with daily children's community nurse visits, a baseline urine sample was collected from the patient on the day of, but prior to commencing, the course of treatment with tobramycin. Further urine samples were collected each day, for the duration of the tobramycin treatment course. A further sample was collected 5-10 days after completion of the treatment course.

In subjects receiving a course of tobramycin treatment at home without daily community nurse visits, a baseline urine sample was collected from the patient on the day of, but prior to commencing, the course of treatment with tobramycin (day 1). Further samples were collected when the patient had their routine monitoring blood tests done. These usually occurred on day 2 (after the first dose of tobramycin) and on day 9 (after the 8th dose of tobramycin). However, some patients had more frequent monitoring blood tests, or not on the days specified, and urine samples were collected on each day that monitoring blood tests were done during the course of tobramycin. Another urine sample was collected on the final day of the course of tobramycin treatment (usually day 14, but courses varied in length). A further sample was collected 5-10 days after completion of the treatment course.

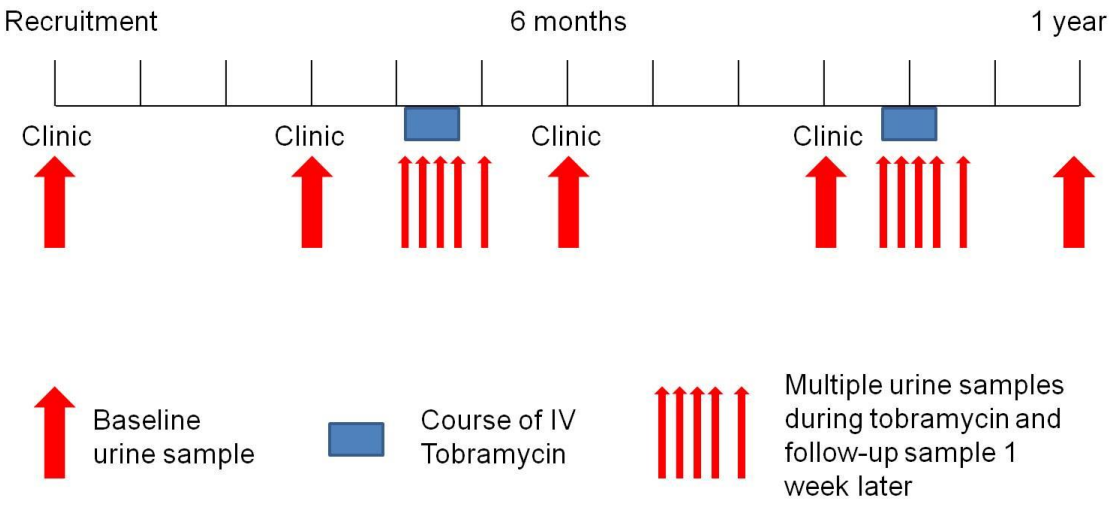


Figure 4.1 – Example patient journey in the URBAN CF study demonstrating routine urine samples collected at clinic visits, and increased frequency of sampling during exposure to IV tobramycin.

Urine samples were collected from each child by an appropriate method dependent on the age of the child. The normally preferred method was a clean catch urine sample into a sterile container. In younger children, samples were collected by placing cotton wool balls into the nappy. Samples were transferred to (if not already collected in) a sterile container

and then transported to the local hospital laboratory. Here samples were centrifuged at 2000g for 4min, and then the supernatant was aliquoted and stored at -80°C.

No additional blood samples or investigations were performed as part of this study. Results of blood investigations done as part of routine clinical care were recorded for each patient. These included serum urea & creatinine, and serum tobramycin levels (all measured in local hospital laboratories).

Study data were collected in paper case report forms, and was then entered into a secure electronic database. Baseline demographic details, including sex, age, and ethnicity, as well as the date of CF diagnosis and CF genotype, were recorded at baseline. Height and weight were measured at baseline, and then at each study visit. Past medical history (including CF related diabetes, ototoxicity or renal disease) and cumulative lifetime exposure to aminoglycosides and colistin was documented from the clinical notes at baseline. Concomitant medications were recorded at each visit.

4.2.2 Determination of urinary biomarkers

Collected urine samples were thawed, mixed and centrifuged (3000rpm, 5min). Biomarker measurements were performed on the resulting supernatants. Urinary KIM-1 and NGAL were measured using the validated electrochemiluminescent assays (Meso Scale Discovery, US) (McWilliam et al., 2014). Urine samples were run in duplicate at a dilution of 1 in 10 in MSD Diluent 37, and repeated at a dilution of 1 in 100 if they remained too concentrated. Biomarker values were normalised to urinary creatinine (uCr) which was determined spectrophotometically as previously described (Waikar et al., 2010). Normalised urinary biomarker values are presented as ng/mg Cr. Laboratory analysis was blinded to participants' clinical characteristics.

4.2.3 Baseline biomarkers

The biomarker values measured in the first urine sample provided by each participant as part of the study were designated as the baseline values for this analysis. KIM-1 and NGAL were measured as described above, and corrected to urinary creatinine. Participants were not receiving intravenous aminoglycoside at the time of this baseline sample. The baseline serum creatinine value was the most recent serum creatinine concentration (µmol/l) recorded before the time of recruitment to the study.

Estimated GFR (eGFR) was calculated from baseline serum creatinine using the Schwartz formula (Schwartz et al., 1976b) following the methodology of Andrieux *et al.* (Andrieux et al., 2010):

$$eGFR\ (Schwartz) = [k * height\ (cm)]\ /\ plasma\ creatinine\ (\mu mol/l)$$

Where k = 40 under 2 years, k = 49 from 2 to 13 years, and above 13 years k = 62 for males and 49 for females.

4.2.4 Sample size

Due to the unavailability of applicable pilot data, it was not possible to undertake power calculations in advance. Our target sample size was 160, estimated using data from a clinical feasibility survey at Alder Hey Children's Hospital, Liverpool, UK. Based on this survey, it was anticipated that if 160 children with CF were recruited, 40 or more would receive at least one course of treatment with tobramycin during the study period.

4.2.5 Statistical analysis

Statistical analysis was completed with in collaboration with Dr A Jorgensen, Department of Biostatistics, University of Liverpool.

All statistical analyses were undertaken in R version 3.2.0 (R Development Core Team, 2011). To explore the association between age, gender, previous exposure to IV

aminoglycoside and eGFR and baseline biomarker values, a multiple linear regression model was fitted with these variables as covariates and log-baseline biomarker value as outcome. Stepwise variable selection was applied to achieve a final regression model ($p<0.05$ significance threshold). For serum creatinine, eGFR was excluded as a covariate as it is used in its calculation. A multiple linear regression model was also used to test for association between age, gender, previous exposure to IV aminoglycoside, baseline KIM-1 and baseline NGAL and eGFR, again with stepwise variable selection applied and assuming a threshold of $p<0.05$ for statistical significance.

For all variables retained in each of the final models, the r-squared value was extracted to allow estimation of the proportion of variability in outcome explained by the variable.

To explore the effect of aminoglycoside exposure on KIM-1 and NGAL levels, profile plots were prepared showing variability per individual across time. A mean line was also fitted using a locally weighted regression (lowess) to demonstrate overall trend.

4.3 Results

4.3.1 Demographics

Table 4.1 Baseline characteristics of children with CF recruited to the URBAN CF study

Demographic		URBAN CF cohort (n=158)
Age (years) [mean (range)]		7.4 (0.04 to 17.7)
Sex [number (%)]	Male	78 (49.7)
	Female	80 (50.3)
Ethnicity [number (%)]	White	156 (98.7)
	Other	2 (1.3)
Genotype [number (%)]	Homozygous DF508	84 (53.2)
	Heterozygous DF508	63 (39.9)
	Other	10 (6.3)
Height (cm) [mean (range)]		117.6 (41 to 181.5)
Weight (kg) [mean (range)]		26.5 (3.88 to 72.9)
Previous exposure to IV aminoglycoside [number (%)]		86 (54.4)
Previous exposure to colistin [number (%)]		91 (57.6)
Previous ototoxicity [number (%)]		2 (1.3)
Existing renal disease [number (%)]		2 (1.3)
Existing CF related diabetes [number (%)]		4 (2.5)

A total of 158 children with CF were recruited to the study. Summary characteristics are presented in

Table 4.1. Thirty-seven of the 158 patients (23.4%) received at least one course of treatment with IV tobramycin during the period of follow-up in the study. Seven patients

withdrew consent during the follow-up period, seven transferred to adult services, and one died. Data for all participants was included up to the point of withdrawal of their consent.

4.3.2 Associations with baseline biomarkers

In the multiple regression model, log-baseline KIM-1 was only associated with the number of previous courses of IV aminoglycoside ($p<0.0001$; $R^2=0.11$; $\beta=0.03$ (95% CI=0.02, 0.05). A scatterplot of log-baseline KIM-1 against number of previous courses (Figure 4.2A) shows that log KIM-1 levels increase with increased previous exposure. For log-baseline NGAL, only gender was found to be statistically significant with males having lower values ($p=0.02$; $R^2=0.03$; $\beta=-0.48$ (95% CI=-0.89,-0.07)) (Figure 4.3D). In the case of serum creatinine, age ($p<0.00001$; $R^2=0.48$; $\beta=0.05$(95% CI=0.04, 0.06) remained in the multiple regression model and so was found to be significantly associated with log-baseline creatinine. A scatterplot (Figure 4.4B) shows that log-baseline creatinine increases with age. As expected, number of courses of aminoglycoside is correlated with age (Pearson's correlation coefficient=0.51). However, the number of aminoglycoside courses was also shown to be associated with KIM-1 independently of the effect of age, since both variables were included as potential covariates in the multiple regression model. In terms of log-baseline eGFR, only age ($p=0.0004$; $R^2=0.11$; $\beta=0.02$ (95% CI=0.01,0.03) was retained in the final model (Figure 4.5).

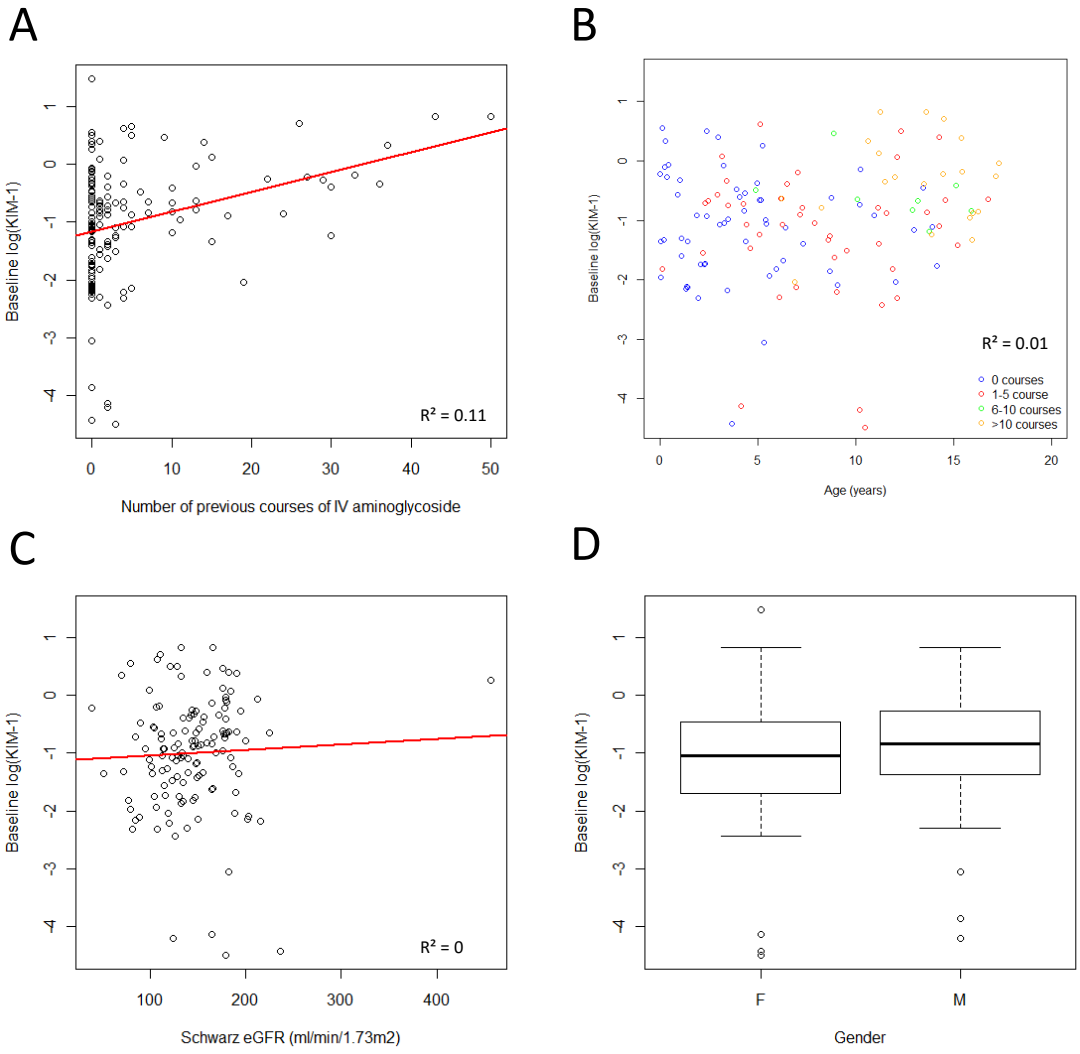


Figure 4.2 – Baseline urinary KIM-1 values. The baseline values are the corrected concentrations (ng/mg Cr) from the first urine sample provided by each participant as part of the study. The log of biomarker value is plotted against number of previous courses of intravenous aminoglycosides (A), age (B, stratified by number of previous courses), Schwartz eGFR (C) and gender (D). Adjusted R-squared values from the univariate regression model are provided. For the box and whisker plots, the bottom and top of the box represent first and third quartile respectively, while the darker line in the box is the median, and outliers are represented by the small circle points.

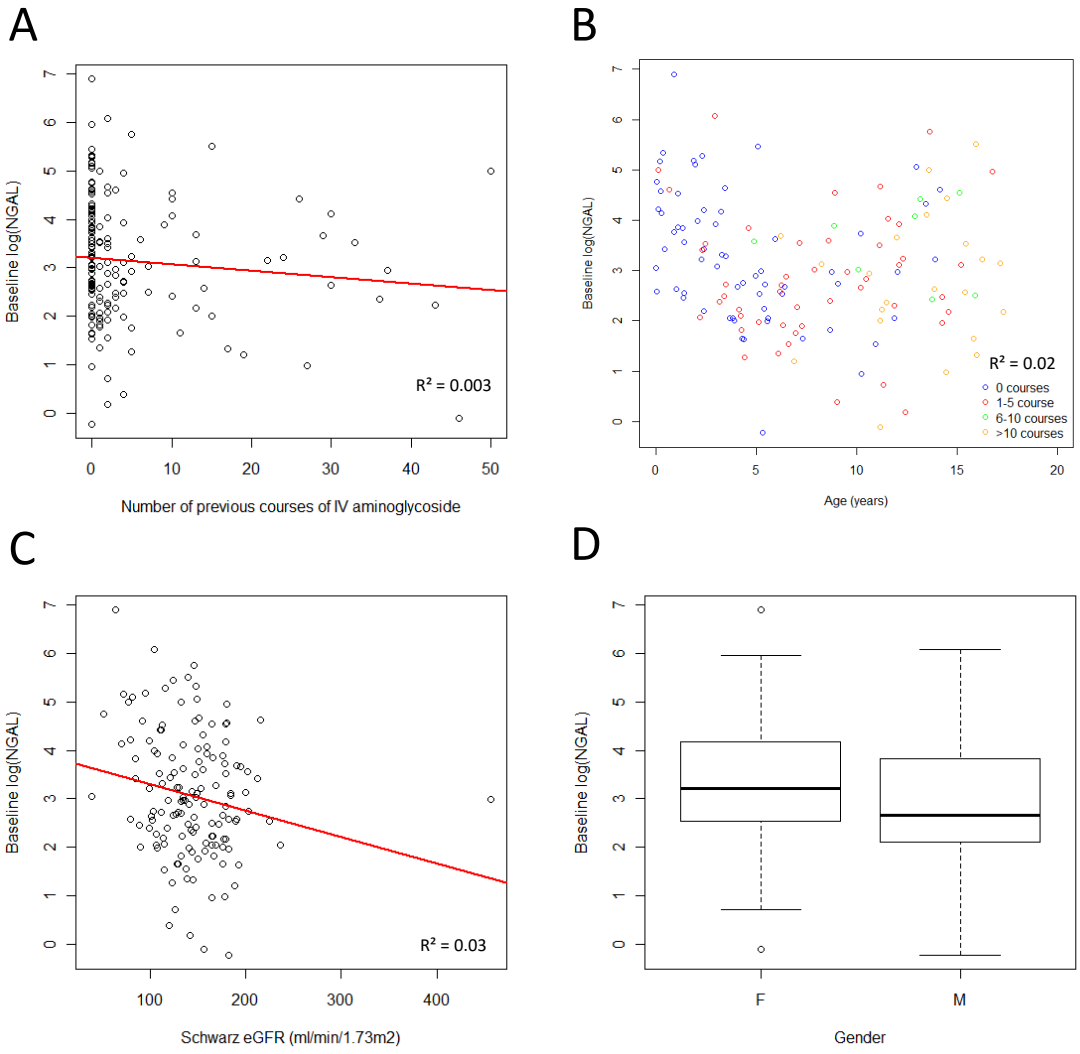


Figure 4.3 – Baseline urinary NGAL values. The baseline values are the corrected concentrations (ng/mg Cr) from the first urine sample provided by each participant as part of the study. The log of biomarker value is plotted against number of previous courses of intravenous aminoglycosides (A), age (B, stratified by number of previous courses), Schwartz eGFR (C) and gender (D). Adjusted R-squared values from the univariate regression model are provided. For the box and whisker plots, the bottom and top of the box represent first and third quartile respectively, while the darker line in the box is the median, and outliers are represented by the small circle points.

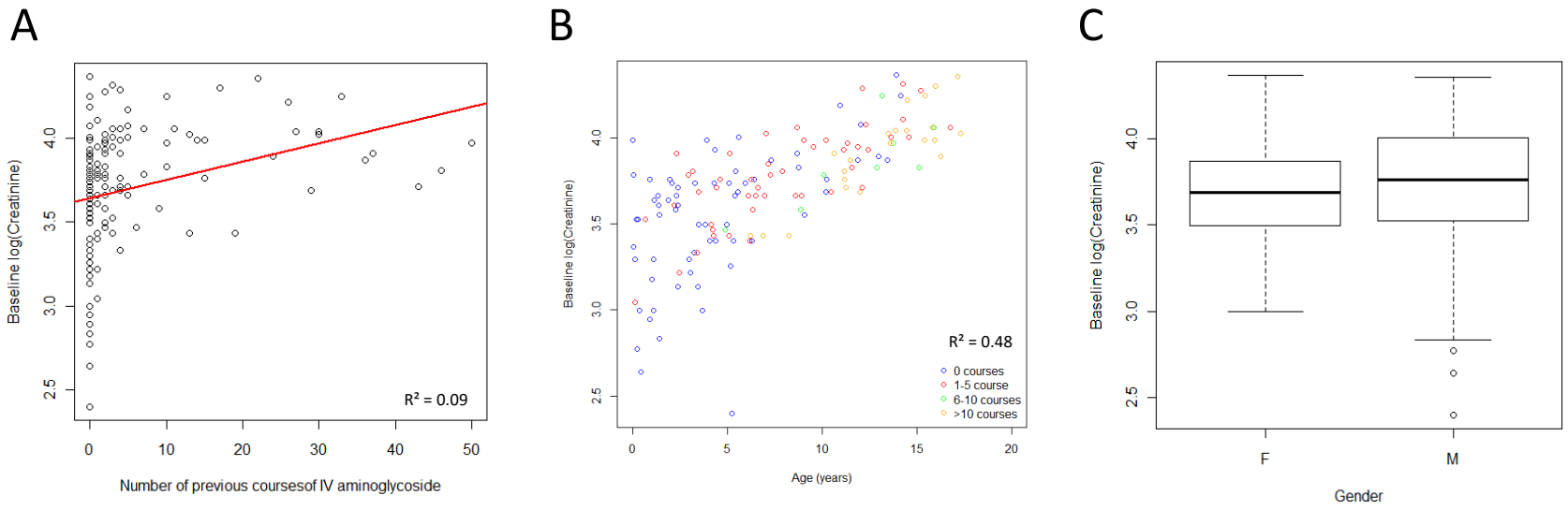


Figure 4.4 – Baseline serum creatinine values. The baseline serum creatinine value was the most recent serum creatinine concentration (µmol/l) recorded at the time of recruitment to the study. The log of serum creatinine is plotted against number of previous courses of intravenous aminoglycosides (A), age (B, stratified by number of previous courses) and gender (C). Adjusted R-squared values from the univariate regression model are provided. For the box and whisker plots, the bottom and top of the box represent first and third quartile respectively, while the darker line in the box is the median, and outliers are represented by the small circle points.

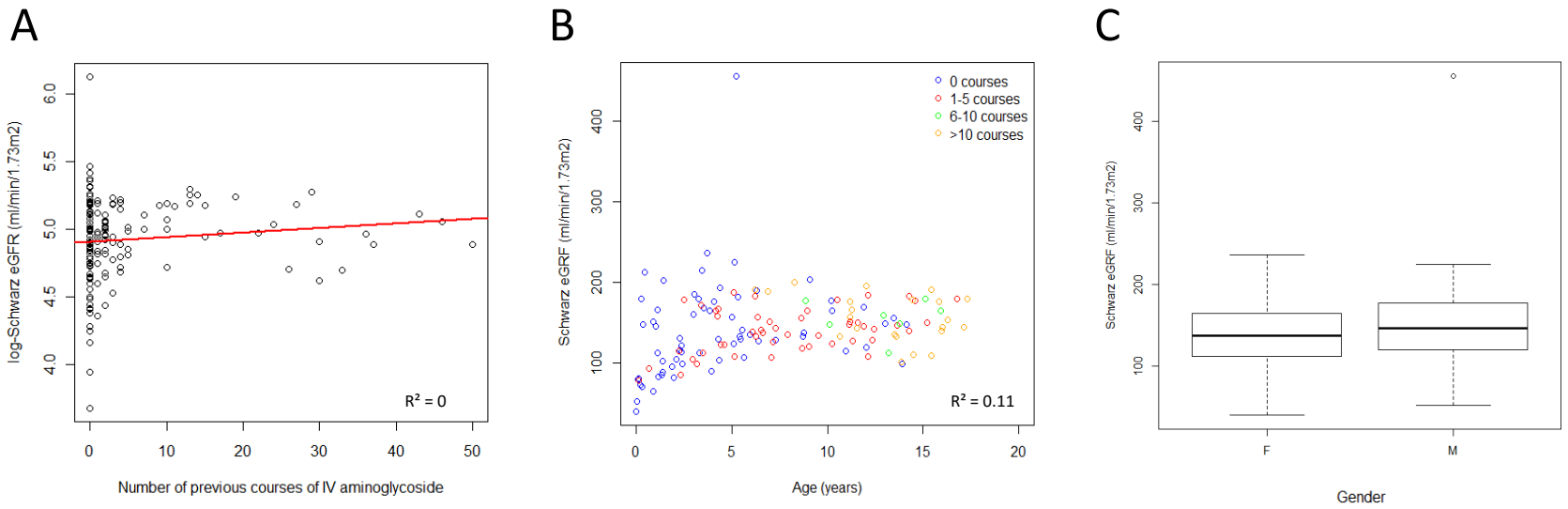


Figure 4.5 – Baseline eGFR. The baseline eGFR was calculated using the Schwartz formula from the most recent serum creatinine measurement at the time of recruitment to the study. The log of eGFR is plotted against number of previous courses of intravenous aminoglycosides (A), age (B, stratified by number of previous courses) and gender (C). Adjusted R-squared values from the univariate regression model are provided. For the box and whisker plots, the bottom and top of the box represent first and third quartile respectively, while the darker line in the box is the median, and outliers are represented by the small circle points.

4.3.3 Biomarkers and aminoglycoside exposure

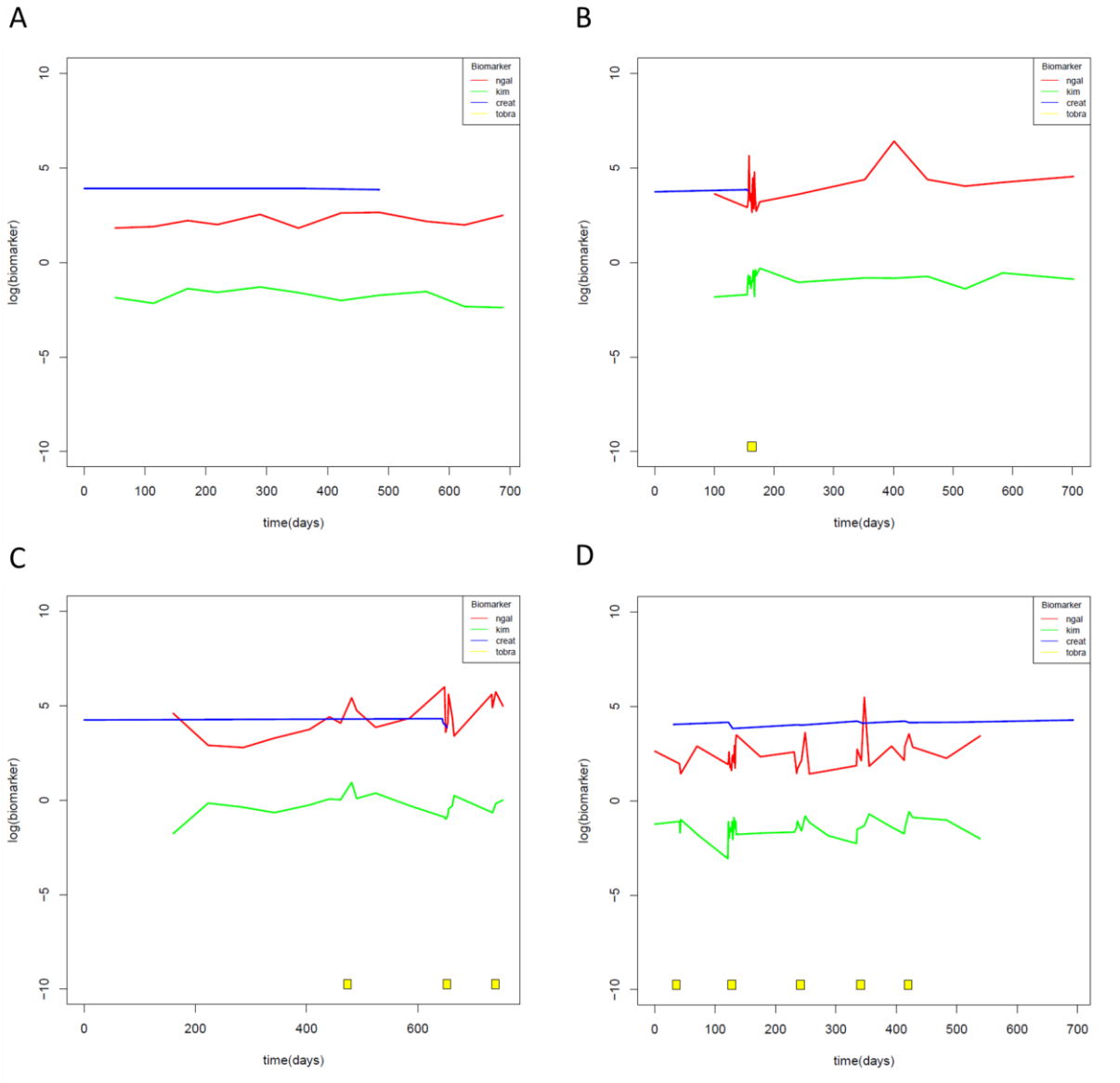


Figure 4.6 – Longitudinal biomarker analysis of children with CF treated with courses of tobramycin. Representative figures demonstrating the longitudinal quantification of the biomarkers KIM-1 (green; ng/mg Cr), NGAL (red; ng/mg Cr) and serum creatinine (blue; µmol/L) for four children treated with tobramycin. Tobramycin treatment episodes and length of treatment (days) are indicated by the yellow boxes on each figure.

Representative longitudinal plots of biomarker concentration are provided for four individuals demonstrating change in biomarker concentration over the period of follow-up

(Figure 4.6). The individual plots do suggest that there is some acute change in biomarker concentration during exposure to tobramycin.

One approach to describing the change in biomarker concentration during exposure to tobramycin is to calculate an absolute change from pre-tobramycin (day 0) value to the peak concentration during treatment. However, due to the wide variation in pre-tobramycin value, a better approach seemed to be to describe a fold-change in the biomarker concentration by dividing the peak value on tobramycin by the pre-tobramycin (day 0) value. For both KIM-1 and NGAL the distribution of peak fold-change is skewed (Figure 4.7). Median peak fold-change for KIM-1 is 2.28 (IQR 2.69) and for NGAL is 4.02 (IQR 7.29).

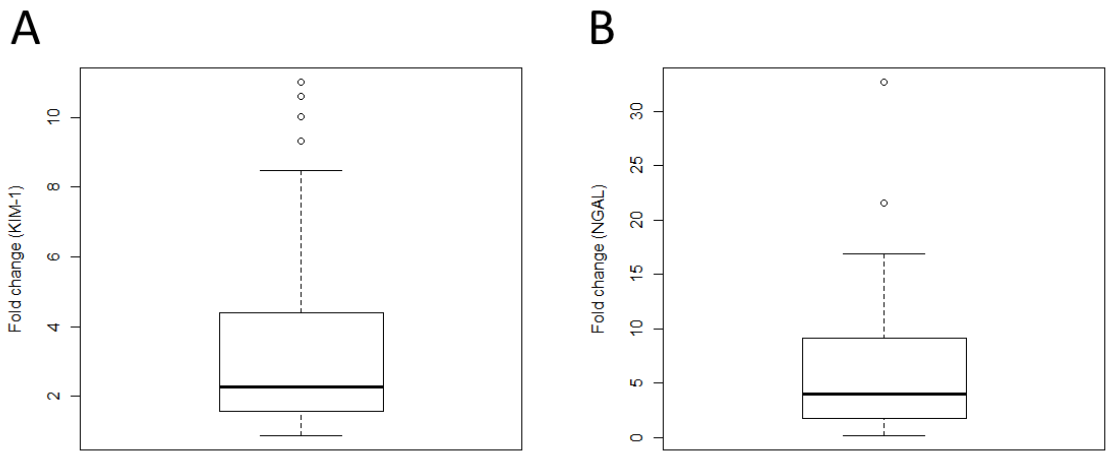
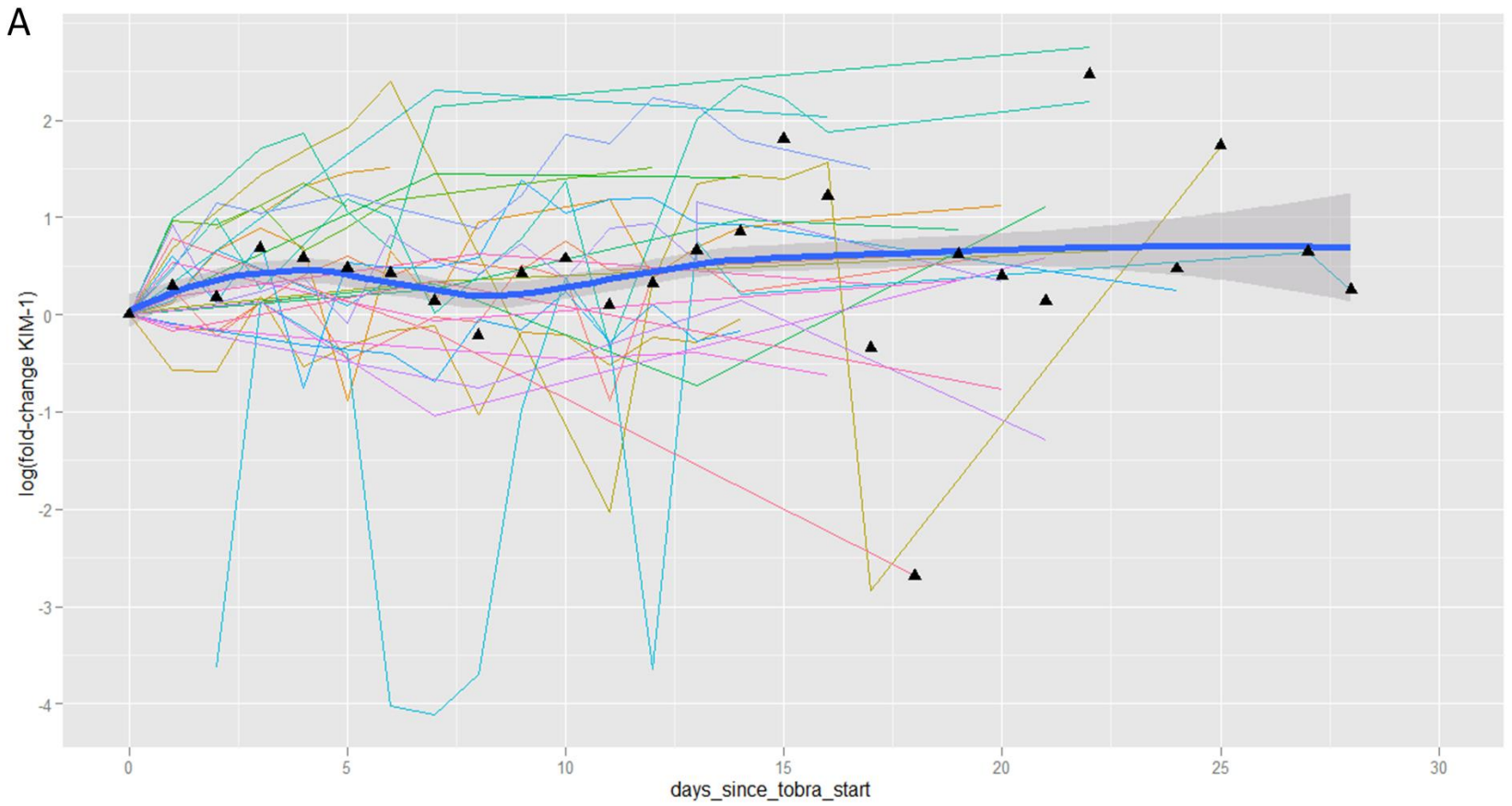
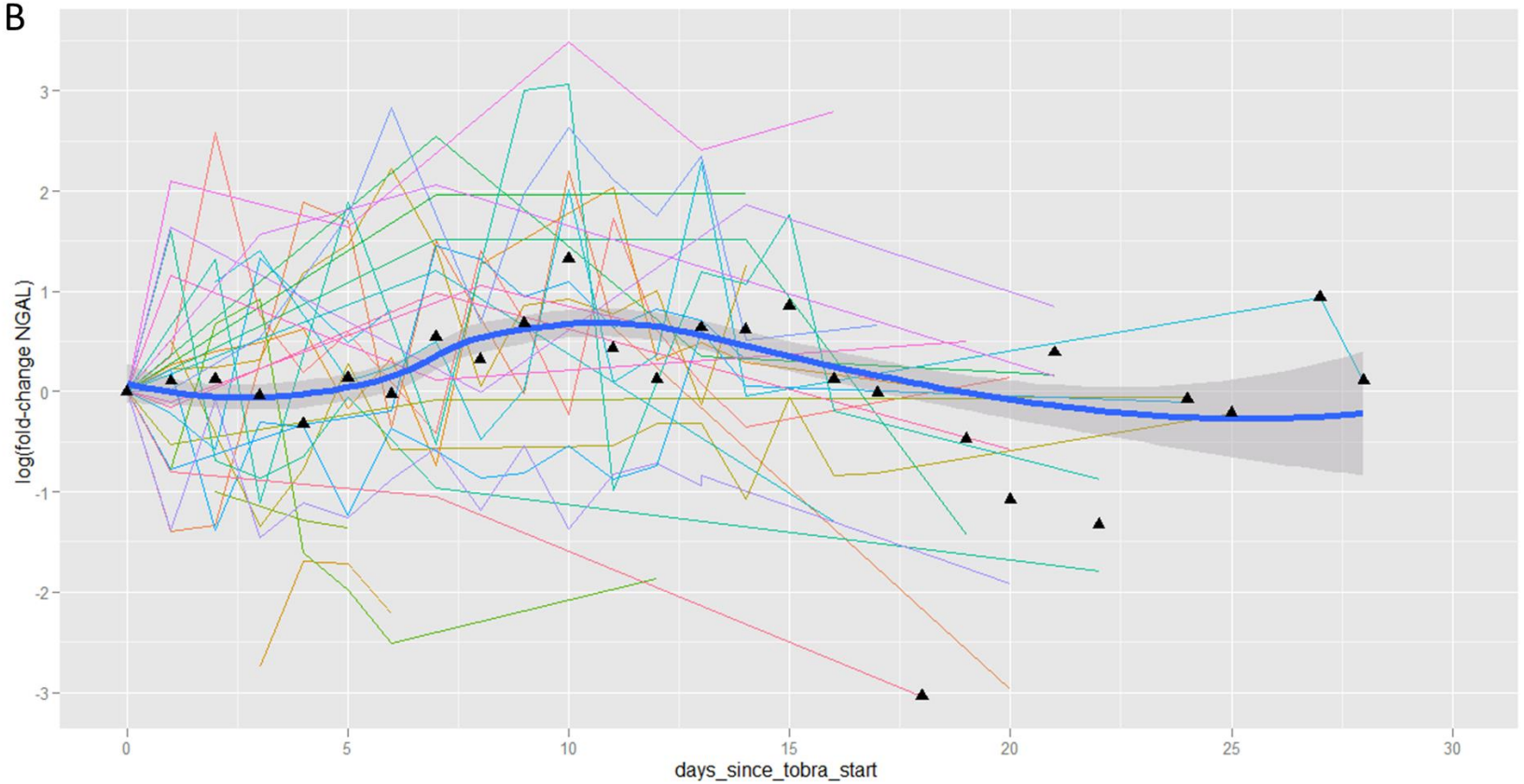


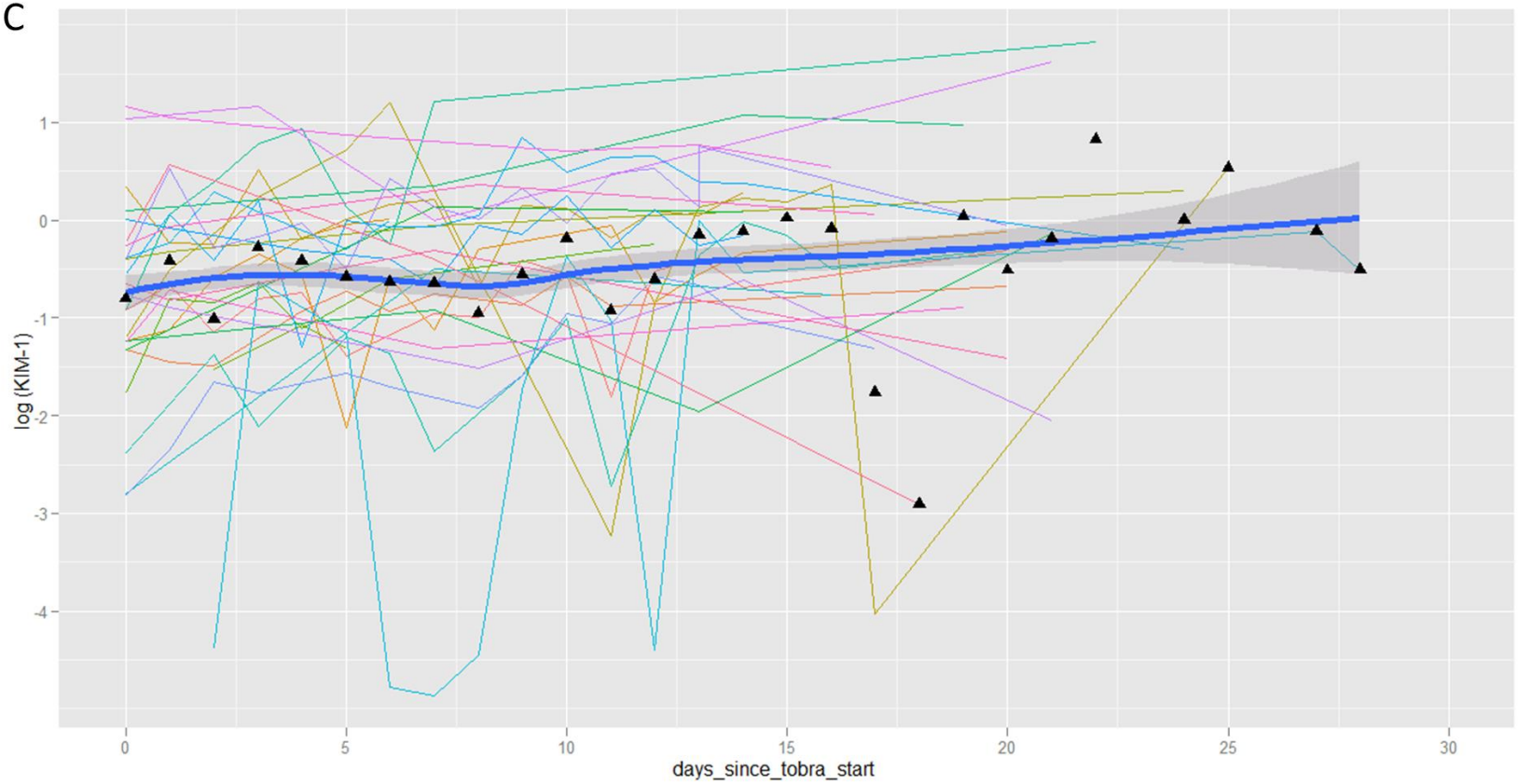
Figure 4.7 – Distribution of peak fold-change in biomarker concentration during exposure to tobramycin. Peak fold-change was calculated for each individual by dividing the peak biomarker concentration measured during tobramycin exposure by their pre-tobramycin (day 0) concentration. Plots are included for KIM-1 (A) and NGAL (B). The bottom and top of the box represent first and third quartile respectively, while the darker line in the box is the median, and outliers are represented by the small circle points.


A
log(fold-change KIM-1)
2
1
0
-1
-2
-3
-4
0
5
10
15
20
25
30
days_since_tobra_start

B


log(fold-change NGAL)
3
2
1
0
-1
-2
-3
0
5
10
15
20
25
30
days_since_tobra_start

C



D

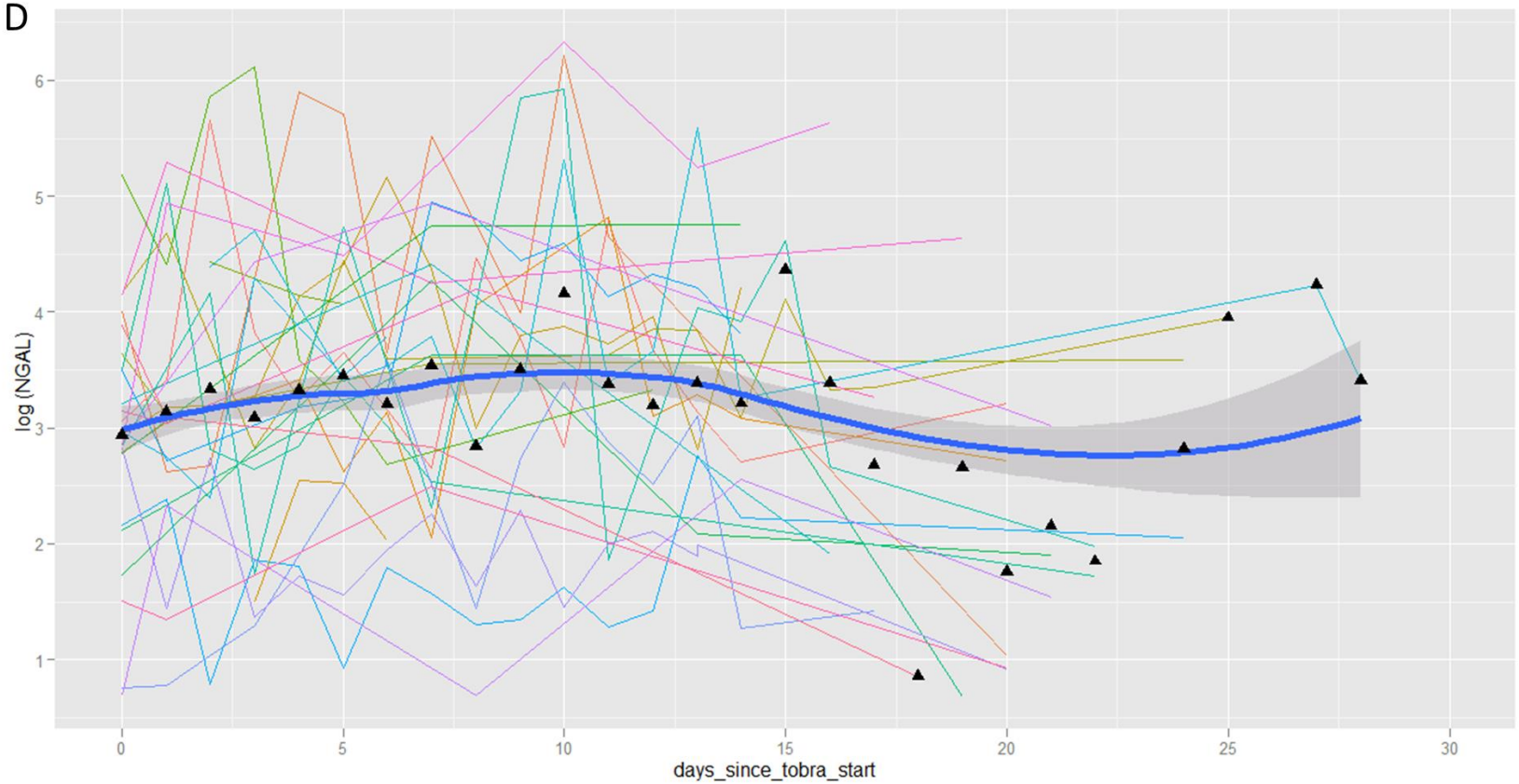


Figure 4.8 – Longitudinal analysis of changes in biomarker values during and after exposure to tobramycin. Plots are from day 0 to day 30 with a course of tobramycin starting on day 0 and usually lasting for 14 days. The day 0 urine sample for biomarker analysis was collected before the first dose of tobramycin was given. Each line represents measured biomarker concentrations for a different individual, and are for their first exposure to tobramycin in this study. Biomarker values are plotted as log of fold change from the day 0 value and as log of absolute value for KIM-1 (A&C) and NGAL (B&D). Daily mean value is plotted (black triangles), and a mean line, produced using locally weighted regression (lowess), with 95% CI is plotted to demonstrate the overall trend.

Longitudinal profile plots (Figure 4.8) demonstrate a high degree of intra-and inter-individual variability. However, the mean trend suggests that both biomarkers increase during exposure to tobramycin. KIM-1 appears to increase earlier, with a peak at 3-5 days, with NGAL peaking later, at 9-11 days. After completing tobramycin (usually at 14 days), NGAL has returned to its pre-tobramycin (day 0) value, whereas KIM-1 remains elevated.

4.4 Discussion

This chapter has demonstrated that, in children with CF, acute changes are seen in both urinary KIM-1 and NGAL during exposure to IV tobramycin. Furthermore, baseline KIM-1 is associated with previous exposure to IV aminoglycosides, with an increasing concentration with increasing number of exposures. In contrast to previously published literature in adults (Al-Aloul et al., 2005a), eGFR was not found to be correlated with previous aminoglycoside exposure.

In accordance with our expectations from the previous study in preterm neonates (McWilliam et al., 2012), elevations in both KIM-1 and NGAL were observed during exposure to tobramycin. Exploratory plots suggest that KIM-1 rises earlier and reaches a peak at 3-5 days, whereas NGAL rises later and reaches a peak at 9-11 days. Furthermore, KIM-1 remains elevated throughout tobramycin treatment and for some time afterwards. NGAL, however, appears to return to its pre-tobramycin level at the end of treatment. Given the large degree of inter- and intra-individual variability it is clear that not all

participants follow the same trend, and the variability is such that it would not be appropriate to make concrete conclusions from these observations. Furthermore, novel statistical techniques employing generalized estimating equations and regression splines could provide more insight, and provide a consideration of the effects of tobramycin exposure on biomarker value, along with the effects of covariates. However, another issue that also needs to be considered is the power of the study, and these effects may become more evident with larger numbers.

A novel finding from this chapter is that baseline KIM-1 in children with CF is associated with cumulative lifetime exposure to IV aminoglycosides. A previous study measuring KIM-1 in children with CF also found a significant correlation with cumulative exposure to aminoglycosides (Lahiri et al., 2014). Our data suggest that KIM-1 becomes elevated during acute episodes of proximal tubule epithelial cell cytotoxicity caused by aminoglycoside exposure, but then remains elevated. Its chronic elevation suggests that the proximal tubule does not fully recover from the acute event, and that KIM-1 is playing a role in this longer term response to toxicity. This interpretation is supported by a growing body of literature which suggests that KIM-1 may be a useful marker for the development of chronic kidney disease (CKD) (Gardiner et al., 2012). In contrast, it may be argued that the relationship between previous aminoglycoside exposure and KIM-1 elevation may not be a causative one. Could KIM-1 simply be a marker for more severe or poorly controlled CF? However, the specificity of KIM-1 for the proximal tubule makes this unlikely.

Baseline urinary NGAL concentration was not associated with previous aminoglycoside exposure. It was associated with sex, which is consistent with our findings in a cohort of healthy children (Chapter 3) (McWilliam et al., 2014), and with published literature in adults (Cullen et al., 2012, Zhang et al., 2013), children (Pennemans et al., 2013), and in

very low birthweight infants (Huynh et al., 2009, Askenazi et al., 2011a). Baseline serum creatinine was strongly correlated with age as has been well described previously in children (Schwartz et al., 1976a).

Estimated GFR was calculated using the Schwartz formula. No correlation was found between eGFR and cumulative lifetime exposure to aminoglycosides. This is in contrast to observations made in a cohort of adult patients with CF (Al-Aloul et al., 2005a). In this previous study, both measured creatinine clearance, and eGFR calculated using the Cockroft-Gault formula (Cockcroft and Gault, 1976), were shown to decrease with increasing lifetime exposure to aminoglycosides. However, the present findings are in agreement with a previous study in a French paediatric CF cohort (Andrieux et al., 2010). In the current paediatric cohort it may be that too few have been exposed to sufficient courses of aminoglycosides for an effect on eGFR to be seen. Perhaps the observed elevation in urinary KIM-1 in children reflects chronic impairment of the proximal tubule epithelial cells which, with time, will result in a global impairment of renal function and a reduction in eGFR. Advances in management, especially the advent of once daily dosing of aminoglycosides, may also account for differences between the current paediatric cohort, and an adult cohort recruited around a decade before (Al-Aloul et al., 2005a). It is also important to note that whilst the Schwartz formula (Schwartz and Work, 2009) is widely used for the calculation of eGFR in children, it is not validated for use in children with CF (Al-Aloul et al., 2007), although it has been used in this population previously (Soulsby et al., 2010, Andrieux et al., 2010). Indeed, estimates of GFR which depend on serum creatinine may overestimate renal function in CF due to the reduced muscle mass in these patients (Al-Aloul et al., 2007). Therefore any further investigation should involve measurement of GFR by a 'gold standard' test (such as Iohexol clearance) alongside calculation of eGFR.

In children with CF significant acute changes occur in the urinary biomarkers KIM-1 and NGAL during exposure to tobramycin. In addition, baseline KIM-1 concentration increases with greater cumulative exposure to aminoglycosides. KIM-1 in particular may be a useful, non-invasive, biomarker of acute and chronic proximal tubular injury associated with exposure to aminoglycosides in children with CF. The clinical utility of KIM-1 should be further evaluated in prospective studies. However, it has immediate potential for use as a surrogate outcome marker in clinical trials looking at interventions to treat or prevent aminoglycoside-induced nephrotoxicity.

5 An *in vitro* model of aminoglycoside toxicity using conditionally immortalised proximal tubule epithelial cells

5.1 Introduction

In vitro models that accurately reflect the *in vivo* situation are key in understanding mechanisms of toxicity and developing potential therapeutic interventions. A variety of animal and human cell lines have been used as *in vitro* models of renal proximal tubule epithelial cells.

The porcine proximal tubule-like cell line, Lilly Laboratories Cell-Porcine Kidney 1 (LLC-PK1), and the Opossum Kidney (OK) cell line are both widely used as a model for the proximal tubule. LLC-PK1 cells demonstrate typical apical and basolateral membrane morphology of proximal tubule epithelial cells, and express some characteristic transport pathways and enzymes (Pfaller et al., 1990). However, LLC-PK1 cells are incapable of gluconeogenesis (Guder and Ross, 1984), a key pathway for normal proximal tubule epithelial cells, and demonstrate an abnormal response to a number of key regulatory hormones (Ausiello et al., 1980). The OK cell line exhibits megalin-mediated endocytosis (Gekle et al., 1997, Xiao Yue et al., 2000) and paracellular permeability comparable to that seen *in vivo* (Bens and Vandewalle, 2008). It has thus been used as a model for aminoglycoside-induced nephrotoxicity (Nagai et al., 2013, Gibbons et al., 2008, Antoine et al., 2010). However, it also lacks gluconeogenesis (Guder and Ross, 1984) and some key proximal tubule epithelial cell enzymes (Gstraunthaler, 1988).

There has been difficulty developing good human proximal tubule cell lines to investigate the mechanism of drug-induced nephrotoxicity in man, and to aid development of reno-protective strategies. A variety of in vitro cell lines have been developed with varying

degrees of human relevance and tubular cell functionality. The HK-2 cell line, derived from a culture of proximal tubule cells taken from the cortex of an adult human kidney (Ryan et al., 1994), has been widely used as a model of nephrotoxicity (Stamps et al., 1994). However, it only expresses a few transporters and, importantly, does not express the important basolateral membrane organic anion (OAT) and cation (OCT) uptake transporters OAT1, OAT3 and OCT2 which are involved in drug transport (Bodnar et al., 1998). Other human proximal tubule epithelial cell lines dedifferentiate after a few passages (Brown et al., 2008).

Recently a novel conditionally immortalised human proximal tubule epithelial cell line (ciPTEC) has been developed and characterised (Wilmer et al., 2010). This group had previously cultured exfoliated proximal tubule epithelial cells from the sediment of midstream urine samples. On light and electron microscopy, these cultured cells exhibit microvilli and tight junctions, demonstrating apical and basolateral polarisation. Furthermore, they demonstrated the presence of megalin, aminopeptidase N, and high levels of alkaline phosphatase activity; all in keeping with epithelial cells of proximal tubular origin (Wilmer et al., 2005). In order to develop a conditionally immortalised cell line, they cultured primary cells from urine samples from healthy volunteers as before, and then immortalised these cells by infection with vectors containing a temperature-sensitive mutant of SV40 large T antigen (SV40T) and the catalytic subunit of human telomerase, human telomerase reverse transcriptase (hTERT). SV40T allows cell proliferation at 33^{o}C, but is inactivated at 37^{o}C, allowing cell maturation (Stamps et al., 1994). hTERT prevents telomere shortening, which would result in replicative senescence (Bodnar et al., 1998). The conditionally-immortalised cell line demonstrated morphology consistent with proximal tubule epithelial cells on microscopy, expressed the proximal tubule specific enzyme aminopeptidase N, and demonstrated alkaline phosphatase activity. They

demonstrated the presence of proximal-tubule specific proteins, aquaporin-1, dipeptidyl peptidase IV and multi resistant protein 4. Albumin uptake was demonstrated using fluorescein isothiocyanate (FITC)-labelled bovine serum albumin, and inhibited by receptor-associated protein (RAP; an inhibitor of megalin-mediated endocytosis), and sodium-dependent phosphate uptake was observed. They also demonstrated the presence and activity of the proximal tubule transporters OCT2 and P-glycoprotein (Pgp) (Wilmer et al., 2010). Sensitivity of ciPTECs to gentamicin has previously been investigated using a spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay, demonstrating a significant increase in cell death with increasing doses of gentamicin (range 100-3000µg/ml) when exposed for 24 hours (Moghadasali et al., 2013).

The aims of this chapter were to:

1. Establish the ciPTEC 14.4 cell line in our laboratory.
2. Determine the utility of this human relevant cell line to investigate the cytotoxic kinetics and dynamics of model aminoglycosides in vitro.

It was hypothesized that the ciPTEC 14.4 cell line would show dose-dependent toxicity when exposed to a range of aminoglycosides.

5.2 Methods

5.2.1 Preparation of cell culture media for ciPTECs

The ciPTEC 14.4 cell line was obtained through a collaborative partner (Wilmer et al., 2010). The ciPTEC media was prepared using sterile procedures in a cell culture hood using a 500ml bottle of DMEM/F-12 (1:1) (1x) + GlutaMAX-1 media (gibco). 50ml of media was removed from the bottle, and the additives then added to the bottle, as per Table 5.1. Media was refrigerated at 4^{o}C.

Table 5.1 – ciPTEC media additives

Additive	Amount per 500ml media	Final
Insulin-Transferrin-Sodium Selenite (ITS)	5ml	I=5µg/ml, T=5µg/ml, S=5ng/ml
Hydrocortisone	360µl	36ng/ml
Epidermal growth factor (EGF)	50µl	100ng/ml
Triiodothyronine	20µl	40pg/ml
Foetal Bovine Serum (FBS)	50ml	10%
Penicillin/Streptomycin	5ml	1%

Additives were prepared as follows:

- Insulin-Transferrin-Sodium Selenite media supplement (ITS, Sigma-Aldrich). The contents of one vial were dissolved in 5mL of acidified water (a few drops of 0.3M hydrochloric acid in 5ml sterile water), and this was then made up to 50mL with sterile water (filtered through 0.22µm). This was divided into 5mL aliquots which were frozen at -20^{o}C.
- Hydrocortisone (Sigma). 1mg was dissolved in 1 ml ethanol, and then made up to 20 ml with DMEM/F-12 medium (50µg/ml). This was divided into 500µl aliquots and frozen at -20^{o}C.
- Epidermal growth factor (EGF, Sigma). 200µg was dissolved in 2 ml sterile water (100µg/ml). This was divided into 100µl aliquots and frozen at -20^{o}C.

- Triiodothyronine (Sigma). 1mg was dissolved in 1ml sodium hydroxide (1M) (filtered through 0.22µm), and then made up to 50ml with DMEM/F-12 medium (20µg/ml). 50µl of this was added to 950µl of medium (1µg/ml). This was divided into 50µl aliquots and frozen at -20^{o}C.
- Foetal Bovine Serum (FBS). Divided into 50ml aliquots and frozen at -20^{o}C.
- Penicillin and Streptomycin (Pen/Strep). Divided into 5ml aliquots and frozen at -20^{o}C.

5.2.2 Cell culture methodology

ciPTEC cells from the 14.4 cell line were cultured in T75 flasks in 12ml of media, and kept in an incubator at either 37^{o}C or 33^{o}C with 5% carbon dioxide as described below. Cells were checked at least every 48 hours by visualisation using a light microscope, and media was changed every 48 to 72 hours. This was done following sterile procedures in a cell culture hood. Fresh media was warmed to 37^{o}C in a water bath in advance. Old media was removed from the flask using a 25ml stripette and placed into a waste pot containing virkon solution. 12ml of fresh media was then added to the T75 flask using a fresh stripette. The flask was then returned to the incubator, and the waste was left for 24 hours before discarding.

When the cells in the flask reached confluence, or at other times for preparation of experiments, the following methodology was followed, using sterile procedures in a cell culture hood. Fresh media was warmed and trypsin was thawed in a water bath at 37^{o}C. The old media was poured out of the T75 flask into the waste pot (virkon). Using a stripette, 5ml of phosphate buffered saline (PBS) was added to the flask. The flask was shaken gently, and then the PBS was removed using the stripette. 4ml of trypsin was added to the flask using a fresh stripette, and the flask placed in the 37^{o}C incubator for 3-5

minutes. The flask was then returned to the cell culture hood and 6ml of ciPTEC media was added to the flask using a fresh stripette to inactivate the trypsin. Using this stripette all the media and suspended cells were drawn up and transferred to a 15ml centrifuge tube. This was then centrifuged at 1600rpm for 4 minutes at 20˚C.

Once centrifuged, the cells formed a pellet at the base of the tube. The media was poured off the cell pellet into the waste pot, and then the cells were resuspended in 1ml of media using a 1ml pipette. A stripette was then used to add a further 9ml of media and to mix the cell suspension. 50µl of the suspension was then removed into a sterile eppendorf (autoclaved) using a pipette for cell counting. The cell count was performed using a Neubauer chamber and the light microscope. A cover slip was placed over the Neubauer chamber and then 10µl of cell suspension was added to each side of the chamber. The Neubauer chamber was placed onto the microscope and cells were visualised on the counting grid using 10x magnification. Cells were counted in the left upper, right upper, left lower and right lower quadrants on both sides of the chamber. The concentration (cells/ml) of the suspension was calculated using the following formula:

$$Concentration\ (cells/ml) = \frac{(Total\ number\ of\ cells\ counted * 10000)}{Number\ of\ squares}$$

Once the concentration of the cell suspension was known, the 10ml cell suspension was then divided into fresh T75 flasks and diluted further (using fresh media) as necessary to give a total volume of 12ml of media per flask. Flasks were mixed gently to ensure an even distribution of cells, and then placed in an incubator. The cell suspension could also be used for setting up experiments, as will be described below.

Aliquots of cells could also be frozen for storage if not required immediately. The procedure above was followed up to the resuspension of the cell pellet in 1ml of media. In

order to prevent damage to the cells caused by water crystal formation during freezing they were frozen in a 10% dimethylsulphoxide (DMSO) solution. This was prepared by adding 800µl DMSO to 6.2ml of fresh ciPTEC media. Eight sterile cryovials were prepared and labelled. A 'Mr Frosty' freezing container was prepared at room temperature with the correct amount of isopropanol. The DMSO solution and cell suspension were mixed together and then immediately aliquoted, 1ml per cryovial. The cryovials were placed into the 'Mr Frosty', and the whole placed in the -80˚C freezer. The 'Mr Frosty' ensures a temperature reduction of 1˚C per minute, which is the optimal rate for cell preservation. After 24 hours the aliquots were removed from the 'Mr Frosty' and kept at -80˚C until required.

When required, frozen aliquots were thawed and then transferred to a 15ml centrifuge tube containing 7ml of fresh ciPTEC media. The resuspended cells were then centrifuged at 1600rpm for 4 minutes at 20˚C. During this time, a fresh T75 flask was prepared by addition of 11ml fresh ciPTEC media. Once centrifuged, the media was poured off the cell pellet into the waste pot, and then the cells were resuspended in 1ml of media using a 1ml pipette. The resuspended cells were added to the flask, which was then mixed gently to ensure an even distribution of cells, and placed in an incubator.

5.2.3 Aminoglycoside dose-response and cell proliferation (MTS) assay

In order to determine the impact of aminoglycosides upon ciPTECs, a number of dose-response experiments were performed. The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega) was used to measure the number of viable cells in these experiments.

The MTS assay is a combination of solutions containing an MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

and PMS, an electron coupling reagent (phenazine methosulfate). MTS is converted into formazan by dehydrogenase enzymes which are present in metabolically active cells. Formazan is soluble in cell culture media, and is measurable by the amount of absorbance at 490nm. Promega assay validation suggests the quantity of formazan product is directly proportional to the number of living cells.

The MTS assay was prepared in advance as follows. The 20ml MTS and 1ml PMS solutions contained in the kit were thawed in a water bath at 37˚C. Once thawed they were combined by addition of the 1ml PMS solution to the bottle containing 20ml MTS solution. This was then thoroughly mixed before being aliquoted into 1.2ml aliquots in 1.5ml eppendorfs. These aliquots were wrapped in tinfoil to protect from light, and then frozen at -20˚C.

ciPTECs were prepared by incubation in a T75 flask. This involved an initial incubation in a T75 flask at 33˚C for 24 hours, followed by a further 6 day incubation at 37˚C. Initial experiments showed no difference in MTS outcome between this protocol, and incubation of cells at 33˚C for 7 days.

After 7 days of incubation, cells were transferred into a sterile 96-well plate for the dose-response experiments. The procedure described above was followed to the point of resuspending cells and performing a cell count. The suspended cells were then diluted as necessary with fresh media to the desired concentration. 100µl of cell suspension was transferred to each well as required. 100µl of media only was transferred to control wells as necessary. The circumferential wells of the plate were generally left as blank wells due to the potential for increased evaporation from these wells, and therefore 100µl sterile water was transferred to these wells to form an evaporation buffer. The plate was then transferred to an incubator at either 33˚C or 37˚C.

After 24 hours, the plates were removed from the incubator and checked using the light microscope to ensure that the cells had adhered to the base of the wells, and that they were around 60-70% confluent. If so, they were ready for dosing. Initial experiments demonstrated that a starting cell suspension concentration of 120000cells/ml gave a suitable confluency after 24 hours.

Dosing concentrations of aminoglycosides were prepared in ciPTEC media or in serum-free media (DMEM/F-12 (1:1) (1x) + GlutaMAX-1 media with no additives). Dosing concentrations for gentamicin and neomycin ranged from 100 to 4000µg/ml, and for tobramycin ranged from 100 to 10000µg/ml. Media was removed from each well of the plate, one column at a time, using a 1ml pipette, taking care not to disturb the cells on the base of the plate, and transferred to the waste pot. This was then replaced with 100µl fresh media with/without aminoglycoside as per the experiment plan. Each plate included media only controls with no cells, and cell controls with media only (no drug). The plate was then returned to the incubator.

The dosing incubation was normally left for 24 hours, except for in a few instances where an incubation of 48 hours was used. To carry out the MTS assay, one aliquot of the MTS assay solution was thawed for each 96-well plate. Using a reagent reservoir and multichannel pipette, 20µl of MTS solution was added to each well. The plate was then immediately wrapped in tinfoil and placed in an incubator at 37˚C in humidified 5% carbon dioxide. Incubation times of 1 or 2 hours were tested and absorbance was greater at 2 hours. Therefore all plates were subsequently read at 2 hours. Plates were read using a plate reader, and the absorbance at 490nm was recorded.

All experiments were performed using ciPTECs between passage 41 and 44, and were derived from a single flask of ciPTECs at passage 40.

5.2.4 Statistical Analysis

Statistical analysis was conducted using Graphpad Prism (version 6.05). One-way ANOVA with Dunnet's multiple comparisons test was used to compare viability at different doses to control. Two-way ANOVA was used to compare the effect of different variables on viability, with Sidak's multiple comparisons test used to compare the difference at each dose. $P<0.05$ was considered significant. A nonlinear dose-response model was used to fit dose-response curves and calculate the 40% of maximum effective concentration (EC40, i.e. a 40% reduction in viability) and 95% confidence intervals (CI).

5.3 Results

5.3.1 Dosing ranges

The initial dosing ranges for gentamicin (0 – 4000 μg/ml), tobramycin (0 – 10000 μg/ml) and neomycin (0 – 4000 μg/ml) were based upon a previous publication demonstrating gentamicin related toxicity in ciPTECs (Moghadasali et al., 2013). Initial experiments demonstrated a dose dependent cytotoxic response using MTS reduction as a read out (Figure 5.1). Ordinary one-way ANOVA demonstrated a significant dose-dependent effect of exposure to gentamicin ($p<0.0001$), tobramycin ($p=0.0131$) and neomycin ($p<0.0001$) compared to control. The first significant dose in which observable cytotoxicity was observed for gentamicin, tobramycin and neomycin was 100, 1000 and 100 μg/ml (0.22, 2.1 and 0.11mM) respectively (Figure 5.1).

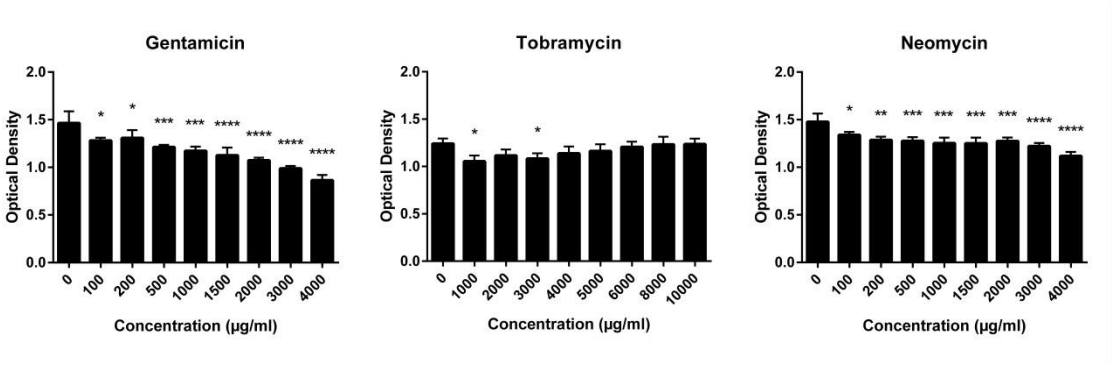


Figure 5.1 – Dose ranging of gentamicin, tobramycin and neomycin. Cells were dosed and incubated for 24 hours at 37˚C. MTS reagent was added 24 hours after dosing, and optical density at 490nm was read at 2 hours. Results are mean (plus standard error) of means of three replicates on three plates run on the same day from the same batch of cells. An Ordinary One-way ANOVA with Dunnett's multiple comparisons test was used to compare optical density at each dose to control. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

5.3.2 Serum free versus ciPTEC media

Although tobramycin is considered less nephrotoxic than gentamicin (Smith et al., 1980, Schentag et al., 1981), some degree of toxicity was expected. It was felt that there could be an inhibitory effect of serum containing media on the toxic effect of tobramycin which might account for the much lower potency of tobramycin seen in vitro compared to gentamicin. This was investigated by using dosing concentrations of the aminoglycosides made up in ciPTEC media or in serum free media, respectively.

Ordinary two-way ANOVA demonstrated a significant difference at low concentrations related to the presence of serum media type for gentamicin ($p<0.0001$) and neomycin ($p=0.0014$), but not for tobramycin. However, it did not change the overall toxicity profile of any of the three aminoglycosides (Figure 5.2).

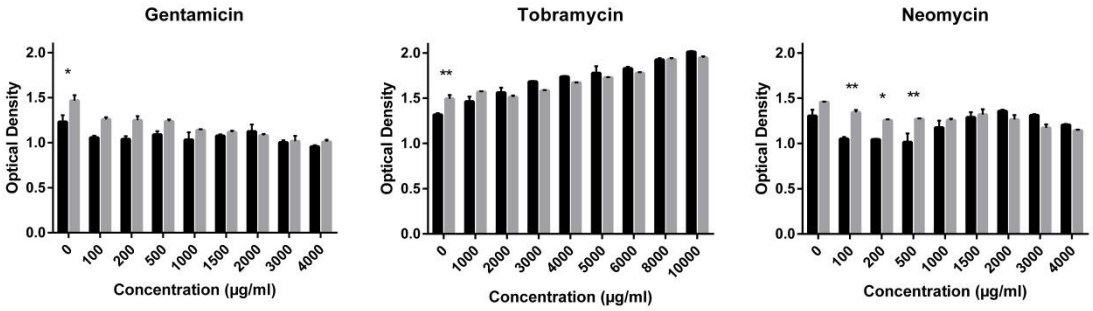


Figure 5.2 - Dose ranging of gentamicin, tobramycin and neomycin: comparison of serum free and ciPTEC media. Dosing concentrations were prepared in both serum free and ciPTEC media. Cells were dosed and incubated for 24 hours at 37˚C. MTS reagent was added 24 hours after dosing, and optical density at 490nm was read at 2 hours. Results are mean (plus standard error) of three replicates on a single plate. An ordinary two-way ANOVA was completed using Sidak's multiple comparisons test to compare the means at each dose concentration to investigate the impact of media type on optical density. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). Serum free media – black bars, ciPTEC media – grey bars.

5.3.3 Correction for media effects

It was felt that there may be an effect of the dosing media at each concentration, possibly related to changes in pH because of high drug concentrations prepared, on the recorded optical density. Therefore plates were run using ciPTEC media and cell-free controls for each dosing concentration. The recorded optical density for the cell free controls was then subtracted from the optical density for the cell containing wells for each dose (Figure 5.3).

Ordinary two-way ANOVA demonstrated a significant difference related to correction for tobramycin ($p<0.0001$) and neomycin ($p=0.0009$), but not for gentamicin. The difference for tobramycin is dramatic, with the correction revealing an inhibitory effect compared to the previously seen augmentation (Figure 5.3).

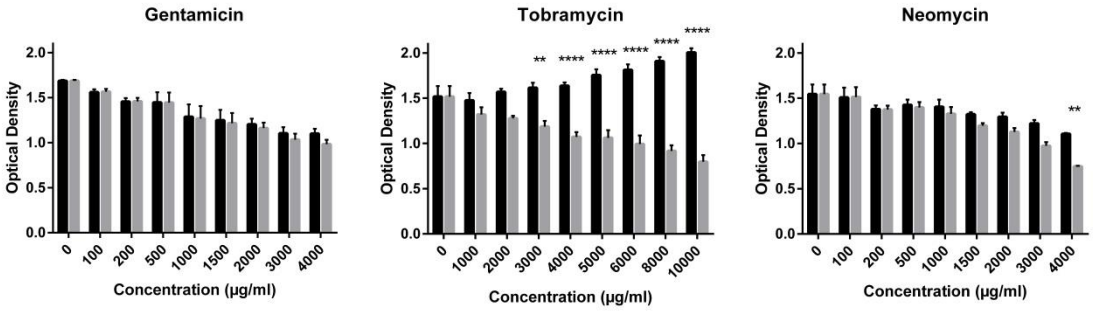


Figure 5.3 – Dose ranging of gentamicin, tobramycin and neomycin with and without correction for background media optical density. Cells were dosed and incubated for 24 hours at 37˚C. MTS reagent was added 24 hours after dosing, and optical density at 490nm was read at 2 hours. Results are mean (plus standard error) of three replicates on a single plate. An ordinary two-way ANOVA was completed using Sidak's multiple comparisons test to compare the means at each dose concentration to investigate the effect of correcting for background media optical density. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). Observed optical density – black bars, corrected optical density – grey bars.

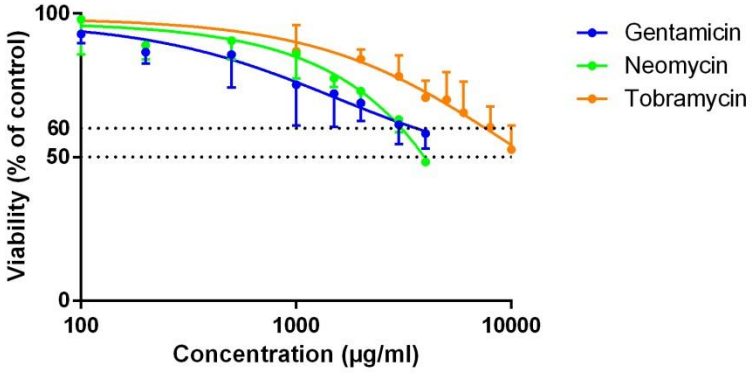


Figure 5.4 – Dose response curves for gentamicin, tobramycin and neomycin following correction for background media optical density. Cells were dosed and incubated for 24 hours at 37˚C. MTS reagent was added 24 hours after dosing, and optical density at 490nm was read at 2 hours. Results are mean (plus standard error) of three replicates on a single plate, expressed as a percentage of control. Dose-response curves were fit using a nonlinear dose-response model in Graphpad Prism version 6.05.

Dose-response curves for the corrected data are presented for gentamicin, tobramycin and neomycin (Figure 5.4). A reduction in viability from control greater than 50% was only achieved using the maximum dose of neomycin. It was therefore not possible to compare the relative potency of each drug by measuring the EC_{50} for cytotoxicity. Instead the EC_{40} (40% reduction in viability) has been compared (**Error! Reference source not found.**). These ata show that the rank order of potency for cytotoxicity of the aminoglycosides in this cell line after 24 hours exposure was neomycin>gentamicin>tobramycin.

Table 5.2 - Effective Concentrations for gentamicin, tobramycin and neomycin following correction for background media optical density.

Aminoglycoside	EC40 (µg/ml) (95% CI)	
	24 hours	48 hours
Gentamicin	3623 (2512-infinite)	4303 (incalculable)
Neomycin	3090 (incalculable)	2607 (2239-2938)
Tobramycin	7599 (6266-infinite)	4320 (3784-4898)

Cells were dosed and incubated for 24 or 48 hours at 37˚C. MTS reagent was added 24 or 48 hours after dosing, and optical density at 490nm was read at 2 hours. Results at 24 hours are of three replicates on a single plate, and at 48 hours of three replicates on three plates run on the same day. Dose-response curves were fit using a nonlinear dose-response model, and EC40s (40% reduction in viability) and 95% confidence intervals (CI) were calculated in Graphpad Prism version 6.05.

5.3.4 Investigation into the time-dependent cytotoxicity effect of aminoglycosides in vitro

A time-course experiment was conducted to compare the effects of incubation of the cells with aminoglycoside-containing media for 24 hours or 48 hours. Cells were plated as before, dosed, and then an MTS assay was completed at 24 or 48 hours post-dosing. Separate plates were used for analysis at 24 and 48 hours. The experiments were conducted using ciPTEC media. Ordinary two-way ANOVA demonstrated a significant difference at 24 and 48 hours for gentamicin (p=0.0001) and tobramycin (p=0.0008), but was not significant for neomycin (Figure 5.5). The rank order of potency for cytotoxicity of

the aminoglycosides investigated in this cell line after 48 hours exposure was neomycin>gentamicin>tobramycin (Table 5.2).

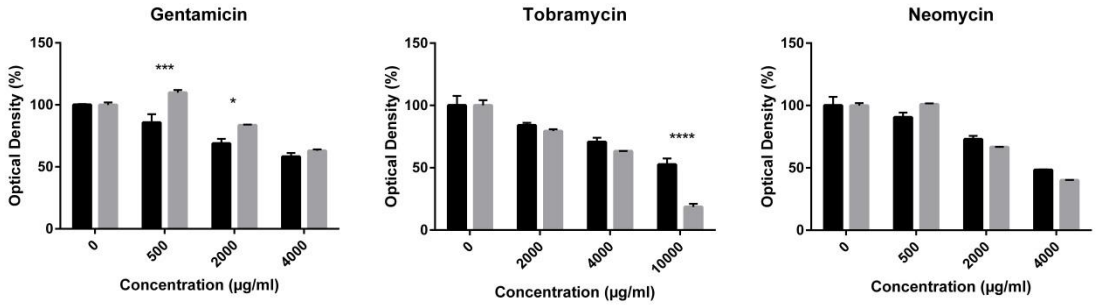


Figure 5.5 – Time-course experiment for gentamicin, tobramycin and neomycin. Cells were dosed and incubated for 24 hours or 48 hours at 37˚C. MTS reagent was added 24 hours after dosing, and optical density at 490nm was read at 2 hours. Results are mean (plus standard error) of the means of three replicates on three plates run on the same day from the same batch of cells and expressed as a percentage of control for each day. An ordinary two-way ANOVA was completed using Sidak's multiple comparisons test to compare the means at each dose concentration to investigate the impact of length of dosing incubation on optical density. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). 24 hours – black bars, 48 hours – grey bars.

5.4 Discussion

Here, dose and time-dependent toxicity of three aminoglycoside antibiotics has been demonstrated in a conditionally-immortalised human proximal tubule epithelial cell line using a cell proliferation (MTS) assay. This work confirms previous evidence of gentamicin-induced toxicity using the same cell line (Moghadasali et al., 2013), but extends this to demonstrate dose-dependent toxicity with neomycin and tobramycin too. At both 24 and 48 hours, the rank order of potency for these aminoglycosides in this cell line was neomycin>gentamicin>tobramycin. This is in accordance with the rank order of nephrotoxicity derived from clinical trials and animal studies (Begg and Barclay, 1995).

A series of experiments were conducted to establish the optimum conditions for the MTS assay, including initial cell suspension concentration, length and temperature of the dosing incubation, the range of doses used, the use of serum-containing or serum-free media, and the length of incubation with the MTS reagent. Most experiments in this chapter were conducted using three replicates on separate plates but run on the same day, using the same batch of cells. In order to confirm these results, it would be necessary to repeat the experiments using independent replications on different days, using different batches of cells.

Previous work with this cell line has demonstrated gentamicin induced cytotoxicity using an MTT assay, with an EC_{50} of 1765µg/ml (Moghadasali et al., 2013). Here we were unable to calculate an EC_{50} but found an EC_{40} of 3623µg/ml for gentamicin. Reasons for this difference in toxicity may relate to differences in the cell viability assay used, the brand of gentamicin used, or to the cell culture methodology and conditions. Cytotoxicity of the three aminoglycosides used was compared using an EC_{40}. This was used rather than the more traditional EC_{50} as a 50% reduction in viability was only achieved at 24 hours using the maximum dose of neomycin (4000µg/ml) (Figure 5.4). It is important to note that the confidence intervals for these EC_{40} values were extremely wide or incalculable. Further work is thus needed to extend the dose ranges of, and length of exposure to, the three aminoglycosides in order to achieve maximum cytotoxicity. This would enable more accurate calculation of EC_{50} values, with tighter confidence intervals, and confirmation of the rank order of toxicity.

Incubation of cells at 33˚C versus 37˚C had relatively minimal impact upon the sensitivity of the ciPTECs to aminoglycosides. Given this, the decision was taken to incubate at 37˚C as this reflects the *in vivo* temperature, and is also the temperature at which ciPTECs are able

to mature, and therefore, in theory, demonstrate characteristics consistent with proximal tubule cells. Results were similar when using serum-containing or serum-free media, suggesting that there is no impact of serum on the availability of the aminoglycosides. Therefore, serum-containing ciPTEC media was used in the following experiments. Incubating for a 48 hour period had little additional impact above the 24 hour incubation.

The drug concentration in the dosing media itself was found to have a significant effect on the observed optical density, possibly related to a pH effect on the colour of the phenol red indicator in the media. This effect had to be corrected for at each dosing concentration in order to reveal the effect of the drug on cell viability. This effect was most marked with tobramycin. Further investigation is required to determine the cause of this dose-related change in media optical density. It may be related to changes in pH of the media caused by the drug, although this has not been confirmed. If this is the case, there is a possibility that the media pH itself may have an impact on cell viability, independent of the drug effect. Further investigation of this would first involve measuring the pH of media containing aminoglycoside at a range of concentrations. Matched pH controls for each of these dose concentrations would then be produced. ciPTECs would then be incubated for 24 hours with either aminoglycoside-containing media or the pH-matched media, and the resulting cell viability compared. This could be done using the MTS assay as before but, as we are aware of the potential for interference with the observed optical density, it would make sense to use an alternative assay of viability such as the adenosine-5'triphosphate (ATP) bioluminescent assay (e.g. Sigma-Aldrich) (Isaksson et al., 1988). This would have the benefit of avoiding the complication of media-related effects on optical density as the assay relies on light emission related to the oxidation of D-Luciferin catalyzed by firefly luciferase.

Further validation of this *in vitro* model would need to confirm toxicity with exposure to aminoglycosides by using different toxicity assays. The MTS assay measures the number of metabolically active cells in culture, as it is these cells that convert the MTS reagent into formazan. The first step would be to measure cell death and damage as opposed to cell survival. This could be done by measurement of lactate dehydrogenase (LDH) activity in the cell supernatant (e.g. Cytotoxicity Detection Kit, Roche) (Antoine et al., 2010). LDH is released from the cytosol of cells when damage to the plasma membrane occurs during cell death and cell lysis (Decker and Lohmann-Matthes, 1988). An alternative assay measuring viable cells is the luminescent ATP assay mentioned above. Apoptosis could be quantified using the luminescent Caspase-Glo assay (Promega), which quantifies caspase activity in cell supernatant (Botto et al., 2010).

In order to establish the ciPTEC model of aminoglycoside toxicity as a good model for proximal tubule epithelial cell damage *in vivo*, further characterisation of the ciPTEC cell line is required. An ideal *in vitro* model should express the same characteristics as the situation *in vivo*. Expression of the key proximal tubule cell proteins can be determined using western blotting, or the polymerase chain reaction (PCR) (Jenkinson et al., 2012). Arguably more important than simply expressing the same markers, is having the same function. Assays for proximal tubule epithelial cell function include: the albumin uptake assay using FITC-labelled bovine serum albumin for megalin-mediated endocytosis (Sidaway et al., 2004); the 5-chloromethylfluorescein diacetate (CMFDA)-uptake assay for function of the multidrug resistance-associated protein (MRP) family of transporters (Jenkinson et al., 2012); and the Hoescht uptake assay for function of the breast cancer resistance protein (BRCP) transporter (Huls et al., 2007). Whilst some of this work has been done by the group which produced the ciPTEC cell line (Wilmer et al., 2010), it is important that this is independently verified.

Future work utilising the ciPTEC model of aminoglycoside-induced toxicity should include investigation of the cellular mechanisms of toxicity. This would include confirmation of the role of megalin-mediated endocytosis by inhibition of this pathway with statins as described previously in OK cells (Antoine et al., 2010). It would also include investigation of the mitochondrial effects of aminoglycosides in ciPTECs by measuring oxygen consumption rates and extracellular acidification rates in real time using a Seahorse Biosciences Analyzer (Nagai et al., 2013). It would be of interest to investigate the measurement of kidney injury molecule-1 (KIM-1), and other known *in vivo* biomarkers of aminoglycoside toxicity, in ciPTECs. Currently there is very poor translation of biomarkers of toxicity from *in vitro* to *in vivo* models. An *in vitro* model that had good translatability to *in vivo* work would then provide a useful tool for the development and initial investigation of potential protective strategies, such as that described previously utilising statins (Antoine et al., 2010).

In conclusion, this chapter demonstrates the utility of an *in vitro* model of aminoglycoside-induced nephrotoxicity using a novel ciPTEC cell line. Toxicity with three aminoglycosides was demonstrated, with a rank order of toxicity in accordance with that previously published (Begg and Barclay, 1995). However, the work presented provides a preliminary insight, and further work is necessary to confirm the validity of this model for aminoglycoside-induced proximal tubule epithelial cell toxicity. This is important as the model potentially holds promise for the investigation of cellular mechanisms of toxicity and the development of renoprotective strategies.

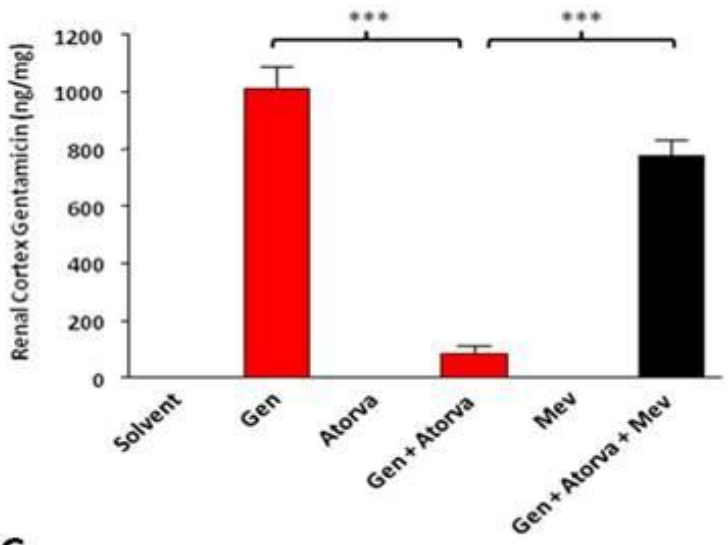
6 Investigation of the potential for statins to inhibit aminoglycoside-induced nephrotoxicity *in vivo*

6.1 Introduction

As previously described, aminoglycoside-induced nephrotoxicity is characterized by selective targeting of the proximal tubule cells within the renal cortex. Accumulation of the drug within the proximal tubule epithelial cells, following glomerular filtration, is thought to be the key mechanistic determinant for the development of toxicity (Mathews and Bailie, 1987). Endocytosis via the multi-ligand receptor megalin has been demonstrated to be the principal pathway for this accumulation – megalin knock-out mice do not exhibit renal accumulation of aminoglycosides (Schmitz et al., 2002). This suggests a potential pathway for reducing renal accumulation of aminoglycosides by inhibition of megalin-mediated endocytosis. Statins inhibit megalin-mediated endocytosis *in vitro* (Verhulst et al., 2004, Sidaway et al., 2004), and thus could inhibit aminoglycoside-induced nephrotoxicity (see section 1.5.2.1, and Figure 1.4). This hypothesis was tested in an *in vitro* proximal tubule model where different statins were shown to prevent aminoglycoside induced cytotoxicity (Antoine et al., 2010). This has more recently also been demonstrated in an *in vivo* model of gentamicin nephrotoxicity in rats using atorvastatin (Figure 1.5).

This chapter seeks to translate this work from these initial *in vitro* and *in vivo* experiments to a point where enough evidence has been obtained to demonstrate whether a first in man study should be attempted. In order to take this forward into man, evidence would be required that statins prevent AG-induced nephrotoxicity *in vivo* in an animal model at plasma concentrations equivalent to those which are attained in man when statins are used therapeutically. Rats have high activity of HMG CoA reductase, and therefore require higher doses of statins for their therapeutic effect, as shown in

A


Cortex Accumulation
Renal Cortex Gentamicin (ng/mg)
1200
1000
800
600
400
200
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Solvent
Gen
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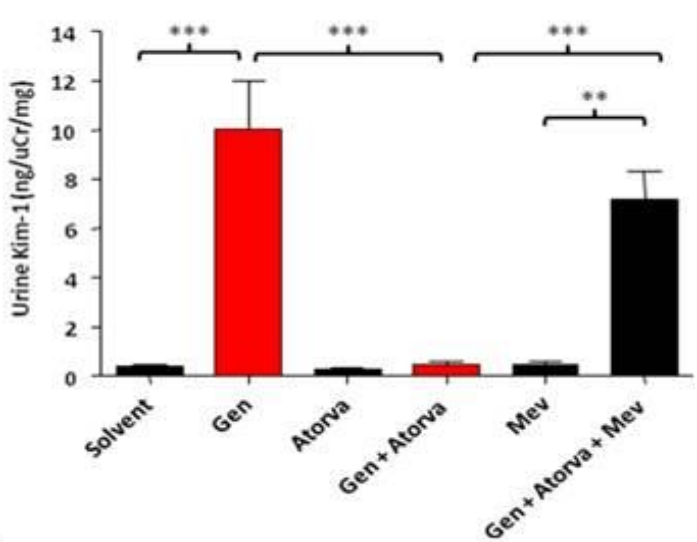
Urinary Kim-1 (Toxicity)
Urine Kim-1 (ng/uCr/mg)
14
12
10
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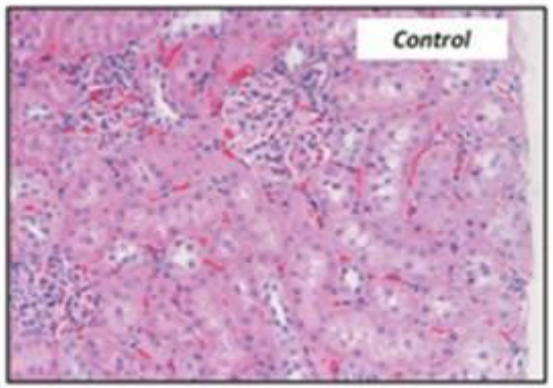
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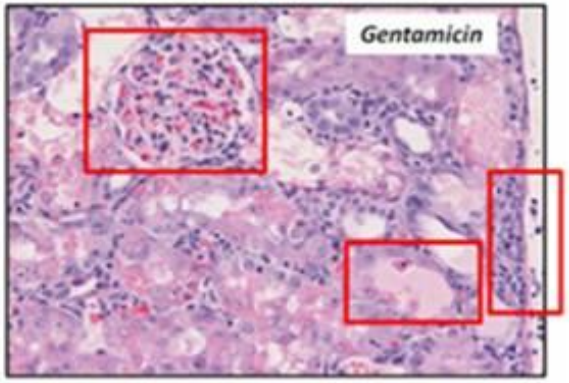
Gentamicin

Figure 1.5. A guinea pig model was chosen as this species demonstrates greater similarity in statin pharmacology to man. Developing a better *in vivo* model would allow comparison of different statins, and dose-ranging work to establish the best statin and dose to take forward into man.

A second aspect of the translational work was to determine whether co-administration of statins with aminoglycosides could have a negative impact on the antibacterial potency of the aminoglycoside. This is important as the ultimate aim of the work is to use statins therapeutically in CF patients receiving tobramycin. Therefore *in vitro* assays were set up to assess the impact of statins on the anti-pseudomonal effect of tobramycin by measurement of the minimum inhibitory concentration (MIC).

The aims of this chapter were to:

1. Investigate the utility of statins to prevent aminoglycoside-induced nephrotoxicity *in vivo*
    a. In an established rat model of gentamicin-induced nephrotoxicity
    b. In a second species (guinea pig) that more closely reflects human statin pharmacology
2. Determine the optimal statin and dose to take forward into human studies using the guinea pig model to inform appropriate dose scaling models
3. Identify any potential inhibitory effects of statins on aminoglycoside pharmacology (bacterial killing) using well established *in vitro* assays

It was hypothesised that:

1. Statins would prevent aminoglycoside-induced nephrotoxicity in both preclinical models via inhibition of megalin-mediated endocytosis in proximal tubule epithelial cells
2. There would be no inhibitory effect of statins on aminoglycoside bacterial killing

6.2 Methods

6.2.1 *In vivo* rat model of gentamicin toxicity and impact of statins

The protocols described were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. Male Sprague Dawley rats received daily intraperitoneal (IP) dosing with gentamicin (200mg/kg/day), rosuvastatin (40mg/kg/day), combined gentamicin and rosuvastatin, or 0.9% saline (2ml/kg, control group) for 9 days at 10am each day. Four animals were included in each group. On day 9, animals were placed into metabolic cages for 2 hours each for collection of urine samples. Urine samples were transferred to 1.5ml eppendorfs and frozen at -80˚C.

Animals were sacrificed at 10am on day 10, 24 hours after the final treatment. Animals were euthanized in their groups, using a rising concentration of carbon dioxide. Blood was obtained from each animal by cardiac puncture. Blood was allowed to clot in the refrigerator for 24 hours. Serum was prepared by the removal of red blood cells by centrifugation at 3000rpm for 1 minute, the supernatant was removed and then frozen at -80˚C for later analysis.

The liver and both kidneys were removed from euthanized animals. Half of the liver and one kidney were placed in 10% formalin. The other half of the liver, and the other kidney were divided into three and immediately frozen in liquid nitrogen, before being transferred to -80˚C.

6.2.1.1 Measurement of urinary NAG

A colorimetric assay was used for the determination of urinary N-Acetyl-β-D-glucosaminidase (NAG) (Roche, Germany). The assay reagent, 3-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide, sodium salt, is hydolysed by NAG, with the release of 3-cresolsulfonphthalein, sodium salt (3-cresol purple) which can be measured photometrically. Prior to running the assay, the reagents were prepared following the manufacturer's instructions.

NAG standard (Roche, Germany) was prepared by dissolving the contents of one bottle in 3ml sterile distilled water to give an initial concentration of 25.40mU/ml. A calibration curve was first prepared from this standard using serial dilutions of 25µl of standard in 25µl distilled water to give further standard concentrations of 12.70, 6.35, 3.18, 1.59, 0.79 and 0.40 mU/ml. A final control calibration standard of distilled water was prepared. Urine samples were removed from the freezer and allowed to thaw at room temperature.

Calibrators and samples were run in triplicate on the same plate. First 100µl of the assay substrate solution (3-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide, sodium salt) was added to each well using a reagent reservoir and a multichannel pipette. The plate was covered and incubated at 37˚C for 5 minutes. Next, 5µl of calibration standard or urine sample was added to each well. The plate was sealed and incubated at 37˚C for 45 minutes, after which 200µl of stop reagent (sodium carbonate) was added to each well. The plate was covered and placed onto a plate shaker at 300rpm for 10 minutes at room temperature. The optical density was then read at 570nm. Sample concentration was calculated using the following formula:

$$Concentration\ (mU/ml) = \ (Absolute - blank\ optical\ density) * 33.33$$

6.2.1.2 Measurement of urinary creatinine

A colorimetric assay was used for the determination of urinary creatinine based on the Jaffe reaction first established in 1886. Prior to running the assay, the assay reagents were prepared as per table 6.1. Reagent A was then wrapped in tin foil to protect it from light.

Table 6.1 Creatinine assay reagents

Reagent	Compound	Preparation	Volume
Reagent A	Sodium hydroxide	1g in 50ml dH2O (0.5M)	50.0ml
	Sodium Phosphate, dibasic	0.7098g in 50ml dH2O (0.1M)	25.0ml
	Sodium borax	1.06g in 50ml dH2O	29.5ml
	Sodium dodecyl sulphate	1.06g in 50ml dH2O	25.0ml
	Picric Acid		45.0ml
	Dimethyl sulphoxide		5.0ml
Reagent B	Acetic Acid		5.0ml
	Concentrated sulphuric acid		1.0ml
	Distilled water		44.0ml

Creatinine standard was prepared by dissolving 50mg creatinine (anhydrous) in 5ml distilled water to give an initial concentration of 10mg/ml (1000mg/dl). A calibration curve was prepared from this standard. 25µl of 1000mg/dl was diluted with 225µl distilled water to give a concentration of 100mg/dl. 100µl of 100mg/dl was added to 150µl distilled water to give a concentration of 40mg/dl. This was the maximum concentration standard for the calibration curve. Serial dilutions were then made of 100µl of the previous standard in 100µl distilled water to give further standard concentrations of 20, 10, 5, 2.5, 1.25 and 0.625mg/dl. A final control calibration standard of distilled water only was prepared. Urine samples were thawed, mixed and centrifuged (3000rpm, 5min). The resulting supernatant was prepared for analysis by ten-fold dilution (20µl sample in 180µl distilled water).

The assay was run in a standard 96 well plate with calibrators and samples run in duplicate on the same plate. First 25µl of standard or sample was added to each well. Next, 125µl of Reagent A was added to each well using a reagent reservoir and multichannel pipette and

incubated at room temperature for 2 minutes. 5µl of Reagent B was added to each well using a reagent reservoir and multichannel pipette. The plate was covered and incubated on a plate shaker at 300rpm for 10 minutes at room temperature. After 10 minutes the optical density was read using a microplate reader at 490nm. A calibration curve was constructed using the measured optical densities for the standard concentrations. The equation for the best fit line was then used to calculate a creatinine concentration from the measured optical density for each sample.

6.2.1.3 Measurement of urinary Kim-1

Urinary Kim-1 was measured using a Rat TIM-1/KIM-1/HAVCR assay kit (Meso Scale Discovery (MSD), US). This assay is a commercially available electrochemiluminescent sandwich immunoassay run on a 96-well plate, and works in the same way as the MSD assays for human urinary KIM-1 and NGAL described in chapter 3.

The assay was run as below, according to the manufacturer's protocol with some slight modification. The assay plate was brought to room temperature. MSD Diluent 5, MSD stock calibrator and urine samples were thawed. 5% MSD Blocker A was prepared by dissolving 1.25g of blocker A powder in 20ml of deionised water, and then adding 5ml of MSD phosphate buffer (5X). Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) was prepared for plate washing. 150µl of Blocker A was added to each well of the plate using a multichannel pipette and reservoir. A reverse pipetting technique was used at all stages in order to avoid the introduction of bubbles. The plate was sealed using an adhesive plate seal, and incubated on a plate shaker at 300rpm at room temperature for 1 hour.

Seven standard concentrations were prepared from the stock calibrator for a standard curve by serial dilutions of the stock calibrator in MSD Diluent 5. The highest calibrator was prepared by adding 10µl of the stock calibrator to 190µl of MSD Diluent 5 to give a

concentration of 10000pg/ml. Six further calibrator concentrations were prepared by serial three-fold dilutions of the previous calibrator (80µl of calibrator in 160µl MSD Diluent 5), to give concentrations of 3333, 1111, 370, 123, 41.2 and 13.7pg/ml. The final calibrator was MSD Diluent 5 only.

Urine samples were thawed, mixed and centrifuged (3000rpm, 5min). The resulting supernatant was prepared for analysis by five-fold dilution (20µl of sample in 80µl MSD Diluent 5).

After 1 hour, the plate was removed from the plate shaker. The plate was inverted over a sink to remove Blocker A, and washed four times with 150µl of PBS-T per well. Following the fourth wash, excess PBS-T was removed from the wells by inverting the plate and banging sharply onto absorbent paper towels on the bench. 25µl of MSD Diluent 5 was added to each well of the plate using a multichannel pipette and reservoir. 25µl of sample or calibrator was then added to each well of the plate. The plate was sealed and incubated on the plate shaker at 300rpm at room temperature for 2 hours.

After 2 hours the plate was removed and washed as before. 25µl of MSD detection antibody solution was added to each well of the plate. This was prepared within 15 minutes prior to use by dilution of 60µl of the 50X stock detection antibody solution in 2.94ml of MSD Diluent 5. The plate was sealed and incubated on the plate shaker at 300rpm at room temperature for 2 hours.

After 2 hours, the plate was removed and washed as before. MSD read buffer T (1X) was prepared during the previous incubation by dilution of 5ml MSD read buffer T (4X) with 15ml deionised water. 150µl of MSD read buffer T (1X) was added to each well of the plate with a reverse pipetting technique using a multichannel pipette and reservoir. The plate

was then immediately read using an MSD SECTOR imager 6000. The plate data was analysed using the MSD DISCOVERY WORKBENCH software (version 4.0), which uses 4-parameter logistic curve fitting to generate the calibration curve.

6.2.2 *In vivo* guinea pig model of gentamicin toxicity and impact of statins

Male Hartley (Crl:HA) guinea pigs (575 – 650g) received IP dosing with gentamicin (0-100mg/kg/day), statin (simvastatin or rosuvastatin, 0.4-40mg/kg/day), combined gentamicin and statin, or 0.9% saline (2ml/kg, control group) in the morning, daily for 9 days. Three to six animals were included in each group. Animals were sacrificed on day 10, 24 hours after the final treatment. Animals were euthanized in their groups, using a rising concentration of carbon dioxide. Blood was obtained from each animal for analysis of serum creatinine and blood urea nitrogen (BUN). Histological analysis of the kidneys was performed. A proximal tubule epithelial cell (PTEC) histology score was used, giving a score from 0 to 5 for assessment of the following: Tubular protein casts; Cortical tubuli which are dilated and devoid of epithelial cells or with necrotic tubular epithelial cells; Focal interstitial mononuclear infiltrates; and Vascular leukocyte recruitment. The protocol described above was developed by us and undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986. The guinea pig model, and the associated analysis of BUN, serum creatinine, and histological samples were conducted by a contract research organisation (Covance).

6.2.3 Impact of statins on MIC of Tobramycin for *Pseudomonas aeruginosa*

The methodology for assessing the effect of statins on the minimum inhibitory concentration (MIC) of tobramycin for *Pseudomonas aeruginosa* was based upon the British Society for Antimicrobial Chemotherapy (BSAC) guidelines (The British Society for Antimicrobial Chemotherapy, 2015). The MIC is the lowest concentration of tobramycin

which inhibits growth of the organism, resulting in an absorbance which does not differ significantly from a blank control.

Broths were made up for four different strains of *Pseudomonas aeruginosa*: PAO1, a widely studied laboratory strain (Stover et al., 2000); LESB58, a resistant epidemic strain (Winstanley et al., 2009); and two clinical isolates from paediatric CF patients, AH13 and AH18. Single colonies of bacteria were transferred from an Agar plate into a glass vial containing 5ml sterile lysogeny broth (LB broth) under aseptic technique. The bacteria containing broth was then mixed and incubated overnight at 37˚C with shaking at 250rpm.

10mM Stock solutions of two statins, atorvastatin and rosuvastatin, were prepared by dissolving 10µM of the dry statin powder in 1ml of dimethylsulphoxide (DMSO). Further concentrations of 1mM, 100µM and 10µM were prepared by serial tenfold dilution of the previous concentration (100µl of previous concentration in 900µl DMSO).

Bacterial suspensions in Iso-sensitest broth (ISB) were prepared from the overnight LB broth incubations as follows to give a 1 in 10 dilution of a 0.5 Macfarlane turbidity standard. 20µl of each incubated LB broth was transferred into 10ml ISB in a sterile glass vial.

For the MIC experiments, a 256µg/ml solution of tobramycin in ISB was prepared by adding 256µl of Tobramycin 10mg/ml standard (aliquots kept frozen at -20˚C) to 10ml ISB in a sterile vial.

100µl of plain ISB broth was added to each well in columns 2 to 11 of a sterile 96 well plate. Then 200µl of tobramycin 256µg/ml ISB was added to each well in column 1. Serial twofold dilutions were carried out by transferring 100µl from each well in column 1 to column 2, then from column 2 to column 3 and so on until column 11. 100µl was then removed from

each well in column 11 and discarded. 100µl of ISB containing the PAO1 strain of *P. aeruginosa* was added to each well in Rows A-D (Columns 1-11), and 100µl of LESB58 ISB was added to Rows E-H (Columns 1-11). A second plate was prepared in the same way for strains AH13 and AH18. 2µl of the statin solution was added to each well in Rows A-C and E-G (Columns 1-11) to give a 1 in 100 dilution of the statin from the starting concentration (i.e. 10mM becomes 100µM, 1mM to 10µM, 100µM to 1µM, and 10µM to 0.1µM). 200µl plain ISB was then added to each well in column 12 to give a blank control (no bacteria or antimicrobial). An example plate layout is given in Figure 6.1.

Column		1	2	3	4	5	6	7	8	9	10	11	12	**Statin**
Pseudomonas Strain 1	A													Statin
	B													
	C													
	D													None
Pseudomonas Strain 2	E													Statin
	F													
	G													
	H													None
Tobramycin Conc (µg/ml)		128	64	32	16	8	4	2	1	0.5	0.25	0.125	Blank	

Figure 6.1 – Example plate layout diagram for MIC experiments using 96-well plate

Separate plates were prepared as above for each statin at each concentration for each strain of *P. aeruginosa* (two strains per plate). The plates were then incubated at 37˚C for 24 hours. After 24 hours, optical density of each well was measured at a wavelength of 600nm.

The effect of statins alone on the growth of the four strains of *P. aeruginosa* was assessed using a modification of the methodology above. Pseudomonal inoculums and statin

concentrations were prepared as before. 100µl of plain ISB broth was added to each well in columns 1 to 8 of a sterile 96 well plate. 100µl of ISB containing one strain of *Pseudomonas aeruginosa* was then added to each well in Columns 1-4, and 100µl of ISB containing a second strain was added to Columns 5-8. A second plate was prepared in the same way for the other two strains. 2µl of atorvastatin solution was added to each well in Rows A-C and 2µl of rosuvastatin solution was added to each well in E-G (Columns 1-8) to give a 1 in 100 dilution of the statin from the starting concentration (i.e. 10mM becomes 100µM, 1mM to 10µM, 100µM to 1µM, and 10µM to 0.1µM). Cells were prepared in triplicate. Blank controls, using 200µl plain ISB, were also prepared (no bacteria or statin). The plates were then incubated at 37˚C. At 24 hours, optical density of each well was measured at a wavelength of 600nm.

6.3 Results

6.3.1 *In vivo* rat model of gentamicin toxicity and impact of statins

Sprague Dawley rats were treated with solvent (control group), gentamicin (200mg/kg/day), rosuvastatin (40mg/kg/day), or gentamicin combined with rosuvastatin for 9 days. Gentamicin-induced nephrotoxicity was confirmed by significantly increased urinary concentrations of NAG and kim-1 at day 9 in the gentamicin treated group versus control. In the rosuvastatin treated group, NAG and kim-1 concentrations were not significantly different from those in the control group, and were significantly lower than in the gentamicin group. The gentamicin and rosuvastatin co-treatment group again had NAG and kim-1 concentrations not significantly different from control, and significantly lower than in the gentamicin group (Figure 6.2).

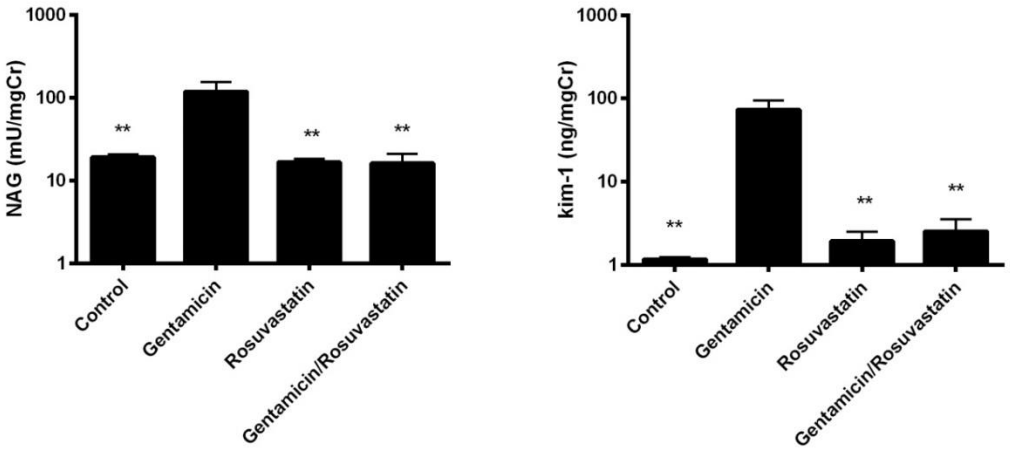

Figure 6.2 - Gentamicin-induced nephrotoxicity in a rat model, and the impact of rosuvastatin. Sprague Dawley (SD) rats (N=4 per group) were treated with solvent (control group), gentamicin (200mg/kg/day), rosuvastatin (40mg/kg/day), or gentamicin & rosuvastatin for 9 days. Nephrotoxicity was measured using urinary NAG and kim-1 on urine samples collected within 24 hours after the final dose. Results are mean (plus standard error) for 4 animals per group. An ordinary one-way ANOVA was completed using Dunnett's multiple comparisons test to compare the means between each treatment group and the gentamicin only group (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

6.3.2 *In vivo* guinea pig model of gentamicin toxicity and impact of statins

An initial experiment was conducted to assess the nephrotoxicity of gentamicin in this model at various doses (0-100mg/kg/day), and to choose an appropriate dose to take forward in subsequent experiments. Nephrotoxicity was assessed using serum creatinine and a PTEC histology score (Figure 6.3). A gentamicin dose of 100mg/kg/day was chosen to take forward to further experiments as it resulted in significant nephrotoxicity by both serum creatinine and histology measures.

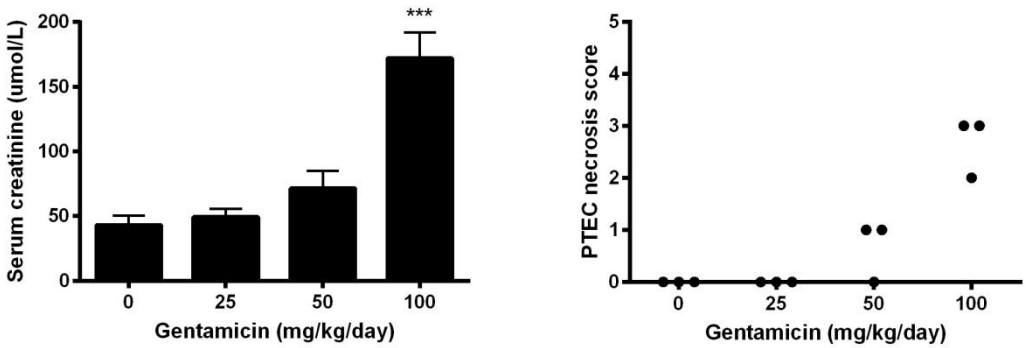

Figure 6.3 - Gentamicin-induced nephrotoxicity in a guinea pig model. Guinea pigs (N=3 per group) were treated with solvent (control group) or gentamicin (25, 50 or 100mg/kg/day) for 9 days. Nephrotoxicity was measured using serum creatinine and a proximal tubule epithelial cell (PTEC) necrosis score. Serum creatinine results are mean (plus standard error) for 3 animals per group. An ordinary one-way ANOVA was completed using Dunnett's multiple comparisons test to compare the means between each treatment group and control (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). PTEC necrosis scores are presented as data points for each animal.

Having established a dose of gentamicin to take forward for this model, the next step was to assess the hypothesis that statins would inhibit gentamicin-induced nephrotoxicity. Simvastatin and rosuvastatin were compared at a range of doses (0.4 to 40mg/kg/day). These doses were chosen with an upper limit equal to the dose used in the rat model. Nephrotoxicity was assessed using serum creatinine and blood urea nitrogen (BUN) (Figure 6.4). Rosuvastatin significantly reduced gentamicin-associated nephrotoxicity, whereas simvastatin did not. The inhibition of nephrotoxicity due to rosuvastatin was dose-dependent, with only a slight reduction at 0.4mg/kg/day, but doses of 4 and 40mg/kg/day resulted in serum creatinine and BUN concentrations no different to control animals.

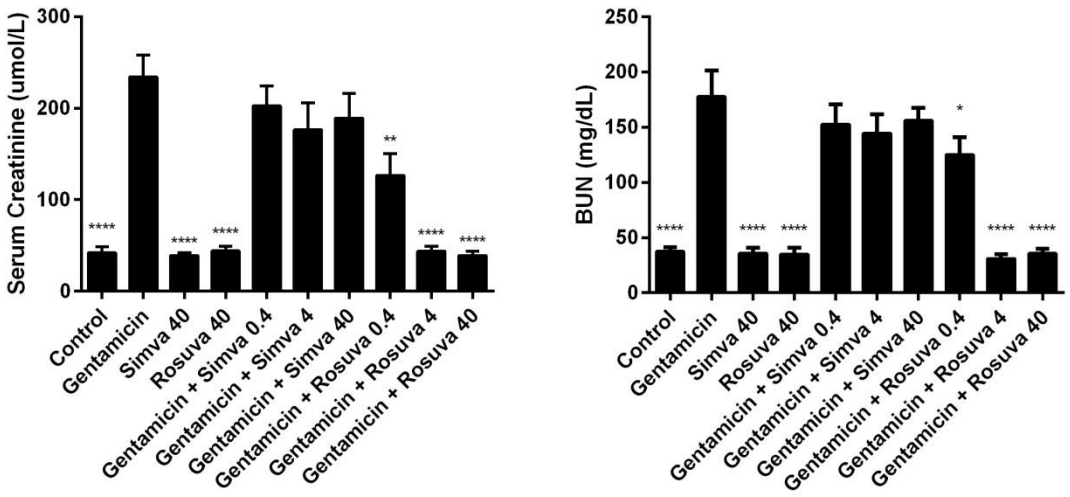

Figure 6.4 - Gentamicin-induced nephrotoxicity in a guinea pig model, and the impact of statins. Guinea pigs (N=6 per group) were treated with solvent (control group), gentamicin (100mg/kg/day), statin, or combined gentamicin and statin (simvastatin or rosuvastatin, 0.4, 4 or 40mg/kg/day) for 9 days. Nephrotoxicity was measured using serum creatinine and blood urea nitrogen (BUN). Results are mean (plus standard error) for 6 animals per group. An ordinary one-way ANOVA was completed using Dunnett's multiple comparisons test to compare the means between each treatment group and the gentamicin only group (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

Having established that inhibition of nephrotoxicity was seen with rosuvastatin, but not with simvastatin, the next step was to determine the lowest effective dose of rosuvastatin in this model. This would inform the choice of dose to take forward into a future clinical trial in human subjects. In guinea pigs, the minimum effective dose lies between 0.4 and 4mg/kg/day (Figure 6.4). Equivalent doses of rosuvastatin in a 40kg child (an approximation for the target population, children with cystic fibrosis aged 10 and above) were calculated using the following dose scaling algorithm (Reagan-Shaw et al., 2008):

$$Human\ dose\ (mg/kg) = Animal\ dose\ (mg/kg) * Animal\ Km/Human\ Km$$
$$Human\ Km\ for\ 40kg\ child\ (BSA\ 1.3m^2) = 30$$
$$Animal\ Km\ for\ Guinea\ Pig = 8$$

Using this formula, doses of 0.4 and 4mg/kg/day in a guinea pig equate to doses of 4.4 and 44mg/day in a 40kg child. Rosuvastatin is licensed at doses of 5 to 20mg/day in children. As these doses lie in the range of interest, equivalent doses for a guinea pig were back-calculated using the same dose scaling algorithm (Table 6.2). These doses were taken forward to a final dose-ranging experiment in the guinea pig model. Nephrotoxicity was assessed using serum creatinine, BUN, and a PTEC histology score (Figure 6.5). Dose-dependent inhibition of nephrotoxicity by rosuvastatin was demonstrated again. The 0.47mg/kg/dose resulted in slightly lower serum creatinine, BUN and PTEC necrosis scores compared to gentamicin alone. However, doses of 0.94mg/kg/day and above resulted in serum creatinine and BUN concentrations and PTEC necrosis scores no different to control animals.

Table 6.2 – Rosuvastatin dose scaling

Dose in 40kg child	Equivalent guinea pig dose
5 mg/day	0.47 mg/kg/day
10 mg/day	0.94 mg/kg/day
20 mg/day	1.88 mg/kg/day
40 mg/day	3.75 mg/kg/day

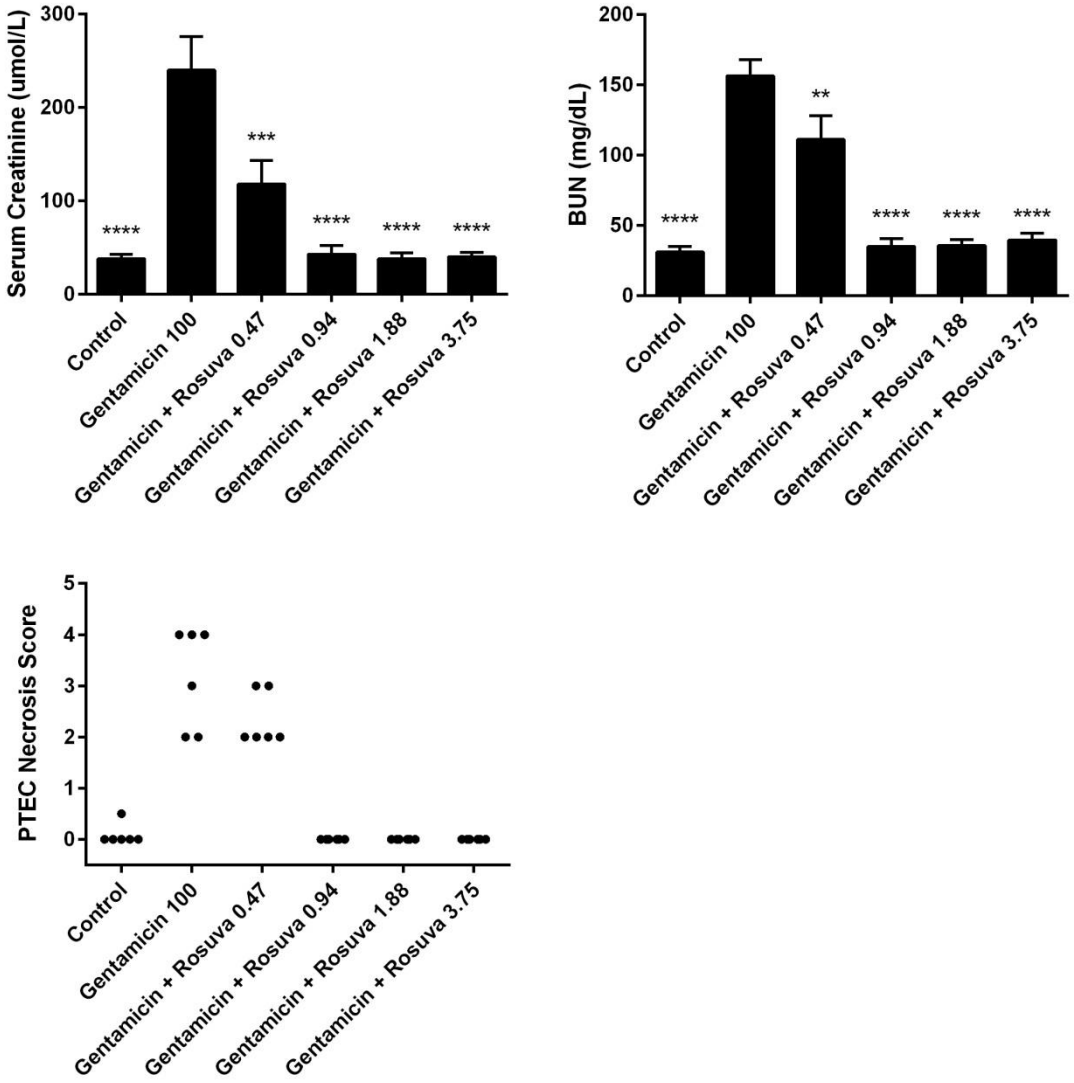

Figure 6.5 – The impact of rosuvastatin on gentamicin-induced nephrotoxicity in a guinea pig model. Guinea pigs (N=6 per group) were treated with solvent (control group), gentamicin (100mg/kg/day), or combined gentamicin and rosuvastatin (0.47, 0.94, 1.88 or 3.75mg/kg/day) for 9 days. Nephrotoxicity was measured using serum creatinine, blood urea nitrogen (BUN) and a proximal tubule epithelial cell (PTEC) necrosis score. Serum creatinine and BUN results are mean (plus standard error) for 6 animals per group. An ordinary one-way ANOVA was completed using Dunnett's multiple comparisons test to compare the means between each treatment group and the gentamicin only group (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

6.3.3 Impact of statins on the MIC of Tobramycin for *Pseudomonas aeruginosa*

MICs of tobramycin for four strains of *P. aeruginosa* (AH13, AH18, PAO1 and LESB58) were derived, and the effect of rosuvastatin and atorvastatin on the MIC was investigated (

Figure 6.6). In the absence of a statin (control), the MIC of tobramycin for AH13 was 4µg/ml, for AH18 was 16µg/ml, for LESB58 was 8µg/ml, and for PAO1 was 1µg/ml. Neither statin, at any concentration, led to a change in the MIC compared to control.

From the initial MIC experiments, there was a suggestion that higher concentrations of the statins appeared to reduce the impact of tobramycin upon the LESB58 strain (resulting in increased absorbance). There was however no change in MIC. A repeat MIC experiment, comparing the effect of atorvastatin 100µM and rosuvastatin 100µM with control confirmed no effect of either statin on the MIC compared to control (Figure 6.7). In the earlier experiment an MIC of tobramycin for LESB58 of 8µg/ml was recorded (

Figure 6.6), but in the repeat experiment was found to be 16µg/ml (Figure 6.7). This is within the acceptable realms of reproducibility as stated in the BSAC guidelines (The British Society for Antimicrobial Chemotherapy, 2015).

Furthermore, there was no impact of either statin (atorvastatin or rosuvastatin) at any concentration on the growth of any of the four strains of *P. aeruginosa* over a 24 hour period (Figure 6.8).

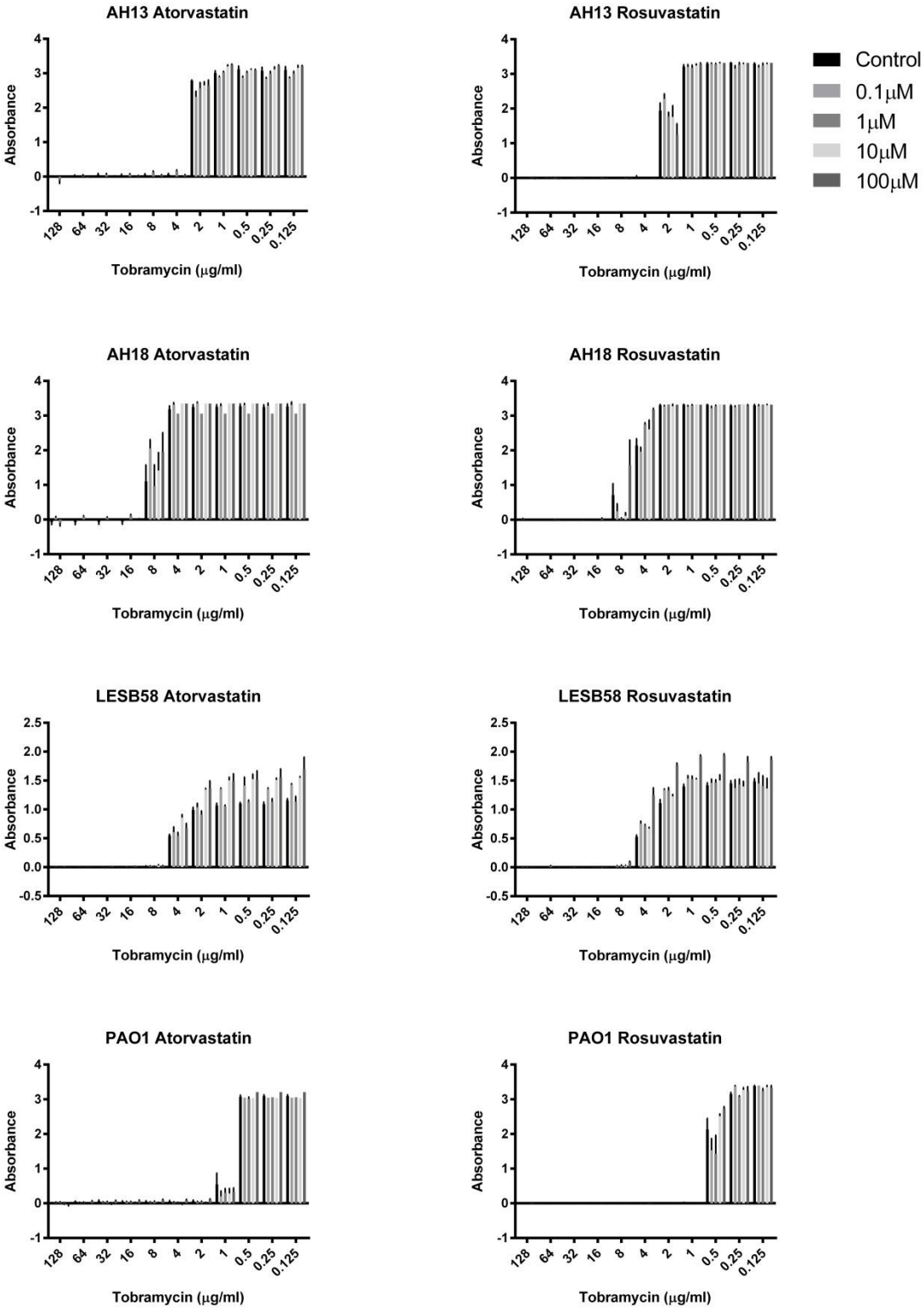
AH13 Atorvastatin
AH13 Rosuvastatin
AH18 Atorvastatin
AH18 Rosuvastatin
LESB58 Atorvastatin
LESB58 Rosuvastatin
PAO1 Atorvastatin
PAO1 Rosuvastatin
Absorbance
Tobramycin (μg/ml)
128 64 32 16 8 4 2 1 0.5 0.25 0.125
Control
0.1μM
1μM
10μM
100μM

Figure 6.6 – The effect of statins on the minimum inhibitory concentration of tobramycin for four strains of Pseudomonas aeruginosa. Inoculums of each strain of Pseudomonas aeruginosa (AH13, AH18, LESB58, PAO1) were exposed to a range of concentrations of tobramycin (0-128μg/ml) in the presence or absence of a statin (atorvastatin or rosuvastatin) at a range of concentrations (0.1-100μM). Absorbance was measured after 24 hours at a wavelength of 600nm. Results are mean (plus standard error) of three replicates on a single plate. In the absence of statin (control), the MIC of tobramycin for AH13 is 4μg/ml, for AH18 is 16μg/ml, for LESB58 is 8μg/ml, and for PAO1 is 1μg/ml. Neither statin, at any concentration, leads to a change in MIC compared to control.

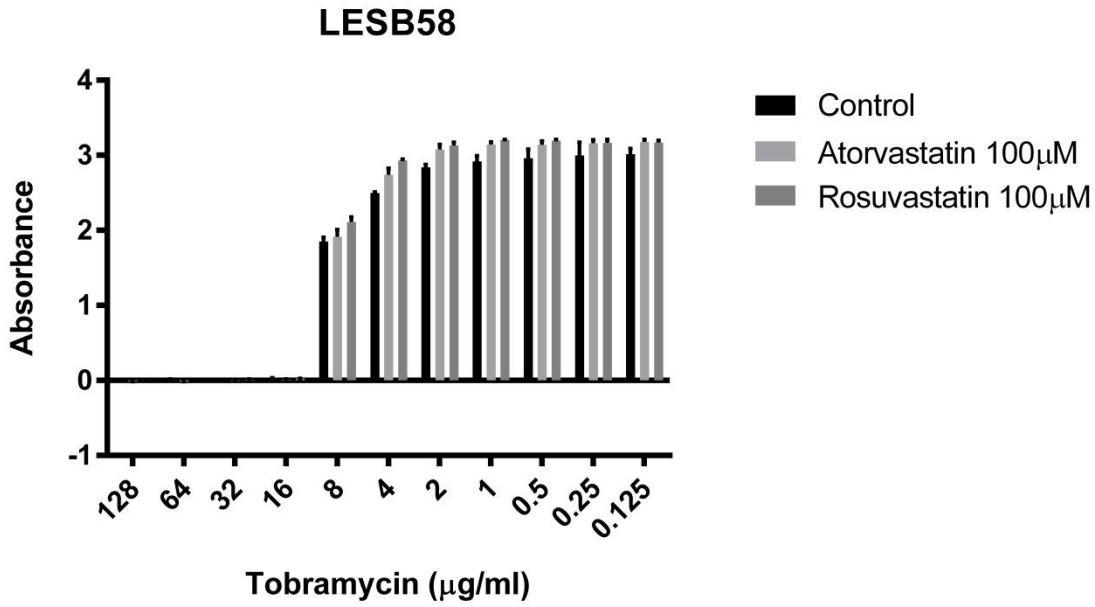

Figure 6.7 – The effect of statins on the minimum inhibitory concentration of tobramycin for the LESB58 strain of Pseudomonas aeruginosa. Inoculums of LESB58 were exposed to a range of concentrations of tobramycin (0-128μg/ml) in the presence or absence of 100μM of a statin (atorvastatin or rosuvastatin). Absorbance was measured after 24 hours at a wavelength of 600nm. Results are mean (plus standard error) of three replicates on a single plate. In the absence of statin (control), the MIC of tobramycin for LESB58 is 16μg/ml. Neither statin, at any concentration, leads to a change in MIC compared to control.

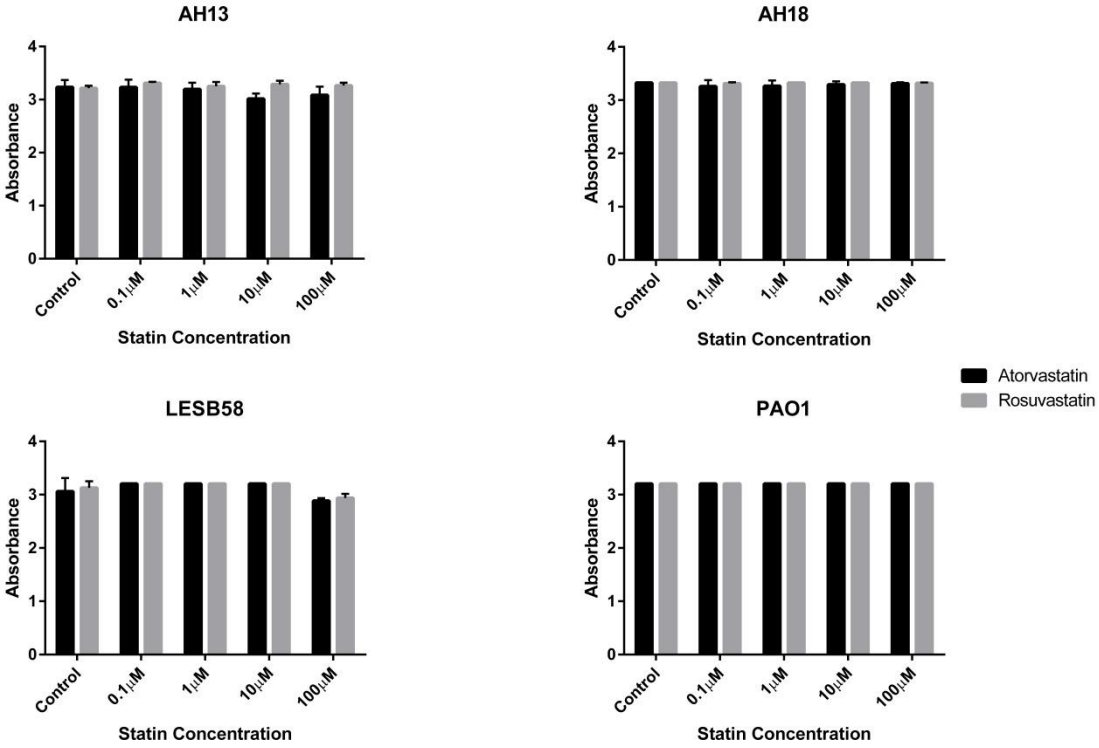

Figure 6.8 – The effect of statins on the growth of four strains of Pseudomonas aeruginosa in the absence of tobramycin. Inoculums of each strain of Pseudomonas aeruginosa (AH13, AH18, LESB58, PAO1) were exposed to a statin (atorvastatin or rosuvastatin) at a range of concentrations (0.1-100μM) and compared to control (no statin). Absorbance was measured after 24 hours at a wavelength of 600nm. Results are mean (plus standard error) of three replicates on a single plate. Two-way ANOVA was undertaken utilizing Dunnet's multiple comparisons test to compare each concentration of statin to control. There was no significant effect of either statin, at any concentration, on the growth of any of the four strains of Pseudomonas aeruginosa.

6.4 Discussion

The data presented in this chapter show that statins inhibit aminoglycoside-induced nephrotoxicity in both rat and guinea pig models. Inhibition of gentamicin-induced nephrotoxicity was demonstrated using both atorvastatin and rosuvastatin in rats, and with rosuvastatin (but not simvastatin) in guinea pigs. The development of a new guinea pig model of gentamicin-induced nephrotoxicity allowed dose-ranging studies which will inform the choice of statin and dose to take forward into clinical trials in humans. *In vitro*

models demonstrated that neither rosuvastatin nor atorvastatin had any effect on the antipseudomonal effects of tobramycin.

Inhibition of aminoglycoside-induced nephrotoxicity has previously been demonstrated *in vitro* (Antoine et al., 2010) and in a rat model using atorvastatin (Figure 1.5). Here, inhibition of gentamicin-induced nephrotoxicity was confirmed in the same rat model using rosuvastatin (Figure 6.2). This builds upon existing literature demonstrating inhibition of gentamicin-induced nephrotoxicity in rats using simvastatin (Chinnapa Reddy et al., 2012, Jabbari et al., 2011). An important aspect of the rat model was the use of urinary kim-1 as an outcome measure for nephrotoxicity. Whilst its use in rat models of aminoglycoside nephrotoxicity is well described (Vaidya et al., 2010), its use here provides translational consistency with the previously described observational studies of aminoglycoside-induced nephrotoxicity in man, and sets a precedent for its use as an outcome measure in a future clinical trial investigating the use of statins to inhibit aminoglycoside-induced nephrotoxicity.

Inhibition of HMG-CoA reductase in rats leads to reduced plasma triglyceride concentrations due to decreased hepatic production (Krause and Newton, 1995), whereas in other species, the principal drug effect is reduced plasma cholesterol. Guinea pigs demonstrate a high degree of similarity to humans in their cholesterol and lipoprotein metabolism, and in their response to drugs acting on these pathways (West and Fernandez, 2004). Therefore the development of a guinea pig model of aminoglycoside-induced nephrotoxicity was a key translational step. The data presented in this chapter shows that inhibition of nephrotoxicity occurred with rosuvastatin, but not with simvastatin. Simvastatin demonstrated greater inhibition of gentamicin-induced cytotoxicity *in vitro* than rosuvastatin (Antoine et al., 2010), but the different pharmacokinetic properties of

these two statins reverse this *in vivo*. Simvastatin is lipophilic, and has over 80% extraction by the liver, mainly by cytochrome P450 3A metabolism (Neuvonen et al., 2008). By contrast, rosuvastatin is more hydrophilic, demonstrates minimal metabolism by cytochrome P450 enzymes, and is 28% renally cleared, with 90% of that accounted for by active uptake by proximal tubule cells (Martin et al., 2003, McTaggart et al., 2001). The fact that simvastatin underwent significant hepatic extraction and does not show renal elimination probably explains why it is unable to exert any real effect at the proximal tubule in the guinea pig model.

Rosuvastatin demonstrates dose-dependent inhibition of gentamicin-induced nephrotoxicity, and the guinea pig model allowed some dose-scaling which will inform the choice of dose to take forward into clinical studies. The target population for the clinical trial will be children with cystic fibrosis aged 10 years and over (as rosuvastatin is not licensed for use in younger children) receiving treatment with tobramycin. Based upon the dose-scaling work completed in the guinea pig model, rosuvastatin, at a dose of 10mg per day will be taken forward into the clinical trial discussed in chapter 7.

Whilst the clinical trial will take place in children receiving tobramycin, the guinea pig model used gentamicin to induce nephrotoxicity. Gentamicin was used to provide continuity with the rat model, with the knowledge that the mechanism of toxicity, and in particular dependence upon megalin-mediated endocytosis, is similar for both compounds (Lopez-Novoa et al., 2011). Furthermore, gentamicin and tobramycin are similar in their potential for nephrotoxicity *in vivo* (Begg and Barclay, 1995). Further continuity between the rat and guinea pig models would have been provided by assessing atorvastatin in the guinea pig model. However, atorvastatin is hepatically extracted, with only around 1% renally excreted (Lennernäs, 2003), and is therefore likely to have a similar effect to

simvastatin. Having demonstrated the effectiveness of rosuvastatin, it was felt that the additional time, cost, and, most importantly, animals, required to undertake this analysis was unnecessary.

Neither rosuvastatin nor atorvastatin had any effect on the minimal inhibitory concentration of tobramycin for any of the four strains of *P. Aeruginosa* (two lab strains and two patient isolates) studied *in vitro*. Neither did these statins alone have any effect on pseudomonal growth. This was important to demonstrate as the planned clinical trial will investigate the co-administration of a statin with an aminoglycoside to children with cystic fibrosis, who are likely to be colonised with *P. Aeruginosa*. Whilst statins have been associated with improved survival from sepsis (Kruger et al., 2013), and community acquired pneumonia (Khan et al., 2013), they have also been associated with an increased incidence of common infections (Magulick et al., 2014). Statins have previously demonstrated no antimicrobial activity against a number of gram negative rods, including *P. Aeruginosa* (Farmer et al., 2013), although other groups suggest that statins may reduce *P. Aeruginosa* virulence (Hennessy et al., 2013, Sarabhai et al., 2015) and modulate the pulmonary cellular immune response (Hennessy et al., 2014).

A weakness of the work presented in this chapter is that we have not assessed the impact of statins on aminoglycoside pharmacology in the same *in vivo* model which we have used to look at nephrotoxicity. Whilst it would be possible to look at the bactericidal effects of the aminoglycosides in the same model, this would have entailed significant additional expense and a larger number of animals. Had the *in vitro* assessment of the effect of statins on the MIC of tobramycin for *P. Aeruginosa* suggested any adverse effect, it would have been important to assess this *in vivo* prior to translation into man.

This translational work has investigated the hypothesis that statins can inhibit aminoglycoside-induced nephrotoxicity by inhibition of megalin-mediated endocytosis in proximal tubule cells. This hypothesis was first tested in an *in vitro* OK cell model (Antoine et al., 2010), and then in rat and guinea pig models. Taken together, all the data confirm the hypothesis that statins may prevent aminoglycoside-induced nephrotoxicity. Importantly, further *in vitro* work demonstrated that there was no detrimental effect of the statins on the antipseudomonal effect of aminoglycosides. The data overall allowed the selection of a statin and dose (rosuvastatin 10mg) to be taken forward into a clinical trial to test this hypothesis in children with cystic fibrosis (chapter 7).

7 Phase IIa, Randomised, Controlled, Open-label Trial of Rosuvastatin for the Prevention of Aminoglycoside-Induced Kidney Toxicity in Children with Cystic Fibrosis

7.1 Protocol Summary

Title: PROteKT

Phase IIa, Randomised, Controlled, Open-Label Trial of Rosuvastatin for the Prevention of Aminoglycoside-Induced Kidney Toxicity in Children with Cystic Fibrosis

Phase: IIa

Study Design: This study is a phase IIa, multi-centre, randomised, controlled, open-label trial of rosuvastatin in children with cystic fibrosis (CF) receiving clinically indicated treatment with the intravenous (IV) aminoglycoside antibiotic, tobramycin. Patients will be randomised equally to either receive rosuvastatin 10mg once daily or no intervention (control), throughout a course of treatment with IV tobramycin (usually lasting 14 days).

Study Objectives:

Primary Objective: This trial will evaluate the effect of rosuvastatin on aminoglycoside-induced nephrotoxicity. This will be assessed using the difference in mean fold-change in urinary KIM-1 from baseline to 'highest value' concentration during exposure to tobramycin between the rosuvastatin treated arm and control arm.

Secondary Objectives:

1. Change in serum concentration of creatinine and eGFR during tobramycin exposure between rosuvastatin treated arm and the control arm.
2. Change in other urinary and plasma biomarkers of renal injury during tobramycin exposure between rosuvastatin treated arm and the control arm.

3. Difference in serious adverse events between rosuvastatin treated arm and the control arm.
4. Difference in tobramycin concentrations between rosuvastatin treated arm and the control arm to identify any pharmacokinetic interaction between rosuvastatin and the tobramycin.
5. Difference in Forced Expiratory Volume in 1 second (FEV1) and C-Reactive Protein, between rosuvastatin treated arm and the control arm to identify any pharmacodynamics interaction between rosuvastatin and the tobramycin
6. Assessment of plasma rosuvastatin concentrations achieved in children randomised to the intervention arm.
7. Difference in biomarkers of *Pseudomonas aeruginosa* between rosuvastatin treated arm and the control arm.
8. Assess the feasibility of collecting DNA for a molecular genetic study of aminoglycoside-induced nephrotoxicity.

Population: Children with cystic fibrosis aged 10 to 18 years receiving clinically indicated treatment with intravenous tobramycin, and who fulfil the inclusion criteria.

Criteria for Inclusion:

1. Age 10 to 18 years inclusive.
2. Diagnosis of cystic fibrosis (established by sweat test or genotype).
3. Planned, clinically indicated, course of treatment with IV tobramycin.
4. Ability to give informed consent.
5. Willingness to comply with all study requirements.
6. Able to take tablets.

Criteria for Exclusion:

1. Existing treatment with a statin.

2. Previous adverse reaction to a statin.
3. Co-enrolment in other drug trials*, or completion of a previous CTIMP within the last 30 days.
4. Previous randomisation in the PROteKT trial.
5. Patients taking any of the following medications: Ciclosporin, Protease Inhibitors, Fibrates, Ezetimibe, Erythromycin (but not other macrolides), Eltrombopag, Dronedarone, Itraconazole, Coumarins, Oral contraceptives, nicotinic acid, fusidic acid.
6. Female participants who are pregnant or lactating or refuse a pregnancy test if of childbearing potential (female participants of childbearing potential must use a barrier method of contraception if sexually active whilst taking rosuvastatin and for 7 days afterwards).
7. Patients of Asian ancestry (Japanese, Chinese, Filipino, Vietnamese, Korean and Indian).
8. Patients with renal disease (eGFR<60ml/min/1.73m^2, using the Schwartz formula, in the 6 months preceding screening).
9. Patients with current elevation in transaminases exceeding 3x the upper limit of normal.
10. Family history, or personal history, of hereditary muscular disorders.
11. Patients with myopathy.
12. Patients with a history of, or active alcohol abuse.
13. Patients with hypothyroidism.
14. Patients with galactose intolerance, the Lapp lactase deficiency, or glucose-galactose malabsorption.
15. Patients who are Hepatitis C positive or HIV-positive.

*Patients who are currently taking part in TORPEDO-CF are allowed to take part in PROteKT as long as their date of randomisation into TORPEDO-CF is not within the previous six months of the screening date for PROteKT.

Study Centres and Distribution: Paediatric cystic fibrosis centres in the UK

Study Duration: The recruitment period will last 18 months

Description of Agent/ Intervention: Oral rosuvastatin 10mg once daily.

Number of participants to be enrolled: Up to 50 participants will be enrolled to account for loss to follow-up. A minimum of 20 participants in each arm of the study is required.

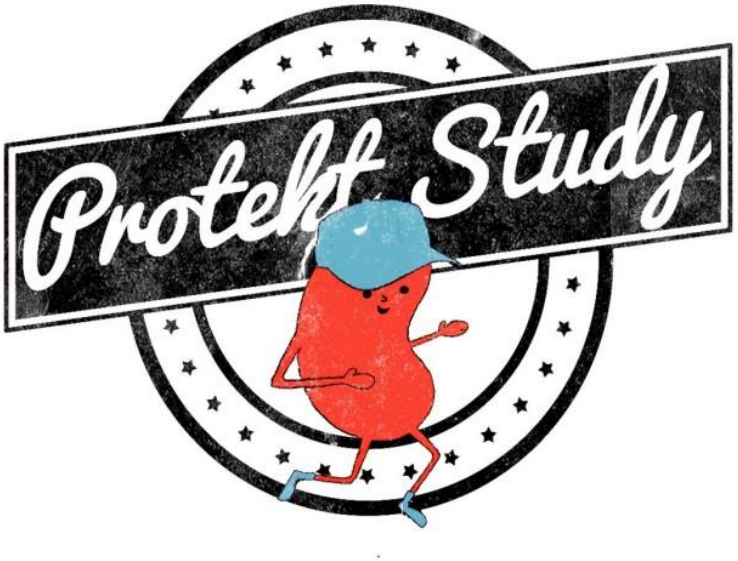

Figure 7.1 - The PROteKT study logo

PROteKT Study Flow chart

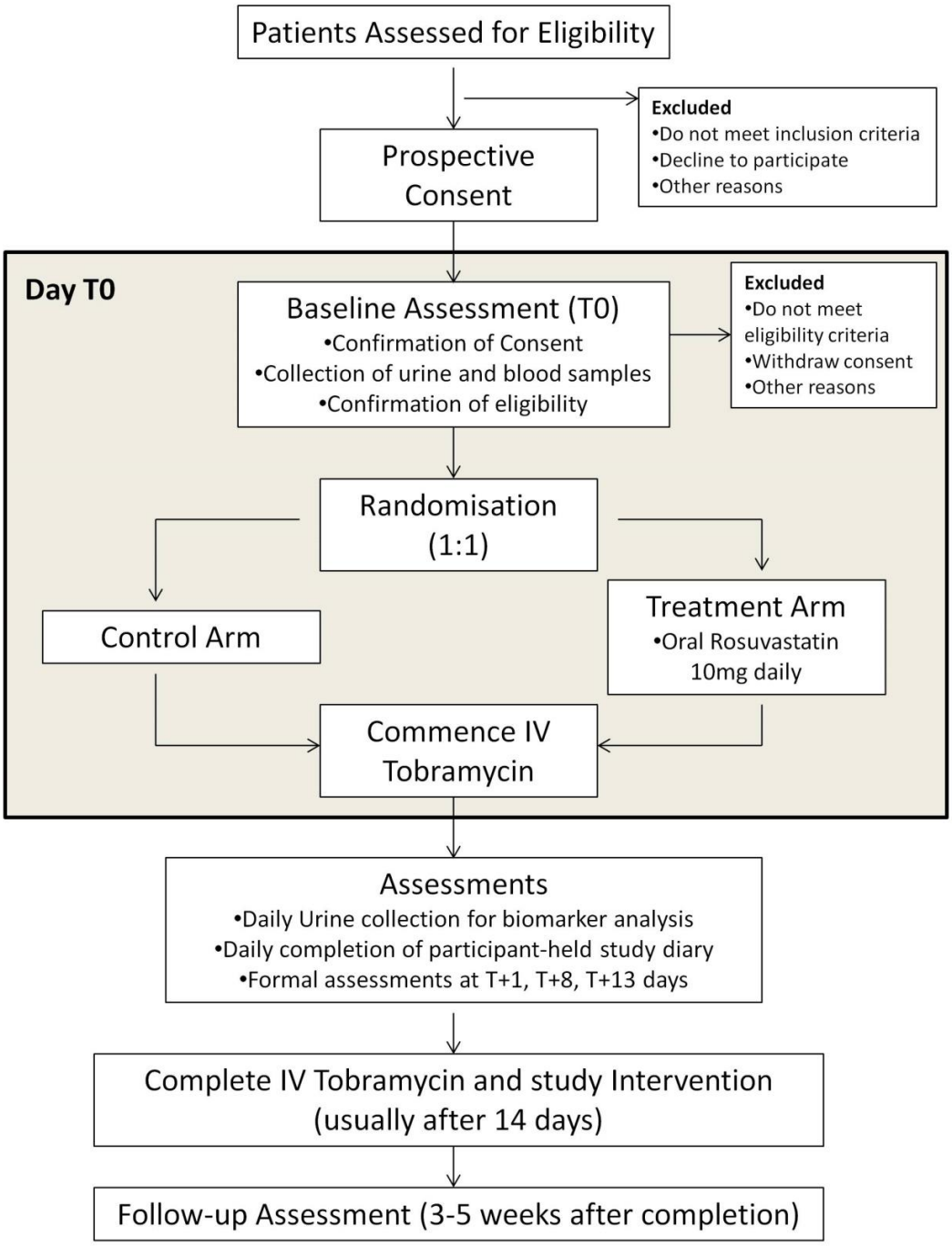

Figure 7.2 – PROteKT study flowchart

7.2 Background Information

7.2.1 Background and Rationale

Introduction: Cystic Fibrosis (CF) is a common, inherited, life-limiting disease which affects around 9000 people in the UK alone (Cystic Fibrosis Trust, 2011). 25% of children with CF aged 12-15 years have chronic pulmonary infection with *Pseudomonas aeruginosa*, and 40% by age 16-19 years (Cystic Fibrosis Trust, 2011). There is improved survival with 2 week courses of intravenous (IV) antibiotics (an aminoglycoside plus a beta-lactam) up to 4 times per year. Aminoglycosides (AGs) are, however, potentially nephrotoxic. A UK national survey of Acute Kidney Injury (AKI) in patients with CF found 24 cases between 1997 and 2004 (Bertenshaw et al., 2007). They estimated an incidence of AKI of 4.6 to 10.5 cases per 10000 CF patients per year. In a follow-on case-control study, they identified an 80-fold increase in the risk of AKI if CF patients received an aminoglycoside within the preceding week (Smyth et al., 2008). Renal impairment (reduced creatinine clearance) is present in 31-42% of adult CF patients, and is associated with cumulative aminoglycoside exposure (P=0.0055) (Al-Aloul et al., 2005a, Pedersen et al., 1987). Current strategies for the prevention of AG-induced nephrotoxicity include extended-interval dosing, and drug trough level monitoring with dose adjustment, but these are only partially effective. Aminoglycosides are an important part of the management of children with CF, helping to improve survival. It is, however, important to develop further strategies to minimise their nephrotoxic consequences.

Assessing aminoglycoside-induced nephrotoxicity: Aminoglycoside-induced nephrotoxicity is characterized by selective targeting of the proximal tubule cells within the renal cortex. Accumulation of the drug within the proximal tubule epithelial cells, following glomerular filtration, is thought to be the key determining mechanism for the development of toxicity

(Mathews and Bailie, 1987). Endocytosis via the multi-ligand receptor, megalin, has been demonstrated to be the principal pathway for this accumulation (Schmitz et al., 2002). Intracellular aminoglycoside can result in apoptosis and necrosis of proximal tubule epithelial cells by a variety of pathways (mitochondrial dysfunction and the release of reactive oxygen species) (Servais et al., 2005, Servais et al., 2008).

Traditional measures of kidney function and of kidney injury rely on measurement of serum markers such as creatinine and blood urea nitrogen. These markers require invasive blood tests, and the information they provide is limited. Serum creatinine (sCr) is used currently to detect acute kidney injury (AKI), but is of limited value since any change in its concentration is a delayed response with levels rising significantly above baseline levels only when 25-50% of renal function has been lost (Askenazi et al., 2009). Furthermore, it is a marker of glomerular filtration, and therefore not specific to damage at other sites in the kidney nephron. Oliguria is, at best, a late sign of AKI, and may not be present in many forms of AKI especially those related to toxins (Karlowicz and Adelman, 1995).

Advantages of KIM-1: There has been increasing interest recently in the development of novel urinary biomarkers, which not only are more sensitive than urea and creatinine, but also delineate damage to different parts of the nephron (Bonventre, 2007, Coca et al., 2008), and have the advantage of being non-invasive. Particularly promising is Kidney Injury Molecule-1 (KIM-1). KIM-1 is upregulated in proximal tubule epithelial cells in response to nephrotoxins, and excreted in the urine (Han et al., 2002, Ichimura et al., 2004). Its upregulation parallels the degree of renal damage seen on histopathology in rodent studies (Zhou et al., 2008). Moreover, recent reports from a multi-site validation investigation in animal models treated with a number of nephrotoxins, suggest that Kim-1 (KIM-1 in man)

can outperform, with respect to sensitivity and specificity, a number of traditional and novel biomarkers of AKI (Vaidya et al., 2010).

In clinical trials, KIM-1 has been shown to be an early diagnostic marker of acute kidney injury (AKI) (Bonventre, 2007). A systematic review found it to be one of the best performing biomarkers for the differential diagnosis of established AKI and for the prediction of mortality risk following AKI (Coca et al., 2008). In our recent work, we measured various urinary biomarkers (KIM-1, Neutrophil Gelatinase-associated Lipocalin (NGAL), and N-acetyl-β-D-glucosaminidase (NAG)) in 41 neonates exposed to gentamicin for treatment of neonatal sepsis. We found a rise in KIM-1 levels during treatment with gentamicin, which remained significant even after adjusting for confounders (McWilliam et al., 2012).

We have recently completed a study (URBAN CF) in healthy children to establish reference intervals for urinary KIM-1 and NGAL, as an important step in the validation of these two biomarkers for use in the paediatric population (McWilliam et al., 2014). A longitudinal study in children with cystic fibrosis is ongoing. A preliminary analysis has demonstrated a significant correlation between baseline KIM-1 and the number of previous courses of aminoglycosides ($R=0.70$, $P<0.002$). During exposure to tobramycin, mean 'highest value' KIM-1 was significantly elevated from baseline (mean 'highest value' KIM-1, 1.24ng/mg Cr, 95% CI, 0.71-1.78ng/mg Cr, $p=0.02$, $n=10$). Mean fold change ('highest value' KIM-1 during tobramycin exposure:pre-treatment baseline KIM-1) was 3.03 (95% CI, 1.89-4.17).

Preventing aminoglycoside-induced nephrotoxicity: Given the problem of aminoglycoside induced nephrotoxicity, particularly in this vulnerable CF population, there is a need for better ways of prevention. Our work in the MRC Centre for Drug Safety Science (CDSS) in Liverpool has been focusing on this question, with a view to developing novel interventions

that could be used in all age groups, with the ultimate aim of improving the benefit-risk ratio of aminoglycoside therapy. Given the mechanism of action of aminoglycosides in causing nephrotoxicity, we identified statins as a possible intervention. Statins are drugs widely used in cardiovascular disease in adults, with proven efficacy and safety. Statins are also used in children having been licensed principally for the treatment of hyperlipidaemia.

In order to test our hypothesis, we first used renal tubular cell models: using a number of statins, we were able to show that statins prevent aminoglycoside-induced cell toxicity *in vitro* (Antoine et al., 2010). This effect occurs through the inhibition of HMG-CoA reductase by statins resulting in a depletion of the cellular sterols required for the megalin-mediated uptake of aminoglycosides into renal proximal tubular cells (Figure 1.4). The next step involved developing an animal model to identify which statin and which dose would be most appropriate in preventing aminoglycoside nephrotoxicity. It is important to note that not all statins are the same because they are handled differently by the body. Initial studies utilised a rat model: although we were able to show inhibition of aminoglycoside toxicity in this model, the doses of statins required were extremely high because of the high activity of HMG CoA reductase in the rat (Figure 1.5). We then progressed to a guinea pig model which does not have the same attributes in terms of HMG-CoA reductase activity as the rat. This was much more successful and was able to show inhibition of gentamicin-induced nephrotoxicity by rosuvastatin (Figure 6.4). However, the effect was not seen with simvastatin.

A rosuvastatin dose-ranging study in the guinea pig model demonstrated inhibition of nephrotoxicity at a dose of 0.94mg/kg (Figure 6.5). Using a dose-scaling algorithm (Reagan-Shaw et al., 2008), this equates to a rosuvastatin dose of 10mg in a 40kg child. We found

that a dose of 0.47mg/kg in guinea pigs (equating to a 5mg dose in a 40kg child) did not inhibit nephrotoxicity.

Our work is an example of translational medicine whereby work at the bench in a laboratory can be transferred to the clinical situation. It is also an example of repurposing of drugs – i.e. rosuvastatin may be useful in an indication which is different from that for which it was originally licensed. Rosuvastatin is already used in children for familial hypercholesterolaemia on a chronic basis, and no increased susceptibility of children to liver or muscle adverse effects has been identified, and there have been no effects on hormonal status and growth reported (Elis et al., 2013).

Need for a clinical intervention study: The next stage of our translational journey is to test the hypothesis that rosuvastatin can inhibit aminoglycoside-induced nephrotoxicity in children with cystic fibrosis. We have therefore chosen to conduct this study using a 10mg dose of rosuvastatin for reasons outlined above. It will use change in urinary KIM-1 during aminoglycoside exposure as its primary outcome measure. This phase IIa study, if positive, will be used to design a national, multi-centre, phase IIb/III study to evaluate the effect of rosuvastatin in preventing aminoglycoside-induced kidney injury.

The development of an intervention strategy to minimise the nephrotoxic consequences of aminoglycosides would enable patients to continue to benefit from their positive impacts, but reduce their risk of suffering acute and long-term adverse consequences.

7.2.2 Objectives

Primary Objective: This trial will evaluate the effect of rosuvastatin on aminoglycoside-induced nephrotoxicity. This will be assessed using the difference in mean fold-change in urinary KIM-1 from baseline to 'highest value' concentration during exposure to tobramycin between the rosuvastatin treated arm and control arm.

Secondary Objectives:

1. Change in serum concentration of creatinine and eGFR during tobramycin exposure between rosuvastatin treated arm and the control arm.
2. Change in other urinary and plasma biomarkers of renal injury during tobramycin exposure between rosuvastatin treated arm and the control arm.
3. Difference in serious adverse events between rosuvastatin treated arm and the control arm.
4. Difference in tobramycin concentrations between rosuvastatin treated arm and the control arm to identify any pharmacokinetic interaction between rosuvastatin and the tobramycin.
5. Difference in Forced Expiratory Volume in 1 second (FEV1) and C-Reactive Protein, between rosuvastatin treated arm and the control arm to identify any pharmacodynamics interaction between rosuvastatin and the tobramycin
6. Assessment of plasma rosuvastatin concentrations achieved in children randomised to the intervention arm.
7. Difference in biomarkers of *Pseudomonas aeruginosa* between rosuvastatin treated arm and the control arm.
8. Assess the feasibility of collecting DNA for a molecular genetic study of aminoglycoside-induced nephrotoxicity.

7.2.3 Potential Risks and Benefits

Rosuvastatin has a marketing authorisation for use in children for the management of hypercholesterolaemia. It can be used at doses up to 20mg/day. Our trial represents an example of drug repurposing, whereby we will use rosuvastatin outside its licence to prevent an important adverse effect. Guidance issued by the MRC, Department of Health and the MHRA on risk-adapted approaches to the management of CTIMPs propose a three

level categorisation for the potential risk associated with the IMP, assigned according to the following categories:

Type A *'no higher than that of standard medical care';*

Type B *'somewhat higher than that of standard medical care';*

Type C *'markedly higher than that of standard medical care'.*

Whilst rosuvastatin has a marketing authorisation, in this study it will be used outside the manufacturer's indication, and therefore the risk associated with the IMP in this trial is categorised as ***Type B 'somewhat higher than that of standard medical care'***. This level of risk informs the risk assessment, regulatory requirements, nature and extent of the monitoring, and the management processes used in the trial.

Participants will be closely monitored for known potential adverse events, and for unexpected adverse events. The management of any symptoms or exacerbations will be in accordance with usual clinical practice and either the local principal investigator (PI) or delegated research staff, will be available throughout the study to discuss specific issues with individuals concerned. Any concerns, which cannot be satisfied at a local level, will be forwarded to the chief investigator (CI). Any participant can withdraw from the study at any time without detriment to their future care. All ethical aspects of the study will be discussed when informed written consent is obtained. Appropriate information leaflets have been developed and will be discussed at the screening consultation. Potential participants will be provided with a copy of the information sheets and their signed consent forms.

7.2.3.1 Potential Risks

A previous trial has been conducted using simvastatin at a dose of 40mg in children with CF for the reduction of airway inflammation (NCT00255242). However, although the trial is recorded as completed, the outcomes have not yet been published. Participation in this trial does not change existing CF treatment protocols. We will also be using rosuvastatin for short periods rather than chronic use which characterises hyperlipidemia.

Short-term rosuvastatin treatment in children who have normal lipid profiles may potentially result in transient hypolipidaemia. We are not aware of any evidence of adverse effects related to short-term hypolipidaemia, but this will be routinely monitored in all patients during the trial. Rosuvastatin can have rare but serious adverse effects involving skeletal muscle and the liver. We will also monitor for these by measuring creatine kinase and liver function tests, and also by monitoring for signs or symptoms daily whilst on rosuvastatin. We will exclude patients taking medications known to interact with rosuvastatin, or with conditions or past history that may lead to an increased susceptibility to adverse effects of rosuvastatin.

One practical issue is the addition to the already existing pill burden in this group of patients; however, rosuvastatin is administered as a single oral tablet taken only once daily, and thus, is likely to cause a minimal increase in the pill burden. There is no further additional burden to participants in the trial.

7.2.3.2 Known Potential Benefits

There are potential benefits if our hypothesis that rosuvastatin can prevent aminoglycoside-induced kidney injury is proven.

7.3 Selection of Centres/Clinicians

Potential study centres will be identified through the Clinical Research Network: Children (CRN: Children). Site set-up will also be facilitated by the CRN. Study centres will be initiated once all global (e.g. local R&D approval) and study-specific conditions (e.g. training requirements) have been met, and all necessary documents completed. Site Initiation visits will take place.

7.3.1 Centre/Clinician Inclusion Criteria

Each participating centre (and Principal Investigator; PI) has been identified on the basis of:

- Being a paediatric cystic fibrosis (CF) treatment centre
- Having at least one lead clinician with a specific interest in, and responsibility for supervision and management of paediatric patients with CF
- Showing enthusiasm to participate in the study
- Ensuring that sufficient time, staff and adequate facilities are available for the trial
- Planning to provide information to all supporting staff members involved with the trial or with other elements of patient management
- Feasibility data confirms that they have a number of eligible patients
- Acknowledging and agreeing to conform to the administrative and ethical requirements and responsibility of the study including adhering to GCP and other regulatory documentation
- Other important criteria are:
    a. Local R&D approval
    b. Completion and return of 'Signature and Delegation Log'
    c. Signed non-commercial agreement between centre and sponsor
    d. Receipt of evidence of completion of (a),(b) and (c)

7.3.2 Centre/Clinician Exclusion Criteria

a. Not meeting the inclusion criteria listed above.

7.4 Trial Design

This study is a phase IIa, multi-centre, randomised, open-labelled trial of rosuvastatin in children with cystic fibrosis (CF) receiving clinically indicated treatment with intravenous (IV) tobramycin.

Patients will be randomised equally to either receive rosuvastatin 10mg once daily or no intervention (control), throughout a course of treatment with IV tobramycin (usually lasting 14 days).

If at the final analysis, a significant reduction in fold-change of KIM-1 during tobramycin exposure is found, rosuvastatin 10mg will be recommended for phase IIb/III.

In a previous study (URBAN CF) urine biomarkers have been measured in children with CF receiving treatment with tobramycin. Preliminary results, specifically the fold-change in urinary KIM-1 from baseline to 'highest value' concentration during exposure to tobramycin, from this study have been used to inform the design of the present study. According to this power calculation, a sample size of 20 in each arm will be recruited (See section 7.9.4).

7.4.1 IV Tobramycin

This study will include only children with CF treated with the aminoglycoside antibiotic tobramycin given intravenously. We will not include those children receiving treatment with other aminoglycoside antibiotics or receiving nebulised aminoglycoside therapy. IV tobramycin is usually given once daily, but can also be given three times per day. Participants may receive tobramycin at either frequency (as decided by the local CF team

on clinical grounds), but this should be specified in the study CRFs, and the time and amount of each dose should be accurately recorded.

7.4.2 Primary Outcome

This trial will evaluate the effect of rosuvastatin on aminoglycoside-induced nephrotoxicity. This will be assessed using the difference in mean fold-change in urinary KIM-1 from baseline to 'highest value' concentration during exposure to tobramycin between the rosuvastatin treated arm and control arm. Urinary KIM-1 will be measured using a validated assay in a GCLP Laboratory at the University of Liverpool. Urinary KIM-1 will be normalised to urinary creatinine.

7.4.3 Secondary Outcome(s)

1. Difference in serum concentration of creatinine and eGFR during tobramycin exposure between rosuvastatin treated arm and the control arm.
2. Difference in other urinary and plasma biomarkers of renal injury during tobramycin exposure between rosuvastatin treated arm and the control arm.
3. Difference in serious adverse events between rosuvastatin treated arm and the control arm.
4. Difference in tobramycin concentrations between rosuvastatin treated arm and the control arm to identify any pharmacokinetic interaction between rosuvastatin and the tobramycin.
5. Difference in Forced Expiratory Volume in 1 second (FEV1) and C-Reactive Protein, between rosuvastatin treated arm and the control arm to identify any pharmacodynamics interaction between rosuvastatin and the tobramycin
6. Relationship between plasma rosuvastatin concentrations achieved in children randomised to the intervention arm and change in urinary KIM-1.

7. Difference in biomarkers of *Pseudomonas aeruginosa* between rosuvastatin treated arm and the control arm.

7.4.3.1 Description of Secondary Outcomes

- Change in traditional markers of renal injury, serum creatinine and estimated Glomerular Filtration Rate, and change in novel urinary and plasma biomarkers of renal injury during tobramycin exposure between the rosuvastatin treated arm and the control arm. Serum creatinine will be measured locally, and novel biomarker analysis (NGAL, cystatin C) will occur centrally at the University of Liverpool.
- To assess safety of the intervention, we will compare the reported expected and unexpected serious adverse events between the rosuvastatin treated arm and the control arm. We will particularly focus on muscle adverse events which have been reported with rosuvastatin. CPK will be measured in these patients through blood that is routinely collected.
- We will assess for interaction between rosuvastatin and tobramycin:
    - Pharmacokinetically, by comparing tobramycin concentrations between the rosuvastatin treated arm and the control arm. Blood samples will be taken at 3 time-points during tobramycin exposure and analysed locally for the tobramycin concentration. Any additional blood samples taken during the study period will also be analysed for tobramycin concentration to give additional data.
    - Pharmacodynamically, by comparing change in percent of predicted Forced Expiratory Volume in 1 second (FEV1), between the rosuvastatin treated and control arms. This is a widely used indirect measure of aminoglycoside treatment outcome in children with CF, and will be measured locally during

study visits. We will also compare change in CRP, a widely used marker of inflammation/infection, between the two groups.

- In the rosuvastatin treated arm, collected blood samples will be analysed centrally for rosuvastatin concentration, in order to assess the pharmacokinetic profile of rosuvastatin in children with CF, to assess compliance, and to relate rosuvastatin concentrations to change in urinary KIM-1.
- We will assess the impact on *Pseudomonas aeruginosa* by measuring biomarkers associated with *P. aeruginosa* quorum sensing which can be used to determine both the presence of *P. aeruginosa* as well as markers of virulence. These markers will be compared between the rosuvastatin treated and control arms

7.4.4 PROteKT substudy - Molecular genetics of aminoglycoside-induced nephrotoxicity

All children who are invited to participate in the PROteKT study will also be invited to participate in a substudy to assess the molecular genetics of aminoglycoside-induced nephrotoxicity.

There exists a considerable degree of inter-individual variability in susceptibility to aminoglycoside-induced nephrotoxicity, and the reasons for this are not clear from the available literature. In particular, whether there is any genetic component to this variability has not been investigated previously in a genome-wide approach.

During this study all participants will be exposed to IV tobramycin as part of their routine clinical care. We will collect a sample for molecular genetic analysis from each child who consents to do so, and identify aminoglycoside-induced nephrotoxicity using novel biomarkers and serum creatinine.

The samples collected in this study will be combined with:

1. Samples collected through the MAGIC study (Molecular Genetics of Adverse Drug Reactions in Paediatric Patients), Research Ethics No: 10/H1002/57.
2. Samples collected through worldwide efforts to evaluate genetic factors predisposing to drug-induced renal injury.

In the future, we will conduct a genome wide association study (GWAS) to identify genetic risk factors for aminoglycoside-induced nephrotoxicity. Any positive hits will be further investigated using functional approaches to elucidate mechanisms and identify causal variants.

We will aim to assess the feasibility of carrying out a molecular genetic study in this group of children, and to address the following objectives:

(1) Identify genetic risk factors for aminoglycoside-induced nephrotoxicity in children in order to provide better preventive strategies in the future.

(2) Identify causal mechanisms of aminoglycoside-induced AKI by exploring genotype/phenotype correlations, in order to provide better interventional strategies in future.

7.5 Study Population

The study will recruit children with cystic fibrosis from paediatric CF treatment centres in the UK.

7.5.1 Inclusion Criteria

1. Age 10 to 18 years inclusive.
2. Diagnosis of cystic fibrosis (established by sweat test or genotype).
3. Planned, clinically indicated, course of treatment with IV tobramycin.
4. Ability to give informed consent.

5. Willingness to comply with all study requirements.
6. Able to take tablets.

7.5.2 Exclusion Criteria

1. Existing treatment with a statin.
2. Previous adverse reaction to a statin.
3. Co-enrolment in other drug trials*, or completion of a previous CTIMP within the last 30 days.
4. Previous randomisation in the PROteKT trial.
5. Patients taking any of the following medications: Ciclosporin, Protease Inhibitors, Fibrates, Ezetimibe, Erythromycin (but not other macrolides), Eltrombopag, Dronedarone, Itraconazole, Coumarins, Oral contraceptives, nicotinic acid, fusidic acid.
6. Female participants who are pregnant or lactating or refuse a pregnancy test if of childbearing potential (female participants of childbearing potential must use a barrier method of contraception if sexually active whilst taking rosuvastatin and for 7 days afterwards).
7. Patients of Asian ancestry (Japanese, Chinese, Filipino, Vietnamese, Korean and Indian).
8. Patients with renal disease (eGFR<60ml/min/1.73m^2, using the Schwartz formula, in the 6 months preceding screening).
9. Patients with current elevation in transaminases exceeding 3x the upper limit of normal.
10. Family history, or personal history, of hereditary muscular disorders.
11. Patients with myopathy.
12. Patients with a history of, or active alcohol abuse.

13. Patients with hypothyroidism.
14. Patients with galactose intolerance, the Lapp lactase deficiency, or glucose-galactose malabsorption.
15. Patients who are Hepatitis C positive or HIV-positive.

Patients who are currently taking part in TORPEDO-CF are allowed take part in PROteKT as long as their date of randomisation into TORPEDO-CF is not within the previous six months of the screening date for PROteKT. Exclusion criteria are based upon those contraindications or cautions outlined in the Summary of Product Characteristics.

7.5.3 Patient Transfer and Withdrawal

In consenting to the trial, patients are consented to trial treatment, follow-up and data collection. If voluntary withdrawal occurs, the patient should be asked to allow continuation of scheduled evaluations, complete an end-of-study evaluation if appropriate and be given appropriate care under medical supervision until the symptoms of any adverse event resolve or the patient's condition becomes stable.

Follow-up of these patients will be continued through the trial research nurses (RN) and the lead investigator at each centre unless the participant explicitly also withdraws consent for follow-up.

7.5.3.1 Patient Transfers

For patients moving from the area, every effort should be made for the patient to be followed-up at another participating trial centre if possible and for this trial centre to take over responsibility for the patient. A copy of the patient CRFs should be provided to the new site. The patient will have to sign a new consent form at the new site, and until this occurs, the patient remains the responsibility of the original centre. The study co-ordinator should be notified in writing of patient transfers.

7.5.3.2 Withdrawal from Trial Intervention

Patients may be withdrawn from the trial treatment rosuvastatin for any of the following reasons:

a. Patient withdraws consent;
b. Clinical decision not to treat with tobramycin;
c. Unacceptable adverse effect of Grade 3/4 which can be attributed to rosuvastatin;
d. Intercurrent illness preventing further treatment;
e. Inability to attend regularly for treatment or assessment;
f. Any change in the patient's condition that justifies the discontinuation of treatment in the clinician's opinion;
g. Failure to comply with the protocol requirements or cooperate with the investigator;
h. The patient starts treatment with any disallowed medication without prior notification and the consent of the investigators;
i. Pregnancy during trial treatment period.

If a patient wishes to withdraw from trial treatment, centres should nevertheless explain the importance of remaining on trial follow-up, or failing this, of allowing routine follow-up data to be used for trial purposes. Generally, follow-up will continue unless the patient explicitly also withdraws consent for follow-up (see Section 7.5.3.3). If tobramycin is stopped before the 2 week period, rosuvastatin must also be stopped (see section 7.7.3.5).

Patients who withdraw from trial treatment but are willing to allow further data collection must have a discussion with the investigator as to whether they will be able to attend subsequent follow-up assessments at specific time-points until the end of their follow-up period. The decision should be based on the patient's own preferences and the clinician.

7.5.3.3 Withdrawal from follow-up

Every effort should be made to collect follow-up data even if participants have withdrawn from trial intervention. In some cases it may not be possible to continue follow-up of trial participants due to transfer to a non-participating centre, loss to follow-up etc. in which case the withdrawal form should be completed. Where a patient is considered as lost to follow-up or if the patient has stated that they do not wish to continue follow-up, the data collected up until that point will be used in the analysis unless the patient explicitly states that they require all data to be removed (see 7.5.3.4).

If patient withdraws consent for blood samples, then participation with just urine tests can continue until end of follow up as long as the patient consents to this.

7.5.3.4 Withdrawal of consent for participation

Patients are free to withdraw consent at any time without providing a reason. If a patient withdraws consent for treatment this should not be considered as withdrawal of consent for follow-up unless the patient explicitly states that they no longer wish to participate in follow-up. Where patients wish to withdraw consent for the trial the data collected up to the time of withdrawal of consent will be included in the analyses unless the patient explicitly states that they require all data collected to be removed. The study co-ordinator should be informed in writing and a withdrawal CRF should be completed in order for the CTRC to ensure that data are removed and not made available for analyses. The patient will not contribute further data to the study.

7.6 Enrolment and Randomisation

7.6.1 Recruitment

Patients who are eligible for inclusion into the trial will be identified and recruited through the paediatric cystic fibrosis centres participating in the study.

Potentially eligible participants will be identified by their clinical care team at each centre via a search of the patient database/s either electronically or manually or by clinic list or clinical record review. A short introductory letter about the study, along with a parent information sheet and age-appropriate information sheet for the child, will be sent out to the family at least 1 week prior to their clinic visit. The introductory letter will ask them to read the information sheets, and will explain that they will be approached by the research team when they come to the clinic to consider consenting to participate in the study.

In the CF outpatient clinic children (and their parents/guardian) who are eligible will be approached by a member of the healthcare team who is also a member of the research team. If they have not had opportunity before coming to the clinic, parents and children will be given time to read the study information sheets. The study will then be verbally presented, and any questions arising from this or the patient information sheets will be answered. If they are willing to participate, informed, written consent will be obtained (see Section 7.11.3 for the consent procedure) from the parents, or the participant if aged 16 years or over. An assent form will be completed by the child if aged under 16 years. This consent will be considered 'prospective' and will be re-confirmed when the child attends for their baseline assessment.

If potential participants feel in need of more time to consider their response, they will be approached again at their next outpatient appointment.

On the rare occasion that a potential participant is due to commence treatment with tobramycin before their next outpatient appointment, they may be approached for consent on the day of attendance to commence their IV antibiotics. However, this should only occur if they have been sent the study information sheets at least 1 week before, and it is clear that they have read and understood this information prior to attending.

7.6.1.1 PROteKT substudy - Molecular genetics of aminoglycoside-induced nephrotoxicity

Information about the substudy will be included in a separate information sheet to the main PRoteKT sheet, and will be sent to eligible participants at the same time as the main information sheet. They will be given at least 1 week to consider their participation in the substudy and will be asked to consent at the time of consenting to the main PROteKT study.

7.6.2 Screening

Children with CF aged 10 to 18 years who are considered likely to require treatment with IV tobramycin during the study period should be approached about the study. A 'Screening Log' will be maintained of all the patients who undergo screening regardless of whether they decide to participate in the study or are found ineligible to participate. Reasons for not being eligible will be recorded. Reasons for declining to participate will be asked routinely but it will be made clear that they do not have to provide a reason unless happy to do so.

7.6.3 Baseline (T0)

On the planned day for beginning tobramycin therapy, participants will be asked to attend early in the day for baseline assessment. The research team should conduct the baseline (T0) assessments and complete the eligibility and baseline case report form (CRF) during the baseline assessment. The baseline assessments include:

1. Verification that the eligibility criteria are fulfilled;
2. Confirmation of consent;
3. Demographic details including age, gender, ethnicity;
4. Full medical and drug history (including concomitant medications such as contraceptives). This will also include an assessment of symptoms normally

associated with rosuvastatin toxicity such as muscle pains in order to get an indication of the prevalence of these symptoms prior to statin exposure;

5. Physical examination;
6. Body weight and height;
7. Pregnancy test for females of childbearing potential (urine): if a potential participant refuses the test, they should not be included in the study;
8. Measurement of FEV1;
9. Collection of blood samples from each patient;
10. Collection of one urine sample from each patient at the same time as the blood samples.

7.6.4 Randomisation

7.6.4.1 Back-up randomisation

The web based randomisation Participants will be randomised equally to either receive rosuvastatin 10mg once daily or no intervention (control) once:

1. Eligibility criteria have been fulfilled;

2. Fully informed written consent has been obtained;

3. Baseline assessments have been completed.

Appropriately delegated research staff at centres will randomise participants using a secure (24-hour) web based randomisation programme controlled centrally by the CTRC. Research staff will be trained to use the randomisation systems as part of the greenlight process for opening centres. Once the site has been initiated, they will be issued with usernames and passwords; new staff can be issued with usernames and passwords if they are delegated with the responsibility for randomising participants.

The randomisation system will randomise equally between the two arms using variable block randomisation.

Participant treatment allocation will be displayed on a secure webpage and an automated email confirmation sent to the authorised randomiser, the PI and the trial manager (TM). The email should be printed out and filed alongside the participant's CRF as this provides information about the randomised treatment, randomisation number and date randomised.

It is the responsibility of the PI or delegated research staff to inform the pharmacy department at their centre prior to randomisation to ensure there is enough supply of the study drugs.

Randomisation: web access https://ctrc.liv.ac.uk/Randomisation/Protekt

If there are any problems with web randomisation, please contact

the trial manager using the following email address: protekt_trial@ucl.ac.uk

Randomisation backup envelopes will be used in case of failure of the randomisation systems outside working hours

In the event of problems with the online randomisation system, the centre should contact the Trial manager at UCL (Monday to Friday between 9:00 to 17:00 excluding bank holidays) to try to resolve the problem.

The online randomisation system should always be used for randomising participants into the study. If there is a system failure that occurs outside UCL/CTRC office hours or when a system failure cannot be resolved in a reasonable time-frame by UCL/CTRC a

randomisation envelope can be used. All centres will be provided with emergency back-up randomisation envelopes for this purpose.

In the event that emergency back-up envelopes are required, the randomising person will select the next sequentially numbered, opaque, pressure-sealed envelope that will give the randomisation allocation. The envelope will be similar to those used for pay slips, which cannot be viewed without fully opening and their construction is resistant to accidental damage or tampering.

As the envelope contains all the details of the randomisation, each page of the open envelope should be scanned and emailed to the trial manager and a photocopy can be sent to CTRC along with completed CRFs. Please ensure that the envelope is stored in the patient's medical notes.

The RN will check to ensure that the correct number of randomisation envelopes is present, that they are intact and that the sequential numbering system is maintained. Any discrepancies should be immediately reported to the trial manager.

Subsequent courses of aminoglycoside

Participants will not be given the option to consent to be re-randomised on subsequent occasions of treatment with IV tobramycin (exclusion criteria 4).

7.7 Trial Treatment

7.7.1 Introduction

Rosuvastatin (marketed as Crestor®; AstraZeneca UK Ltd) is indicated for clinical use in the treatment of hypercholesterolaemia. However, the current trial will use rosuvastatin outside the manufacturer's indication.

Participants recruited into the study will be randomised to one of the following arms:

Control Arm: Non intervention arm

Treatment Arm: oral rosuvastatin 10 milligram (mg) dose, once daily, for the duration of a treatment course of IV tobramycin (usually 14 days)

7.7.2 Formulation, Packaging, Labelling, Storage and Stability

The rosuvastatin used in the trial will be sourced via usual local NHS procurement arrangements once the sites have been initiated. The size of the procurement of investigational drug at each site will be pre-determined based on the patient recruitment target for that individual site. Recruitment will be monitored centrally and drug procurement will be tailored in liaison with the respective pharmacies to ensure that pharmacies always hold adequate supplies of trial treatment.

7.7.2.1 Description and Composition of the Drug Product

Rosuvastatin:

Generic name – Rosuvastatin (Brand name: Crestor®).

Rosuvastatin is a selective and competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate, a precursor for cholesterol. It is used to treat hypercholesterolaemia, and to prevent cardiovascular events in those who are at risk.

Rosuvastatin tablets are for once-daily treatment, and may be given at any time of day, with or without food.

Crestor® 10 mg oral tablets: Each tablet contains 10 mg rosuvastatin (as rosuvastatin calcium). Each tablet contains 91.3 mg lactose monohydrate. It is a Round, pink coloured, Film-coated tablet intagliated with 'ZD4522' and '10' on one side and plain on the reverse.

Crestor® 10 mg oral tablets contain the following excipients: Tablet core; Lactose monohydrate, Microcrystalline cellulose, Calcium phosphate, Crospovidone, Magnesium stearate; Tablet coat; Lactose monohydrate, Hypromellose , Triacetin, Titanium dioxide (E171), Ferric oxide, red (E172).

Crestor® is manufactured by AstraZeneca UK Ltd.

7.7.2.2 Packaging and Labelling

Crestor® 10 mg tablets are packaged in blisters of aluminium laminate/aluminium foil of 7, 14, 15, 20, 28, 30, 42, 50, 56, 60, 84, 90, 98 and 100 tablets (although not all packages may be marketed).

Blister packs will be labelled and dispensed for trial treatment locally at each site in accordance with Annex 13 of Good Manufacturing Practice.

7.7.2.3 Stability and Shelf life

Rosuvastatin should be stored as per the Summary of Product Characteristics (SmPC). Please refer to the reference SPCs provided as a separate document to this protocol.

The product should be stored below 30ºC in the original package to protect the tablets from moisture. The 10 mg tablet has a shelf life of 3 years. The IMP will be stored in local pharmacy departments in line with the conditions of the SmPC. No special requirements above normal pharmacy practice for temperature monitoring or other processes have been identified.

7.7.3 Preparation, Dosage and Administration of Study Treatment/s

7.7.3.1 Dispensing

For each randomised patient who receives the intervention arm (Rosuvastatin), treatment will continue for the duration of their treatment course of IV tobramycin (usually 14 days).

The trial treatment can start immediately after randomisation and must be given on the same calendar day and usually prior to the first dose of IV tobramycin. The PI or delegated other will issue a prescription based on the patient's randomisation status. For participants in the treatment arm, rosuvastatin will be dispensed at a dose of 10mg once daily, for the duration of their treatment course of IV tobramycin, unless interruption or discontinuation is warranted (see sections 7.5.3.2 and 7.7.3.4).

For participants receiving the trial treatment as an inpatient, the prescription will be written on an inpatient prescription chart. For participants receiving the trial treatment at home, the prescription will be written on an outpatient prescription chart.

The respective pharmacy will dispense the trial treatments labelled as described in section 7.7.2.2.

The medications will be dispensed upon production of a valid, signed trial prescription to either the RN or directly to the patient as detailed below:

7.7.3.1.1 Dispensing for the Control Arm:

For patients randomised to the Control Arm (the non-intervention arm), no prescription will be given.

7.7.3.1.2 Dispensing for the Treatment Arm: Oral rosuvastatin 10 mg, once daily

For those who are randomised to the treatment arm, sufficient trial treatment (usually 14 tablets) consisting of blister packs of rosuvastatin 10mg will be dispensed on receipt of a valid trial prescription.

7.7.3.2 Lost or Damaged Medications

In the event of loss or damage to the tablet pack they are currently using, the participant (or their parent/guardian) or the inpatient ward should contact the RN who will arrange

that the patient can be dispensed a replacement prescription in sufficient time to ensure there is no break in medication.

7.7.3.3 Administration

The patient will be instructed in the correct use of the medications dispensed. Further guidance will be provided throughout the remainder of the trial where necessary.

The trial treatment has only one route of administration:

Oral (rosuvastatin) - One tablet is to be administered daily, and may be given at any time of day, with or without food.

The daily dose of rosuvastatin should be given prior to the dose of IV tobramycin.

7.7.3.4 Dose Modifications

Rosuvastatin dose modifications will not be allowed. Those who show adverse effects as a result of the trial intervention may be withdrawn from the trial treatment. The decision to withdraw the patient from trial treatment is based on the patient's current clinical presentation. The decision to interrupt or discontinue trial therapy is at the discretion of the treating physician using their informed clinical opinion. Doses may be interrupted or discontinued at any point during the trial period for reasons such as unacceptable adverse effects, intercurrent illness, development of serious disease or any change in the patient's condition that the physician believes warrants a change in medication. Any changes must be documented in the CRF along with the justification for those changes. Follow-up should be continued until the end of the trial as per the study visit schedule. If the patient is withdrawn from trial treatment, the dispensed medications will be returned to pharmacy for disposal via their local procedures.

7.7.3.5 Duration of Treatment

For those in the treatment arm, treatment with rosuvastatin will continue for the full duration of the course of IV tobramycin received. Usually this will last for 14 days (14 once daily IV doses), in which case the participant would receive 14 once daily doses of rosuvastatin on each day of the IV tobramycin course. Treatment with oral rosuvastatin should continue for the duration of the course of IV tobramycin. Therefore, if the IV tobramycin course is shorter than 14 days, rosuvastatin should be discontinued after the final dose of IV tobramycin has been given. If the course of IV tobramycin is longer than 14 days, then additional oral rosuvastatin should be dispensed, in order to continue until the final day of the IV tobramycin course.

7.7.4 Unblinding

The study is an open label trial, therefore unblinding is not required. This is a phase IIa study, and we are using the change in an objective biomarker (KIM-1) as an end-point, which is not going to be affected by whether the trial is blinded or not. However, the laboratory measurement of KIM-1 will be blinded.

7.7.5 Accountability Procedures for Study Treatment/s

As the rosuvastatin used in the trial will be sourced via usual NHS procurement arrangements, pharmacy will liaise with the local procurement department to ensure that the site has the following in place and will report any problems to the study co-ordinator:

- A record of deliveries and dispensing of rosuvastatin - A system in place that allows for the retrieval if the manufacturer issues a recall - local procedures should be used.
- Enough rosuvastatin within shelf life assigned to be used in the study (see Section 7.2.3).

- Rosuvastatin is used in compliance with the protocol requirements and accountability records are maintained.
- Batch number of the product dispensed should be recorded on the prescription form, and these forms should be filed in a trial folder to permit retrospective verification.

7.7.6 Assessment of Compliance with Study Treatment/s

Once the participant has been informed of their treatment allocation, treatment compliance with the study protocol should be recorded in the study CRFs (this will include a daily record of the time of taking the study IMP, completed by the participant in their patient-held study diary. Where patients are still in hospital, the diary can also be completed by the research nurse). At the T+13/final day assessment, a count of any unused tablets will be performed, and these will be returned to the local site pharmacy

7.7.7 Concomitant Medications/Treatments

7.7.7.1 Medications Permitted

Details of concomitant medications will be collected at the screening visit (T0) and recorded on the CRF. They will be reviewed daily during admission for IV tobramycin, and at all subsequent study visits.

The participants in this study are likely to be taking a number of concomitant medications for the management of their CF. The trial treatment has few adverse interactions with other medicinal products, therefore concomitant medications, with the exception of those listed in Section 7.7.2, are permissible.

7.7.7.2 Aluminium and magnesium hydroxide containing antacids

The SmPC for rosuvastatin states that aluminium and magnesium hydroxide containing antacids can cause reduced absorption of rosuvastatin. However, this interaction can be

avoided by ensuring that the antacid is given at least 2 hours after rosuvastatin. Providing this advice is followed, then participants on these antacids may participate in the study.

7.7.7.2.1 Clarithromycin and Azithromycin

Erythromycin is contraindicated with rosuvastatin, but the other macrolides are permitted. Unlike other statins, Rosuvastatin is not metabolised through CYP3A4. With other statins erythromycin results in increased plasma levels of the statin due to the interaction via CYP3A4. However, with Rosuvastatin erythromycin results in lower plasma levels, possibly secondary to it increasing gut motility. There is no published data on interactions between clarithromycin or azithromycin and rosuvastatin. However, there is not thought to be a class effect, and therefore there is no reason for concomitant use of these to be contraindicated.

7.7.7.3 Medications Not Permitted

The following are not permitted for the duration of the trial period (from SmPC & BNFc):

1. Ciclosporin (Contraindicated with Rosuvastatin, SmPC)
2. Protease Inhibitors (strongly increase rosuvastatin levels, SmPC)
3. Fibrates (increase rosuvastatin levels, SmPC)
4. Ezetimibe (small increase in rosuvastatin level, SmPC)
5. Erythromycin, but not other macrolides (see 7.7.1.2, reduced rosuvastatin level, SmPC)
6. Eltrombopag (increase rosuvastatin levels, SmPC)
7. Dronedarone (increase rosuvastatin levels, SmPC)
8. Itraconazole (increase rosuvastatin levels, SmPC)
9. Coumarins (increased anticoagulant effect with rosuvastatin, SmPC)
10. Oral contraceptives (increased levels of ethinylestradiol, norgestimate, and norgestrel with rosuvastatin, SmPC)

11. nicotinic acid (BNFc)
12. fusidic acid (BNFc)

7.7.7.4 Precautions required

Pregnancy: A pregnancy test should be carried out in all female participants of childbearing potential (all who have reached menarche) at the time of enrolment to the study before taking rosuvastatin. If a potential participant refuses the test, they should not be included in the study. Rosuvastatin is not recommended in pregnancy; therefore, for those who are on the treatment arm, barrier contraception must be *strongly* advised to all women of childbearing age for the duration of the trial, if sexually active. Sensitivity will need to be exercised by the researcher addressing this issue with the potential participant, and care may be required to avoid embarrassment to the child in front of their parents. An appropriate approach should be discussed beforehand with the clinical team who know the patient and their family. This issue has been discussed with the CRN: Children, and is in line with their recommendations.

7.7.7.5 Data on Concomitant Medication

The dose and name of all concomitant medications, including over the counter and alternative medicines should be documented on the CRF at T0. This will be reassessed on a daily basis during tobramycin treatment, and at each follow-up visit by the PI/RN. Any new medications introduced or any changes to current medications should be documented on the CRF.

7.7.8 Co-enrolment Guidelines

To avoid potentially confounding issues, patients recruited into another CTIMP should not be enrolled in this study. Where recruitment into another study is considered to be appropriate and without having any detrimental effect on this study, this must first be discussed with the Chief Investigator. We have received confirmation from the TORPEDO-

CF trial Co-Chief Investigator (Professor Alan Smyth) that patients already taking part in the TORPEDO-CF trial may be approached about participation in the PROteKT trial but only when a period of six months have lapsed since patients were randomised onto the TORPEDO-CF trial. If the same patient can be approached for either trial, then the TORPEDO-CF trial should take precedent over PROteKT since the eligibility criteria is more restrictive in TORPEDO-CF.

7.7.9 Pharmacy Manual

Further details will be provided to each participating site in a PROteKT study Pharmacy Manual.

7.8 Assessments and Procedures

Participating centres will be expected to each maintain a file of essential trial documentation (Site File), and copies of all completed case report forms (CRFs) for the trial. Data collection will use paper CRFs.

All paper CRFs should be completed by personnel named on the delegation log as authorised to do so. Once completed, original CRFs should be sent to the CTRC, and copies should be kept in the local site file.

Participant details including name, initials and date of birth will be reported on the consent form, separate to clinical data. Once written informed consent has been obtained from the participant, the participant will be invited to attend a baseline screening visit on the day of admission for IV tobramycin treatment. At this visit, consent will be confirmed, the RN will collect the baseline characteristics, and the participant will be randomised and followed-up in the trial. For screening, baseline and randomisation procedures refer to section 7.6. For details of procedures associated with trial treatments refer to section 7.7. Data similar to

that collected at baseline (see section 7.6.3) should be recorded on the appropriate follow-up CRFs.

7.8.1 Schedule for Follow-up

See schedule of study procedures, Table 7.1.

Patients with CF may receive their treatment course of IV tobramycin as an inpatient or at home. Participants should attend for their baseline (T0) visit as described, before commencing treatment with IV tobramycin. They may then either be admitted or will go home to commence their IV tobramycin. Daily urine sample collections will be completed either on the inpatient ward, or at home (see section 7.8.2.1). Assessments at T+1, T+8 and T+13 where blood sample collection is required will normally require the subject to attend the study site, unless facilities exist for bloods and lung function to be done at the patient's home. The T+1 assessment should be completed on that day. The T+8 assessment should be completed between days T+7 and T+9 inclusive (allowing it to be planned to coincide with clinically indicated bloods more easily). The T+13 assessment should be completed on day T+13 or the final day of tobramycin treatment if this occurs earlier. If treatment with IV tobramycin extends beyond 14 days, a further assessment, completing the same information as the T+13 day assessment, should be completed on the final day of therapy, and daily assessments should be continued until this point.

Scheduled study visits are designed to fit with routine hospital visits where possible. The 4 week follow-up visit should be conducted between 3 and 5 weeks after the completion of tobramycin treatment. Participants withdrawn from trial treatment will be asked to continue with scheduled follow-up visits. If the participant misses the scheduled follow-up visit, the RN should conduct the follow-up at the earliest convenient date. If a participant

does not wish to continue in the study, a withdrawal CRF will be completed to capture the date and reason for trial withdrawal as detailed in section 7.5.3.3.

7.8.1.1 Scheduled Assessments

Baseline assessments will be completed at the T0 visit as described in section 7.6.3. During treatment with IV tobramycin the T+1, T+8 and T+13 assessments will include a review of concomitant medications, assessment of adverse events, a symptom-directed physical examination if required, and collection of a urine sample for biomarker analysis.

Urine samples for biomarker analysis will be collected daily during treatment with IV tobramycin as described in section 7.8.2.1. If the participant is an inpatient, samples will be refrigerated and then sent to the local laboratory for processing and storage. If the participant is receiving IV tobramycin at home, samples will be stored in the home refrigerator in a sealed container until the next scheduled study visit (T+8 or T+13) when they will be sent to the local laboratory.

Participants will complete a daily diary during IV tobramycin treatment in which they will record the times of their IV tobramycin dose, rosuvastatin dose, and urine sample collection. They will also have a telephone number to contact the study team in case of any symptoms of a suspected adverse event.

Assessment at T+1day will include the routine daily assessment, plus collection of blood samples for analysis as described in sections 7.8.2-7.8.4.

Assessments at T+8 and T+13days, will include the routine daily assessment, plus height and weight measurements, measurement of FEV1, and collection of blood samples for analysis as described in sections 7.8.2-7.8.4.

The 4 week (+/- 1 week) follow-up visit will include collection of a urine sample for biomarker analysis, height and weight measurements, measurement of FEV1, and collection of blood samples for analysis as described in sections 7.8.2-7.8.4.

7.8.1.2 Unscheduled Assessments

If treatment with IV tobramycin extends beyond 14 days, a further assessment, completing the same information as the T+13 day assessment, should be completed on the final day of therapy.

If treatment with IV tobramycin is completed before 14 days, a final assessment (akin to the T+ 13 assessment) should be completed on the final day of therapy. Patients randomised to the treatment arm will also need to stop taking Rosuvastatin on the same day as IV Tobramycin is completed.

In circumstances where a decision is made to stop IV tobramycin which does not leave sufficient time to complete a final day assessment, the assessment may be completed the following day.

During IV tobramycin participants will be reviewed daily for symptoms or signs of adverse events. If there exists clinical concern of an adverse event, part of the assessment would include blood samples for all blood tests described in sections 7.8.2-7.8.4.

If additional blood samples are collected during IV tobramycin therapy, on days other than the T+1, T+8 and T+13 assessments, details should be completed on the relevant day's CRF.

Following the completion of IV tobramycin and before the scheduled 4 week follow-up visit, some participants may need to attend for unscheduled study visits. In these instances, the PI or RN should complete the Unscheduled Visit CRF.

7.8.2 Procedures for Assessing Efficacy

Efficacy of trial treatments will be assessed throughout the period of the study using objective measures.

7.8.2.1 Assessment of Urine Biomarkers

The primary outcome measure for the trial will be fold-change in urinary KIM-1 during tobramycin exposure.

Urine samples will be collected from each child at baseline, and on each day of tobramycin treatment. A further sample will be collected at the 4 week follow-up visit.

The normally preferred method of collection will be a clean catch urine sample into a sterile container. Samples will then be transferred to (if not already collected in) a sterile universal sample container (white top).

If the participant is an inpatient, the daily samples must be stored at fridge temperature (4^{o}C) for 1 week and then sent to the local laboratory for processing and further storage at -80^{o}C (or -20 ^{o}C for a maximum of 6 months). If the participant is receiving IV tobramycin at home, samples will be stored in the home refrigerator in a sealed container until the next scheduled study visit (T+8 or T+13). In the latter case, when the study team receive the samples, they need to ensure that they are stored at fridge temperature for a total of one week (including time spent at patient's home) and then sent to the local laboratory for processing and further storage at -80^{o}C (or -20^{o}C for a maximum of 6 months). KIM-1 is stable in urine stored at 4^{o}C for up to 1 week (Chaturvedi et al., 2009, Pennemans et al., 2012).

Urine samples will be centrifuged and then aliquoted into smaller volumes at the local site. These aliquoted samples will then be frozen (at -80^{o}C or -20^{o}C for a maximum of 6 months) and stored at the local site. Batched samples will be couriered on dry ice to a GCLP

registered laboratory at the University of Liverpool for subsequent storage and analysis (here samples will be stored at -80^{o}C). All measurements of biomarkers will be undertaken in a standardised manner, with the laboratory analysis being blinded to timing of samples and clinical outcomes of patients. When samples are analysed for urine biomarkers, urinary creatinine will also be measured, as this is used to standardise urinary biomarker values. Analysis of the primary outcome measure, KIM-1, and urinary creatinine, will be undertaken using validated assays in a GCLP laboratory at the University of Liverpool. Analysis of other urinary biomarkers will be undertaken in other laboratories at the University of Liverpool. Samples will be kept until they have been used up.

7.8.2.2 Assessment of Serum creatinine

Serum creatinine measurements will be performed at T0 and T+1 day, T+8 days, T+13 days, and the 4 week follow-up visits. The blood sample mentioned in Section 8.4 will be analysed at the laboratory serving the local research site for this purpose.

7.8.3 Procedures for Assessing Safety

Adverse event reporting is detailed in Section 7.10 (Pharmacovigilance) and will occur from the point of randomisation and throughout the trial treatment period up until the final follow-up visit 3-5 weeks after the patient has taken the final dose of investigational medicinal product.

7.8.4 Other Assessments: Special Assays or Procedures

Blood samples will be collected from each participant at the T0, T+1 day, T+8 days, T+13 days, and the 4 week follow-up visits at the respective sites. A minimum of two 1.2ml samples in Lithium/Heparin blood tubes, and one 1.2ml sample in a serum blood tube will be collected at each timepoint. Plasma and serum will be extracted locally at each site, aliquoted and stored at -80ºC. Wherever possible, the timing of study bloods should coincide with any clinically indicated bloods being taken on that day in order to minimise

the burden to the patient. The method of blood collection will most likely need to be by venepuncture given the blood volumes required.

7.8.4.1 Assessment of effect of statin on cholesterol

Serum lipid levels (total cholesterol, triglycerides, LDL-c and HDL-c) will be performed at T0 and T+1 day, T+8 days, T+13 days, and the 4 week follow-up visits. The blood sample mentioned above in Section 7.8.4 will be analysed at the laboratory serving the local research site for this purpose.

7.8.4.2 Assessment of effect of statin on liver and muscle

Liver Function Tests (Alanine transaminase (ALT), aspartate transaminase (AST), Alkaline Phosphatase, and bilirubin) and creatine kinase will be performed at T0 and T+1 day, T+8 days, T+13 days, and the 4 week follow-up visits; analysis will be carried out at the laboratory serving the local research site using the same blood sample as mentioned above (see section 7.8.4).

7.8.4.3 Assessment of novel serum biomarkers

Plasma and serum will be extracted from blood samples and will be stored at -80^{o}C until sent to the Wolfson Centre for Personalised Medicine, University of Liverpool for laboratory analysis of novel biomarkers. Samples will need to be sent on dry ice by courier.

7.8.4.4 Assessment of C-reactive protein

C-reactive protein will be performed at T0 and T+1 day, T+8 days, T+13 days, and the 4 week follow-up visits; analysis will be carried out at the laboratory serving the local research site using the same blood sample as mentioned above (see section 7.8.4).

7.8.4.5 Assessment of response to tobramycin

Forced Expiratory Volume in 1 second (FEV1) (percent predicted) will be measured using spirometry at T0 , T+8 days, T+13 days, and the 4 week follow-up visits.

7.8.4.6 Assessment of tobramycin concentrations

Tobramycin concentrations will be measured at T+1 day, T+8 days, and T+13 days; analysis will be carried out at the laboratory serving the local research site using the same blood sample as mentioned above (see section 7.8.4).

7.8.4.7 Assessment of rosuvastatin concentrations

For patients in the treatment arm of the study, Rosuvastatin concentrations will be measured at T+1 day, T+8 days, and T+13 days. Plasma and serum will be extracted from blood samples and will be stored at -80ºC until sent to the Wolfson Centre for Personalised Medicine, University of Liverpool for laboratory analysis of rosuvastatin concentrations. Samples will need to be sent on dry ice by courier.

7.8.4.8 Assessment of Pseudomonas aeruginosa biomarkers

Biomarkers including molecules associated with *Pseudomonas aeruginosa* quorum sensing can be used to determine both the presence of *P. aeruginosa* as well as markers of virulence. These markers will be quantified from the serum/plasma and urine samples sent to the University of Liverpool as described above.

7.8.4.9 Assessment of changes in sputum microbiome

Sputum samples will be collected at the baseline T0, T+8 days, T+13 days and the 4 week follow-up visit. Ideally a sample of greater than 200ul is required. If clinically indicated samples are being collected at these times, then the remainder of the sample can be kept for the study. Samples should be frozen at -80^{o}C at the local site as soon as possible after collection. They will be stored at the local site until sent to the Wolfson Centre for Personalised Medicine, University of Liverpool for storage prior to laboratory analysis. Samples will need to be sent on dry ice by courier. These samples will be used for analysis of the sputum microbiome, and for investigation of the underlying mechanisms behind changes in the microbiome during exposure to tobramycin with or without rosuvastatin.

This will include analysis of biomarkers of *Pseudomonas aeruginosa* quorum sensing in sputum as per 7.8.4.8. Bacteria will also be isolated from the samples for routine microbiology analysis such as typing and antibiotic susceptibility testing.

Cough swabs and Bronchoalveolar lavage (BAL) fluid if collected for clinically indicated reasons during the study, and sputum samples collected for clinical reasons at times other than those prescribed above, can also be stored for analysis as part of the study, providing accurate time and date of collection is provided. BAL samples should be frozen at -80^{o}C in the same way as sputum samples.

Details of previous microbiology results and sensitivities of previously isolated organisms will be recorded at baseline. Any new results during the course of the study will also be recorded.

7.8.5 Additional Blood Tests

During the study period, participants may have blood samples taken for a clinically – indicated reason on days other than T+1, T+8 and T+13. In this case, any left-over blood samples would be used for the assessment of tobramycin and rosuvastatin concentrations.

7.8.6 PROteKT substudy - Molecular genetics of aminoglycoside-induced nephrotoxicity

7.8.6.1 DNA Collection

The aim is to collect 5ml of whole blood in an EDTA blood tube to allow for DNA extraction. At a minimum a blood sample should contain 2 ml. If a blood sample cannot be collected then a Saliva sample will be collected (2 x 2ml).

All of our participants will be having blood samples taken as part of the PROteKT study. Therefore, an additional blood sample, for DNA collection, will be taken at the same time as other study bloods. Usually this will be done at the Baseline Assessment (T0), but it can

be taken at any study visit when other bloods are being done. However, if the patient does not wish to have an additional blood test carried out we will use a saliva sample.

7.8.6.2 Laboratory analysis

All samples will be posted/ delivered to the Wolfson Centre for Personalised Medicine, University of Liverpool. Here samples will be processed, DNA extracted and stored securely. We have instituted a new bar coding system in the Department that adds further security in terms of sample tracking and also for confidentiality purposes. All biological samples stored within the Department are in keeping with the Human Tissue Act (license held by the University of Liverpool).

The samples collected in this study will be combined with:

1. Samples collected through the MAGIC study (Molecular Genetics of Adverse Drug Reactions in Paediatric Patients), Research Ethics No: 10/H1002/57.
2. Samples collected through worldwide efforts to evaluate genetic factors predisposing to drug-induced renal injury.

In the future, we will conduct a genome wide association study (GWAS) to identify genetic risk factors for aminoglycoside-induced nephrotoxicity.

7.8.7 Loss to Follow-up

If any of the trial patients are lost to follow up, contact will be attempted through the RN and lead investigator at each centre. Wherever possible, information on the reason for loss to follow-up will be recorded (see section 7.5).

7.8.8 Trial Closure

The end of the trial is defined to be the date on which data for all participants has been finalised and all data has been entered onto the database with data entry privileges withdrawn (data lock).

Table 7.1 – Schedule of study visits

	Pre-clinic	Clinic	Baseline T0	Daily during IV Tobramycin	T+1day	T+8days (between day	T+13days/Final day assessment	Follow-up visit (4 weeks after final dose of	Additional assessment in case of
Screening for potential participants	x								
Information sheet sent to patient	x								
Identification of eligible patients		x							
Signed Informed consent		x							
Assessment of Eligibility Criteria		x							
Confirmation of consent and eligibility criteria			x						
Review of Medical History			x						
Review of Concomitant Medications			x		x	x	x		
Urine pregnancy test			x						
Randomisation			x						
Physical Exam - Complete			x						
Dispense study medication			x						
Study Intervention			x	x					
Treatment diary				x					
Physical Exam - Symptom-Directed					x	x	x		x
Assessment of Adverse Events					x	x	x		x
Check study compliance					x	x	x		
Return unused medication							x		
Height			x			x	x	x	
Weight			x			x	x	x	
Collection of urine sample for biomarker analysis			x	x				x	
Measurement of FEV1			x			x	x	x	
Blood samples:			x		x	x	x	x	x
Serum Creatinine			x		x	x	x	x	x
Lipid Profile			x		x	x	x	x	x
Liver Function			x		x	x	x	x	x
Creatine Kinase			x		x	x	x	x	x
CRP			x		x	x	x	x	x
Tobramycin					x	x	x		x
Rosuvastatin (Treatment arm)					x	x	x		x
Plasma biomarkers			x		x	x	x	x	x
DNA			x						
Sputum			x			x	x	x	x

7.9 Statistical Considerations

7.9.1 Introduction

A separate and full statistical analysis plan will be developed prior to the final analysis of the trial. This would include verification of the assumption that fold-change in KIM-1 is normally distributed, and use of an appropriate transformation (logarithmic) if necessary.

7.9.2 Method of Randomisation

Participants will be randomised using a web-based randomisation tool. This will randomise equally between two arms using variable block randomisation.

7.9.3 Outcome Measures

7.9.3.1 Primary Outcome

The primary outcome measure will be the difference in mean fold-change in urinary KIM-1 from baseline to 'highest value' concentration during exposure to tobramycin between the rosuvastatin treated arm and control arm.

7.9.3.2 Secondary Outcomes

- Change in traditional markers of renal injury, serum creatinine and estimated Glomerular Filtration Rate, and change in novel urinary and plasma biomarkers of renal injury during tobramycin exposure between the rosuvastatin treated arm and the control arm.
- To assess safety of the intervention, we will compare the reported expected and unexpected serious adverse events between the rosuvastatin treated arm and the control arm. We will particularly focus on muscle adverse events which have been reported with rosuvastatin.
- We will assess for interaction between rosuvastatin and tobramycin:

- Pharmacokinetically, by comparing tobramycin concentrations between the rosuvastatin treated arm and the control arm.
- Pharmacodynamically, by comparing change in percent of predicted Forced Expiratory Volume in 1 second (FEV1) and change in CRP, between the rosuvastatin treated and control arms.

- In the rosuvastatin treated arm, collected blood samples will be analysed centrally for rosuvastatin concentration, in order to assess the pharmacokinetic profile of rosuvastatin in children with CF, to assess compliance, and to relate rosuvastatin concentrations to change in urinary KIM-1.
- We will assess the impact on *Pseudomonas aeruginosa* by measuring biomarkers associated with *P. aeruginosa* quorum sensing which can be used to determine both the presence of *P. aeruginosa* as well as markers of virulence. These markers will be compared between the rosuvastatin treated and control arms

7.9.4 Sample Size and Power Requirement

A power calculation for the study has been completed using the following assumptions: The fold-change in KIM-1 ('highest value' measurement following treatment / baseline) is normally distributed in each arm with a common standard deviation. A mean fold change in KIM-1 of 3.03, with a standard deviation of 1.84 was derived from an early analysis of samples in the URBAN CF study from 10 participants receiving a single course of treatment with tobramycin. The same data was also inspected to assess that the assumption of normality is reasonable. Using these assumptions and utilizing a 2-sample t-test, a sample size of 20 in each arm would have a power of 0.92 to detect a difference in fold-change between the groups of 2, at a two-sided significance level of 0.05. We plan to include 50 patients in the trial in order to compensate for loss to follow up.

7.9.5 Analysis Plan

The primary outcome will be analysed using the method of analysis of covariance (ANCOVA). The outcome measure will be the 'highest value' of KIM-1 during exposure to tobramycin and the covariates will be treatment group and the baseline KIM-1 value. To explore the secondary objectives of identifying change in biomarkers in the active arm in comparison to the control, linear mixed effect models will be used to fully exploit the serial nature of these outcomes. These models can estimate the effect of treatment and the timing of any effect on each biomarker while accounting for variability within patients of the various biomarkers. We will then be able to assess which biomarker has the largest and/or earliest change in response to treatment. The evaluation of beneficial and adverse biomarkers in relation to rosuvastatin treatment will be examined using joint modelling approaches accounting for informative loss to follow up or censoring. All modelling will be adjusted for the covariate effects. The secondary outcomes of pharmacokinetics will be analysed using the relevant modelling approaches. Expected and unexpected adverse events will be analysed as the standard MHRA guidelines. A separate and full statistical analysis plan will be developed prior to the final analysis of the trial.

7.9.6 PROteKT substudy - Molecular genetics of aminoglycoside-induced nephrotoxicity

7.9.6.1 Statistical Methods

The samples collected in this study will be combined with:

1. Samples collected through the MAGIC study (Molecular Genetics of Adverse Drug Reactions in Paediatric Patients), Research Ethics No: 10/H1002/57.
2. Samples collected through worldwide efforts to evaluate genetic factors predisposing to drug-induced renal injury.

Statistical methodology for genetic association studies is a rapidly developing field, and the most up to date methods will be applied to bring the most powerful statistical methods to bear on the data analysis, and thus extract the maximum information possible from the genotype data. A detailed statistical analysis plan will be prepared prior to starting the analysis.

Prior to the association analyses, a test for Hardy Weinberg equilibrium will be undertaken at each SNP, using Fisher's exact test. Any marker found to deviate significantly ($p<0.001$) will be flagged and the reasons for deviation explored. Population substructure will also be tested for, and adjusted for in the analysis if any is detected. The extent of missing genotype data per SNP and per patient will be examined and the reasons explored. Tests to ensure that any missing genotype data is at random will also be conducted. Multiple imputation methods will be used should missing genotypes be extensive.

For assessing association between a SNP and the risk of an ADR, two tests for association will be undertaken to compare genotype frequencies between cases and controls. The first will be a Chi-squared test, which makes no assumption regarding the underlying mode of inheritance, and the second will be a Cochrane-Armitage test for trend, which assumes an additive mode of inheritance. In the event that it is necessary to adjust for the effect of potential confounding factors, two logistic regression models will be fitted – the first including covariates to represent the confounding factors only and the second including covariates to represent both the confounding factors and the SNP – and a likelihood ratio test used to assess for association. The regression analysis will be conducted twice under the two different assumptions regarding mode of inheritance. In addition to the p-value, the false discovery rate will be calculated to assess for statistical significance whilst accounting for the multitude of tests undertaken.

7.10 Pharmacovigilance

7.10.1 Terms and Definitions

The Medicines for Human Use (Clinical Trials) Regulations 2004 (SI 2004/1031) definitions:

Adverse Event (AE) - Any untoward medical occurrence in a subject to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product.

Adverse Reaction (AR) - Any untoward and unintended response in a subject to an investigational medicinal product which is related to any dose administered to that subject.

Unexpected Adverse Reaction (UAR) - An adverse reaction the nature and severity of which is not consistent with the information about the medicinal product in question set out in:

- In the case of a product with a marketing authorization, in the summary of product characteristics for that product
- In the case of any other investigational medicinal product, in the investigator's brochure relating to the trial in question.

Serious Adverse Event (SAE), Serious Adverse Reaction (SAR) or Suspected Unexpected Serious Adverse Reaction (SUSAR) - Any adverse event, adverse reaction or unexpected adverse reaction, respectively, that:

- results in death
- is life-threatening* (subject at immediate risk of death)
- requires in-patient hospitalisation or prolongation of existing hospitalisation**
- results in persistent or significant disability or incapacity, or
- consists of a congenital anomaly or birth defect

- Other important medical events***

*'life-threatening' in the definition of 'serious' refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

**Hospitalisation is defined as an inpatient admission, regardless of length of stay, even if the hospitalisation is a precautionary measure for continued observation. Hospitalisations for a pre-existing condition, including elective procedures that have not worsened, do not constitute an SAE.

***Other important medical events that may not result in death, be life-threatening, or require hospitalisation may be considered a serious adverse event/experience when, based upon appropriate medical judgment, they may jeopardise the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition

7.10.2 Notes on Adverse Event Inclusions and Exclusions

7.10.2.1 Trial specific definitions

- Myalgia and myopathy (including myositis) are expected adverse reactions related to rosuvastatin. In addition to monitoring for symptoms, we will also measure creatine kinase as part of the study. If creatine kinase is increased above 4 times the upper limit of normal, rosuvastatin should be discontinued, and the local site should report as a serious adverse reaction. If creatine kinase is less than 4 times the upper limit of normal and muscular symptoms are absent or mild, this should be recorded as an adverse reaction, but rosuvastatin can be continued and the patient should be closely monitored. If muscular symptoms are severe and cause daily symptoms, even if creatine kinase is less than 5 times

the upper limit of normal, rosuvastatin should be discontinued, and the local site should report as a serious adverse reaction.

- Increases in liver transaminases are an expected adverse reaction related to rosuvastatin. Liver transaminases will be measured as part of the study. If liver transaminases are increased above 3 times the upper limit of normal, after the start of rosuvastatin, then rosuvastatin should be discontinued, and the local site should report as a serious adverse reaction.
- Minor side-effects identified in SmPC that if occur, the impact would be relatively non-substantial in this patient group: Headache, Dizziness, Constipation, Nausea, Abdominal pain, Myalgia, Asthenia, Pruritis, Rash, Urticaria. These should be recorded as Adverse Reactions.
- Side effects identified in the SPC that could have a substantial impact in this patient group: Thrombocytopenia, Hypersensitivity reactions including angioedema, Polyneuropathy, Memory loss, Diabetes Mellitus, Pancreatitis, Jaundice, Hepatitis, Rhabdomyolysis, Stevens-Johnson syndrome, Immune-mediated necrotising myopathy. These should be recorded and reported as Serious Adverse Reactions.

7.10.2.2 Include

- An exacerbation of a pre-existing illness
- An increase in frequency or intensity of a pre-existing episodic event/condition
- A condition (even though it may have been present prior to the start of the trial) detected after trial drug administration
- Continuous persistent disease or symptoms present at baseline that worsens following the administration of the study/trial treatment

- Laboratory abnormalities that require clinical intervention or further investigation (unless they are associated with an already reported clinical event).
- Abnormalities in physiological testing or physical examination that require further investigation or clinical intervention

7.10.2.3 Do Not Include

- Medical or surgical procedures- the condition which leads to the procedure is the adverse event
- Pre-existing disease or conditions present before treatment that do not worsen
- Situations where an untoward medical occurrence has occurred e.g. cosmetic elective surgery
- Overdose of medication without signs or symptoms
- The disease being treated or associated symptoms/signs unless more severe than expected for the patient's condition

Children with cystic fibrosis may require hospitalisation for exacerbations related to their condition. Therefore any readmission which occurs during the study pharmacovigilance period does not require expedited reporting as 'serious' unless it is felt there is evidence to suggest that exposure to rosuvastatin may have had a causative effect.

7.10.3 Reporting Procedures

Adverse reactions and all serious adverse events should be recorded. Depending on the nature of the event the reporting procedures below should be followed. Adverse reactions and all serious adverse events will be recorded from the point that the participant provides informed consent and throughout the trial treatment period up until the date of the follow-up assessment (3-5 weeks after the patient has taken the final dose of investigational medicinal product).

7.10.3.1 Non serious ARs/AEs

All adverse reactions (non-serious events suspected to be related to any dose administered of rosuvastatin) should be recorded, whether expected or not. Adverse reactions should be recorded on the study CRFs. Non-serious adverse events do not need to be reported.

7.10.3.2 Serious ARs/AEs/SUSARs

SARs, SAEs and SUSARs should be reported within 24 hours of the local site becoming aware of the event to the trial manager (scan report, encrypt and attach to email: protekt_trial@ucl.ac.uk). The SAE form asks for the nature of event, date of onset, severity, corrective therapies given, outcome and causality. The responsible investigator should sign the causality of the event. Additional information should be sent within 5 days if the reaction has not resolved at the time of reporting.

The trial manager will ensure that all serious adverse events are reviewed by the Chief Investigator or another senior clinician nominated by the Sponsor. For each serious adverse event, the CI, or his deputy, will make a judgment about whether the nature or severity of the circumstances surrounding the event are consistent with adverse events associated with the underlying condition, or with rosuvastatin. In the event of a serious adverse event that has a causal relationship to rosuvastatin and is not consistent with the information contained in the Summary of Product Characteristics the event will be labelled as a Suspected Unexpected Serious Adverse Reaction (SUSAR). All events labelled as SUSARs will be reported to the sponsor by the trial manager within 24 hours of the decision being made. All SUSARs will be reported by the trial manager to the competent regulatory authority (MHRA) in accord with the Sponsor's Standard Operating Procedures. The trial manager should then supply a copy of the SUSAR to the CTRC in parallel with submitting to the MHRA.

All investigators will be informed of all SUSARs occurring throughout the study.

7.10.4 Responsibilities – Investigator

The Investigator is responsible for reporting all ARs that are observed or reported during the study. The Investigator is also responsible for reporting all SAEs observed or reported during the study, regardless of their relationship to study product.

All SAEs must be reported immediately by the investigator on an SAE form unless the SAE is specified in the protocol as not requiring immediate reporting.

7.10.5 Reporting of Pregnancy

Study participants will be tested for pregnancy as part of the trial screening process. Any pregnancy which occurs during the study should be recorded in the study CRF and the participant should be instructed immediately to stop taking study drugs. The sponsor should be informed. All pregnancies that occur during treatment need to be followed up until after the outcome using the SAE form. Consent to report information regarding these pregnancy outcomes should be obtained from the mother prior to completion and emailing of the SAE Form. Any SAE experienced during pregnancy must be reported on the SAE form. The investigator should contact the participant to discuss the risks of continuing with the pregnancy and the possible effect to the foetus. Appropriate Obstetric care should be arranged.

7.10.6 Notes on Severity / Grading of Adverse Events

The assignment of the severity/grading should be made by the investigator responsible for the care of the participant. Regardless of the classification of an AE as serious or not, its severity must be assessed according to medical criteria alone using the following categories:

Grade 1 (Mild): does not interfere with routine activities

Grade 2 (Moderate): Interferes with routine activities

Grade 3 (Severe): impossible to perform routine activities

A distinction is drawn between serious and severe AEs. Severity is a measure of intensity (see above) whereas seriousness is defined using the criteria in section 10.1, hence, a severe AE need not necessarily be a Serious Adverse Event.

7.10.7 Relationship to Trial Treatment

An initial assignment of the causality should be made by the investigator responsible for the care of the participant using the definitions below.

Following reporting, a further assessment of causality will be made by the Chief Investigator. In the case of discrepant views on causality between the Chief investigator and others, the MHRA will be informed of both points of view.

7.10.7.1 Definitions of Causality

Unrelated - There is no evidence of any causal relationship. N.B. An alternative cause for the AE should be given

Unlikely - There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial medication). There is another reasonable explanation for the event (e.g. the participant's clinical condition, other concomitant treatment).

Possibly - There is some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after administration of the trial medication). However, the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant treatments).

Probably - There is evidence to suggest a causal relationship and the influence of other factors is unlikely.

Almost certainly - There is clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out.

7.10.8 Expectedness

An AE whose causal relationship to the study drug is assessed by the investigator as "possible", "probable", or "definite" is an Adverse Drug Reaction. All events judged by the designated investigator to be possibly, probably, or almost certainly related to the IMP, graded as serious and unexpected (see section 7.10.2 and SmPC for list of Expected Adverse Events) should be reported as a SUSAR.

7.10.9 Follow-up After Adverse Events

All adverse events should be followed until satisfactory resolution or until the investigator responsible for the care of the participant deems the event to be chronic or the patient to be stable.

When reporting SAEs and SUSARs the investigator responsible for the care of the participant should apply the following criteria to provide information relating to event outcomes: resolved; resolved with sequelae (specifying with additional narrative); not resolved/ongoing; ongoing at final follow-up; fatal or unknown.

7.11 Ethical Considerations

7.11.1 Ethical Considerations

We consider the specific ethical issues relating to participation in this trial to be:

7.11.1.1 Informed consent process

The study will be conducted in a paediatric population. We will provide age-specific patient information leaflets, which will be sent by the clinical team to the patient's home address at least 1 week prior to them being approached for consent. Consent will be requested from parent/guardian with child assent form for participants <16 years old. Participants aged 16-18 years will consent for themselves. Informed consent will be sought according to methods approved by an independent REC and local NHS management organisation, and will be in accordance with the Declaration of Helsinki. The researchers will have experience in taking informed consent from parents and children, and will have up-to-date GCP training.

7.11.1.2 Allocation of participants to control arm

Half of recruits will be allocated to the non-intervention control arm. These patients will not receive any investigational drug and therefore do not get any direct benefit of the intervention, if any; however such a non-intervention comparator arm is necessary for the identification of a positive drug effect in the treatment arm. This will have no impact on their standard CF treatment as rosuvastatin is used as an addition to standard care.

7.11.1.3 Risk of adverse effects

There are known adverse effects associated with rosuvastatin that are outlined in the SmPC. Whilst serious adverse effects are rare, we will monitor participants receiving rosuvastatin to identify these early.

7.11.1.4 Increased medication burden to the participants

There will be further increase in the medication burden to the participants of this trial. However, we do not envisage this to be a major issue since the intervention is available as a single tablet that needs to be taken only once daily for a period of only 2 weeks.

7.11.1.5 Contraception during the treatment period

A pregnancy test should be carried out in all female participants of childbearing potential (all who have reached menarche) at the time of enrolment to the study before taking rosuvastatin. If a potential participant refuses the test, they should not be included in the study. Rosuvastatin is not recommended in pregnancy; therefore, for those who are on the treatment arm, barrier contraception must be *strongly* advised to all women of childbearing age for the duration of the trial, if sexually active. Sensitivity will need to be exercised by the researcher addressing this issue with the potential participant, and care may be required to avoid embarrassment to the child in front of their parents. An appropriate approach should be discussed beforehand with the clinical team who know the patient and their family. This issue has been discussed with the NIHR Medicines for Children Research Network, and is in line with their recommendations.

7.11.1.6 Additional visits required for the trial

We have taken steps in designing this study to minimise the need for participants to attend for extra visits. Participants will need to attend for baseline assessment, but they would normally need to attend on this day for their IV line to be sited and bloods taken. During the treatment phase daily assessments and collection of urine samples, may occur on an inpatient unit, or through a home visit if the participants is having IV tobramycin at home. Blood samples may also be collected at home if this is normal local practice. The 4 week follow-up visit will usually be additional, although efforts will be made to combine this visit with another clinical visit if possible.

7.11.1.7 Additional Blood tests

The protocol is designed so that the majority of study bloods will be collected at the same time as routine clinical bloods. However, it is anticipated that children participating in the study will receive two additional blood tests to those required for routine clinical care.

When blood tests are done, an additional volume of at least 4mls of blood will be required. This is well within the volume limits recommended by the European Medicines Agency. If clinically indicated blood samples are being taken at other times, an additional sample will be taken for study bloods. Where possible we will seek to minimise the volumes of blood taken by using left-over blood from clinical samples.

7.11.1.8 Conflict of interest

The members of the research team who complete the urine biomarker analysis will not be involved in the medical care of participants, and will be blinded to study arm allocation. Those involved in recruiting participants will be members of the team providing medical care to participants. There are no other conflicts of interest.

7.11.2 Ethical Approval

The trial protocol will be submitted to a multi-centre Research Ethics Committee (MREC) and will also undergo independent review at the R&D offices at participating sites. The local R&D office should be sent the appropriate site specific information form complete with the necessary authorisation signatures, plus any other documentation requested for review. Local Research & Development (R&D) approval is required before the site is initiated and patients recruited.

Consent from the patient should be obtained prior to participation in the trial, after a full explanation has been given of the treatment options, including the conventional and generally accepted methods of treatment. Patient Information and Consent Forms should also be implemented. The right of the patient to refuse to consent to participate in the trial without giving a reason must be respected. After the patient has entered the trial, the clinician must remain free to give alternative treatment to that specified in the protocol, at any stage, if he/she feels it to be in the best interest of the patient. However, the reason

for doing so should be recorded and the patient will remain within the trial for the purpose of follow-up and data analysis according to the treatment option to which they have been allocated. Similarly, the patient remains free to withdraw at any time from the protocol treatment and trial follow-up without giving reasons and without prejudicing their future treatment.

7.11.3 Informed Consent Process

Informed consent is a process initiated prior to an individual agreeing to participate in a trial and continues throughout the individual's participation. In obtaining and documenting informed consent, the investigator should comply with applicable regulatory requirements and should adhere to GCP and to the ethical principles that have their origin in the Declaration of Helsinki.

Discussion of objectives, risks and inconveniences of the trial and the conditions under which it is to be conducted are to be provided to patients and their parents by staff with experience in obtaining informed consent. Information sheets which outline the trial and possible risks and benefits will be sent to the patient and their parents/guardian at least one week prior to their routine clinic visit once they are identified by the delegated clinical staff at respective recruitment centres to meet the eligibility criteria. This will enable the patient and their parent/guardian to go through relevant information pertaining to the trial and associated risks and benefits in advance. Patient information sheets describing in detail the trial interventions/products, trial procedures and risks will be approved by an independent ethical committee and the patient will be asked to read and review the document.

Potentially eligible participants will be identified by the clinical team at each centre via a search of the patient database/s either electronically or manually or clinic list review. A

short introductory letter about the study, along with a parent information sheet and age-appropriate information sheet for the child, will be sent out to the family at least 1 week prior to their clinic visit. The introductory letter will ask them to read the information sheets, and will explain that they will be approached by the research team when they come to the clinic to consider consenting to participate in the study.

In the CF outpatient clinic, children (and their parents/guardian) who are eligible will be approached by a member of the research team. If they have not had opportunity before coming to the clinic, parents and children will be given time to read the study information sheets. The designated member of the research team will explain the trial to the patient. This information will emphasise that participation in the trial is voluntary and that the participant may withdraw from the trial at any time and for any reason. They will discuss the objectives of the study and all potential benefits and inconveniences of taking part. They will clearly outline all of the responsibilities the patient will be expected to meet if they agree to participate, including attendance at study visits and compliance with trial medications. All participants and their parents/guardian will be given opportunity to ask any questions that may arise, and will have the opportunity to discuss the study and time to consider the information prior to agreeing to participate. A contact point where further information about the trial may be obtained will be provided.

The parent (or the patient if aged 16 or over) will sign and date the informed consent document. The person taking consent must also personally sign and date the form. A copy of the informed consent document will be given to the patient for their records. The original copy will be filed in the participant's notes and a further copy of the signed consent form will be stored in the Investigator Site File. An assent form will be completed by the child if aged under 16 years. This consent will be considered 'prospective' and will be re-

confirmed when the child attends for their baseline assessment. If a child aged 15 years has given assent and their parent has given consent then the child will not be re-consented if they reach the age of 16 years during the short follow up period unless they request to do so.

The researcher delegated to obtain informed consent will be determined on a site by site basis, depending on the experience and knowledge of the individual staff at that site. Only personnel deemed competent to do so by the PI and delegated the duty on the site signatory and delegation log will be able to obtain informed consent. This can include PIs, other delegated investigators and RNs. Where informed consent is being obtained by a RN, the patient should have access to a clinician with expertise in management of children with cystic fibrosis if they have any concerns about participation or any further questions that the RN is unable to sufficiently answer.

Where the informed consent discussion is conducted through a translator (i.e. the patient is non-English speaking), the translator will also sign the consent form to confirm that a full and accurate account of the study information has been provided to the patient.

Patients will be given sufficient time to consider their decision and consent to the trial. The patient may, without being subject to any resulting detriment, withdraw from the trial at any time by revoking the informed consent. The rights and welfare of the patients will be protected by emphasising to them that the quality of medical care will not be adversely affected if they decline to participate in this study.

7.11.4 Study Discontinuation

In the event that the study is discontinued, participants will be treated according to standard clinical care. The process for participants who withdraw early from trial treatment or from the trial completely is described in Section 7.5.3.

7.12 Trial Monitoring

7.12.1 Risk Assessment and Trial Monitoring Plan

Monitoring procedures for the PROteKT trial will be determined from the trial specific risk assessment. The risk assessment exercise will inform how monitoring should be conducted and the details of the monitoring activities will be documented in the related trial specific monitoring plan. The risk assessment is completed in partnership with the Chief Investigator, Sponsor and CTRC.

Guidance issued by the MRC, Department of Health and the MHRA on risk-adapted approaches to the management of CTIMPs propose a three level categorisation for the potential risk associated with the IMP, assigned according to the following categories:

Type A *'no higher than that of standard medical care';*

Type B *'somewhat higher than that of standard medical care';*

Type C *'markedly higher than that of standard medical care'.*

7.12.1.1 What are the particular hazards of the trial?

Rosuvastatin is indicated for clinical use in the treatment of hypercholesterolaemia. However, in this study rosuvastatin will be used outside the manufacturer's indication, and therefore the risk associated with the IMP in this trial is categorised as ***Type B 'somewhat higher than that of standard medical care'***. This level of risk informs the risk assessment, regulatory requirements, nature and extent of the monitoring, and the management processes used in the trial. Important factors for consideration include:

- The study will be conducted in a paediatric population, therefore close monitoring of consent/assent procedures will be a priority.

- A robust, centralised, web-based randomisation tool will be used to avoid the potential for compromise of random allocation in this open-label trial.
- Rosuvastatin has some known potential adverse effects. Rare but serious adverse effects involve skeletal muscle and the liver. We will monitor specifically for these by measuring creatine kinase and liver function tests respectively.
- Rosuvastatin will be used in a population who have normal lipid profiles, which may potentially result in transient hypolipidaemia. We are not aware of any evidence of adverse effects related to short-term hypolipidaemia, but this will be routinely monitored in all patients during the trial.

7.12.2 Source documents

Source data: All information in original records and certified copies of original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents (original records or certified copies). (ICH E6, 1.51).

Source documents: Original documents, data, and records (e.g., hospital records, clinical and office charts, laboratory notes, memoranda, subjects diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate copies, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories and at medico-technical departments involved in the clinical trial). (ICH E6, 1.52).

In order to resolve possible discrepancies between information appearing in the CRF and any other participant related documents, it is important to know what constitutes the source document and therefore the source data for all information in the CRF. The

following data recorded in the CRF should be consistent and verifiable with source data in source documents other than the CRF (e.g. medical record, laboratory reports and nurses' notes).

The following parameters that will be documented in the CRF are not source data:

- Relevant medical history and diagnosis (medical notes are source documents)
- Data for evaluation of eligibility criteria (medical notes are source documents)
- Physical examinations and assessments (medical notes are source documents).
- Concomitant medications (including changes) and diagnoses (medical notes are source documents)
- Dispensing of trial medication (pharmacy records are source documents)
- Adverse events (medical notes are source documents)

For data where no prior source documentation exists and which will be recorded directly in the CRF the CRF will be considered the **source document.**

In addition to the above source documentation the date(s) of conducting informed consent discussion, date of provision of patient information, randomisation number, treatment allocation and the fact that the patient is participating in a clinical trial should be added to the patient's medical record.

7.12.3 Data Capture Methods

Data will be collected on paper case report forms (CRFs) and via participant completed diaries. CRFs will be sent into CTRC for data entry into the study specific database by members of the Data Management Staff delegated with data entry responsibilities.

7.12.4 Central Monitoring

At the time of data entry data will be checked for missing or unusual values (range checks) and these will be raised as data queries with the centres. Data queries will be issued to centres in order to address problematic/missing data. Central monitoring reports will highlight where there are issues with missing data, non response to data queries and missing CRFs. The frequency for producing central monitoring reports will be determined from the risk assessment and specified in the monitoring plan.

7.12.5 Clinical Site Monitoring

The trial manager from UCL and staff from CTRC will be conducting routine on-site monitoring visits for the PROteKT trial. In addition there may also be 'triggered' visits to centres should central monitoring highlight any issues.

The specific monitoring activities to be carried out at site visits will be described in the monitoring plan. As members of the CTRC and the trial manager at UCL will need to access patient records, laboratory reports and other confidential medical information this fact is included in the patient information sheet and consent form.

7.12.6 Confidentiality

UCL will be undertaking activities requiring the transfer of some identifiable data: Verification that appropriate informed consent is obtained will be enabled by the provision of participant's signed informed consent form supplied to the trial manager at UCL by recruiting sites. This involves the transfer of participant names. This process is disclosed in the consent form. UCL will preserve the confidentiality of participants taking part in the study. Completed consent forms will be stored securely in a location separate to the clinical data collected.

7.12.7 Records Retention

The investigator at each investigational site must make arrangements to store the essential trial documents, (as defined in Essential Documents for the Conduct of a Clinical Trial (ICH E6, Guideline for Good Clinical Practice)) including the Investigator Site File and Pharmacy Site File, until the CTRC informs the investigator that the documents are no longer to be retained, or for a maximum period of 15 years (whichever is soonest). In addition, the investigator is responsible for archiving of all relevant source documents so, that the trial data can be compared against source data after completion of the trial (e.g. in case of inspection from authorities). The investigator is required to ensure the continued storage of the documents, even if the investigator, for example, leaves the clinic/practice or retires before the end of required storage period. Delegation must be documented in writing.

The CTRC undertakes to store originally completed CRFs, except for source documents pertaining to the individual investigational site, which are kept by the investigator only. The CTRC will archive the documents in compliance with ICH GCP utilising the Records Management Service of the University of Liverpool. All electronic CRFs and trial data will be archived onto an appropriate media for long term accessible storage. Hard copies of data will be boxed and transferred to specially renovated, secure, premises where unique reference numbers are applied to enable confidentiality, tracking and retrieval.

7.13 Research Governance, Sponsorship and Financial Arrangements

7.13.1 Research Governance

7.13.1.1 Sponsorship

PROteKT will be sponsored by The Joint Research Office, which has been set up by Liverpool Health Partners, a collaboration between the University of Liverpool and 9 NHS Trusts.

7.13.1.2 Trial support

The PROteKT study will be run with the input of the Clinical Trials Research Centre (CTRC), University of Liverpool, and a Trial Manager has been appointed, based at University College London (UCL). The CTRC is a fully registered Clinical Trials Unit (CTU) with the UKCRC and the national Medicines for Children trials unit. This CTU has extensive experience in conducting multi-centre clinical trials including investigational medicinal products regulated by the Medicines and Health care Products Regulatory Agency. The study will be adopted onto the NIHR portfolio and will receive support from the CRN: Children.

7.13.1.3 Independent Data and Safety Monitoring Committee (IDSMC)

The IDSMC will comprise of a statistician, clinician specialising in care of patients with CF, and a chairperson who are independent of the applicants and have no other involvement in the trial. The IDSMC will be responsible for reviewing and assessing recruitment, interim monitoring of safety and effectiveness, trial conduct and external data. The IDSMC will first convene prior to trial initiation and will then define frequency of subsequent meetings (at least annually). The IDSMC will provide a recommendation to the Trial Management Group concerning the continuation of the study.

7.13.1.4 Day to day trial management

The Trial Management Group (TMG) will be responsible for the day-to-day running and management of the trial; they will meet monthly throughout the trial. The Chief Investigator (MP) will meet with other trial staff (e.g. Trial manager, data manager, trial administrator, and trial statisticians) as required. We will also include a patient or parent representative onto this group in order to provide advice on patient recruitment, if one can be identified.

Recruitment and quality of data will be monitored by the trial team:

The data manager will be responsible for chasing overdue CRFs and following-up data queries until resolution as per routine practice.

The data manager alongside the trial manager and statistics team will also contribute towards the production of central monitoring reports. Typically these reports will highlight:

- Adverse event reporting rates between centres
- Protocol violations between centres
- Missing critical data items between centres
- Screening, recruitment and dropout rates between centres

These central monitoring reports will be reviewed by the TMG and, in accordance with the monitoring plan, will identify when additional intervention, e.g. triggered site visits, should be undertaken.

The CTRC has processes in place to ensure that the trial will not open to recruitment until appropriate approvals and authorisations have been obtained from the MHRA, independent REC, and NHS Research & Development departments.

7.13.1.5 Trial Management division of responsibilities

The trial manager, based at UCL, will undertake the administration of all safety reports from the PIs at sites and provide data to the CTRC for the Independent Data and Safety Monitoring Committee. The IDSMC, and TMG meetings will be organised by the trial manager. CTRC SOPs will be used for trial management. The trial master file will be at UCL, who will provide a truncated TMF for the CTRC (e.g. ethics and CTA, contracts, current protocol etc).

CTRC will provide support and advice for efficient recruitment, robust data management, quality control, statistical analysis, effective monitoring of recruitment targets and reporting activities in compliance with governance requirements. CTRC SOPs will be used for statistics and data management.

The trial manager and CI should produce the Development Safety Update Report (DSUR). A template DSUR report document and the data required for summary tables can be agreed up front with the CTRC. SUSARs will be submitted to UCL (trial manager) for processing. The trial manager will liaise with the CI for medical oversight and ensure any expedited reports are submitted appropriately. The trial manager should then supply a copy of the SUSAR to the CTRC in parallel with submitting to the MHRA. CTRC Statisticians will provide summary data and contribute to the report, and the clinical perspective will be led by the CI. Guidance on SUSARs will be available from a CTRC Senior Trial Manager. CTRC are not responsible for this reporting other than to ensure we have accurate and up to date data on trial participants for summarising for safety monitoring for IDSMC/DSUR.

7.13.2 Insurance

The University of Liverpool professional indemnity and clinical trials insurance, and NHS indemnity schemes, will apply as appropriate

7.13.3 Funding

Funding for the study has been awarded by The J P Moulton Charitable Foundation. Additional funding to support this study is available through the North West England MRC Clinical Pharmacology and Therapeutics Fellowship awarded to Dr S McWilliam.

7.14 Regulatory Approval

This trial falls within the remit of the EU Directive 2001/20/EC, transposed into UK law as the UK Statutory Instrument 2004 No 1031: Medicines for Human Use (Clinical Trials) Regulations 2004 as amended. This trial will be registered with the MHRA for a Clinical Trial Authorisation (CTA). The EudraCT number is 2014-002387-32.

7.15 Publication

7.15.1 Publication Policy

The study findings will be published in an appropriate peer-reviewed journal.

8 General Discussion

8.1 Introduction

This thesis presents a novel, translational, approach to aminoglycoside-induced nephrotoxicity in children. Two key objectives have been established: firstly, to develop improved diagnostic tools for the early identification of aminoglycoside-induced nephrotoxicity in children; and secondly, to develop a novel intervention to prevent aminoglycoside-induced nephrotoxicity. These two objectives were supported by two hypotheses: that KIM-1 represents a novel urinary biomarker with clinical utility as a biomarker of aminoglycoside-induced nephrotoxicity in children; and that statins can prevent aminoglycoside-induced nephrotoxicity in children with CF. The ultimate aim of this thesis was to combine these two objectives in a first-in-man clinical trial to investigate the potential of statins to prevent aminoglycoside-induced nephrotoxicity in children with CF, using KIM-1 as the primary outcome measure.

8.2 Aminoglycoside-induced nephrotoxicity in children

Four studies from the US have provided an up to date assessment of aminoglycoside-induced nephrotoxicity in children (Goldstein et al., 2013), including preterm infants (Rhone et al., 2014), and those with CF (Downes et al., 2014, Downes et al., 2015).

A retrospective review of nephrotoxin exposure in 107 preterm neonates at one US Centre documented gentamicin exposure in 86.0% (Rhone et al., 2014). In this cohort, 26.2% developed AKI. Whilst this study cannot demonstrate causation, it does highlight the potential adverse impact of exposure to nephrotoxins in this population. This is particularly important in view of growing evidence that those born pre-term are at an increased long-term risk of CKD (Carmody and Charlton, 2013).

Three further studies have been produced by one research group based in Cincinnati. First, in a study of all admissions involving aminoglycoside exposure, they defined AKI by the pRIFLE criteria (a 25% or greater increase in estimated creatinine clearance) (Goldstein et al., 2013). Using daily serum creatinine monitoring, they identified AKI in 25% of unique patients exposed to aminoglycosides, and during 31% of admissions. The second study assessed the impact of daily monitoring of serum creatinine during treatment with aminoglycosides in children with CF (Downes et al., 2014). AKI was defined as a rise in serum creatinine by ≥0.3 mg/dL within 48 h or a 1.5-fold increase in the baseline serum creatinine level. Daily monitoring not only led to more cases of AKI being identified (in 21 of 103 courses (20%)), but these were also identified earlier. The study suggested that daily monitoring also led to changes in management in an attempt to prevent or ameliorate AKI, although a randomized trial would be required to assess whether there was any impact on patient outcomes. In a third study, which had a case-control design, they identified 593 admissions in which children were treated with an aminoglycoside for an exacerbation of CF (Downes et al., 2015). Of these, 131 (22%) developed AKI. 49 of these were excluded by the authors as early onset AKI (occurring <72 hours after admission) as they were felt to be unlikely to be caused by aminoglycoside exposure. This left 82 cases of AKI (14%). Risk factors associated with aminoglycoside-induced AKI included: days of aminoglycoside therapy (odds ratio (OR) 1.10, 95% confidence interval (CI) 1.02-1.20), receipt of a previous aminoglycoside within 90 days prior to admission (OR 2.28, 95% CI 1.10-4.72), low serum albumin (OR 3.45, 95% CI 1.41-8.45), and concomitant receipt of trimethoprim/sulfamethoxazole (OR 3.27, 95% CI 1.75-6.10). AKI risk was lower if infection with *Staphylococcus aureus* was present (OR 0.47, 95% CI 0.25-0.90). The authors were unable to explain this finding but hypothesized that this group of patients may have had lower cumulative aminoglycoside exposure.

It would be informative to repeat this study in other clinical settings to confirm the importance of these risk factors in other CF populations. In future studies, it will be helpful to assess lifetime cumulative aminoglycoside exposure as a risk factor, and to include measurement of baseline biomarkers to assess their predictive value for AKI.

These interesting studies have certainly added to the evidence that aminoglycoside-induced nephrotoxicity is important in children. Length of treatment and previous recent exposure to aminoglycosides were highlighted as risk factors for AKI (Smyth et al., 2008, Lopez-Novoa et al., 2011). It is interesting that low serum albumin is a risk factor for aminoglycoside-induced AKI, as albumin is a primary substrate for megalin. One hypothesis is that lower serum albumin results in lower renal tubular albumin concentrations, which leads to less competitive inhibition of megalin-mediated uptake of aminoglycoside. This mechanism could be explored further in an *in vitro* model such as the ciPTEC model that has been investigated in this thesis (chapter 5).

Emerging evidence suggests that the cystic fibrosis transmembrane conductance regulator (CFTR) may also play a role in the renal accumulation of aminoglycosides (Raggi et al., 2011). Mice with non-functional CFTR demonstrated a 15% reduction in renal gentamicin accumulation compared to controls. *In vitro* studies using cultured proximal tubule cells from these mice demonstrated defective endocytosis, reduced uptake of gentamicin and albumin, and a slight reduction in megalin expression (Raggi et al., 2011). This association of CFTR with megalin-mediated endocytosis in proximal tubule epithelial cells may reflect the underlying mechanism for the increased renal clearance of aminoglycosides seen in CF (Touw, 1998), and may confer some renal protection. However, as we have seen, this does not prevent individuals with CF from experiencing aminoglycoside-induced nephrotoxicity.

These findings support the importance of megalin-mediated endocytosis as a key pathway in the development of aminoglycoside-induced nephrotoxicity, and confirm the necessity of investigating therapeutic interventions to inhibit it.

8.3 Novel biomarkers for aminoglycoside-induced nephrotoxicity

8.3.1 KIM-1

In line with preclinical data (Vaidya et al., 2010), KIM-1 outperformed other biomarkers (NGAL, NAG and serum creatinine) in the identification of aminoglycoside-induced nephrotoxicity in preterm neonates (Chapter 2). KIM-1 also increased acutely in children with CF during exposure to tobramycin, and remained elevated following the withdrawal of tobramycin (Chapter 4). In children with CF, baseline KIM-1 concentration was associated with previous exposure to IV aminoglycosides (Chapter 4).

The utility of KIM-1 for the identification of aminoglycoside-induced nephrotoxicity in preclinical models has been established previously (Vaidya et al., 2010, Zhou et al., 2008). However, it has not yet been widely studied for this indication in humans. One study has measured KIM-1 in children with CF, their primary aim being to assess changes in biomarkers associated with exposure to ibuprofen.They found no significant association with ibuprofen, but did show a significant correlation between KIM-1 and previous courses of aminoglycosides ($r=0.35$, $p=0.012$) (Lahiri et al., 2014). A published abstract also reported elevated KIM-1 concentrations in children with CF receiving aminoglycosides (Uluer et al., 2010).

A growing body of literature suggests that KIM-1 may be a useful marker for the development of chronic kidney disease. IgA nephropathy is a common form of glomerulonephritis characterized by glomerular deposition of IgA antibodies. Acute disease progresses to chronic kidney disease in some patients. The utility of urinary KIM-1

as a biomarker for IgA nephropathy has been investigated, and been shown to track disease progression and predict severity (Xu et al., 2011, Xu et al., 2014, Peters et al., 2011, Kwon et al., 2013, Lin et al., 2014), reflect histological changes (Lin et al., 2014), and track response to treatment (Seo et al., 2013). Diabetic nephropathy is the most common cause of CKD worldwide. Urinary KIM-1 is elevated in diabetic nephropathy (De Carvalho et al., 2015), and lower concentrations predict disease regression (Vaidya et al., 2011). KIM-1 is also measurable in blood (Sabbisetti et al., 2014). Plasma KIM-1 is elevated acutely after renal ischaemia associated with cardiac bypass, and serum KIM-1 is elevated in CKD of various aetiologies (Sabbisetti et al., 2014).

The dual utility of KIM-1 as a biomarker of both AKI and CKD, is supported by growing evidence of its mechanistic role in the response to kidney injury and repair (Huo et al., 2010). It plays an important protective role in the response to AKI, promoting tubular epithelial cell regeneration (Huo et al., 2010). It also confers a phagocytic phenotype on epithelial cells which may be important in clearing kidney tubules of apoptotic and necrotic cell debris (Ichimura et al., 2008) resulting in a downregulation of innate immunity and the inflammatory response (Yang et al., 2015). However, it has been hypothesised that in CKD, KIM-1 may lead to excessive epithelial cell proliferation and have a role in the development of tubular fibrosis (Huo et al., 2010).

8.3.2 NGAL

Urinary NGAL increased during aminoglycoside exposure in both preterm neonates (chapter 2) and children with CF (chapter 4). In neonates, the increase in NGAL was not significant once confounders had been taken into account. In children with CF, the increase in NGAL appeared to be later than for KIM-1. There was no association of NGAL with previous aminoglycoside exposure. In contrast to KIM-1, therefore, NGAL does not

demonstrate as much promise as a biomarker for aminoglycoside-induced nephrotoxicity. Currently no other published studies have evaluated NGAL as a biomarker for aminoglycoside-induced nephrotoxicity in any human population.

NGAL has consistently been demonstrated to be elevated in inflammatory conditions and sepsis. In preterm neonates, serum and urinary NGAL correlate with other inflammatory markers (Suchojad et al., 2015), and urinary NGAL has shown promise as a potential biomarker for sepsis (Pynn et al., 2015, Parravicini et al., 2010). If NGAL is already raised due to sepsis, it may not be useful as a biomarker for nephrotoxicity caused by an aminoglycoside prescribed to treat that sepsis. This may explain why the increase in NGAL in neonates was no longer significant after adjustment for confounding factors (chapter 2).

Given its potential as an inflammatory biomarker, NGAL has been investigated as a marker of pulmonary exacerbations in CF (Zughaier et al., 2013). Serum NGAL was elevated in CF patients compared to healthy controls. In patients with CF, there was no difference in serum NGAL during pulmonary exacerbations compared to when the patients were well. In septic non-CF patients, serum NGAL was elevated above all other groups. The lack of change in serum NGAL during exacerbations in CF means that it is not useful as a marker of exacerbations. This does mean that measuring urinary NGAL in CF patients may be more likely to reflect renal injury, and less likely to reflect the pulmonary status. However, the possibility that any elevation of urinary NGAL seen in patients with CF may be due to sepsis cannot be excluded.

8.3.3 Summary and future work

Referring back to the list of characteristics of an ideal biomarker in section 1.4.2, this thesis has added evidence for a number of these. Both KIM-1 and NGAL can be measured in the population of interest from minimally invasive urine samples, and robust assays have been

developed to measure them. For KIM-1 translation from preclinical to clinical utility has been demonstrated.

This thesis adds evidence for the potential of KIM-1 as a biomarker of acute and chronic proximal tubular injury associated with exposure to aminoglycosides, particularly in children with CF. KIM-1 shows more promise as a biomarker of aminoglycoside-induced nephrotoxicity than NGAL.

Our study in preterm infants contributes to the existing evidence of acute increases in markers of proximal tubule toxicity during aminoglycoside exposure (Kent et al., 2014). It has also been shown that all three biomarkers measured were most raised in those neonates with AKI. However, a recent review of the literature questions whether studies, such as the one presented in chapter 2, have yet demonstrated a convincing link between elevation of tubular biomarkers and clinical outcomes (Kent et al., 2014). Another recent review identified urinary NGAL and KIM-1 as having potential as markers of nephrotoxicity and AKI in neonates (Mussap et al., 2014). Whilst numerous short-term studies have been conducted, there are currently no good studies focusing on long-term renal outcomes in preterm neonates.

Further studies are required to establish the optimal use of KIM-1 as a biomarker for aminoglycoside-induced nephrotoxicity in children with CF. It has not yet been demonstrated that it is predictive of clinically relevant outcomes for this indication. No patients in the URBAN CF cohort developed AKI during the study follow-up. Therefore, larger cohorts of patients would be required to demonstrate an ability to predict AKI (defined by pRIFLE or AKIN criteria) as a result of aminoglycoside-induced nephrotoxicity.

Qualification of these biomarkers for clinical use requires further studies in large, multicenter, cohorts, with longer periods of follow-up to demonstrate any association with both AKI and CKD, amongst other important clinical outcome measures. In any such study, it would be important to incorporate measurements of renal biomarkers alongside the use of 'gold standard' measures of renal function (such as Iohexol). Demonstrating a clear association with clinically relevant outcomes will inform future translation into clinical practice.

In children with CF, KIM-1 may have potential to inform three key areas of clinical decision making. First, baseline KIM-1 may allow risk stratification so that above a certain cut-off threshold, aminoglycoside exposure is avoided or reduced, or additional preventative measures are initiated to prevent further renal injury. Second, the measurement of KIM-1 acutely during aminoglycoside exposure may allow early diagnosis of AKI, allowing withdrawal of the aminoglycoside and other early therapeutic intervention. Thirdly, KIM-1 may have value in predicting renal outcomes in CF, and in monitoring response to therapeutic interventions.

An important issue however will be to ensure that the risk-benefit is maintained, and that the antibiotic is not stopped too early in the face of a complicated lung infection. The work of the PSTC in qualifying a number of renal biomarkers for preclinical use has already been highlighted (Section 1.4.2.1). The ongoing work of the PSTC will further address the evidence for the utility of these biomarkers in clinical settings, and their association with clinically relevant outcomes. This will provide evidence to allow the regulators to approve some of these biomarkers for clinical use, and to give clinicians confidence in interpreting these biomarkers to inform clinical decision making. This multicentre, consortium, approach is able to provide assessment of larger numbers of patients with more robust and

standardised analysis than can be achieved by multiple, small, single centre studies. This work will also help inform whether use of single biomarkers or of biomarker panels will be more helpful in different clinical scenarios (Pickering and Endre, 2012).

Clinical decision making is limited by a lack of therapeutic interventions to prevent renal injury. The development of robust biomarkers will actually aid the development of novel therapeutics. The qualification of renal biomarkers for preclinical use will have benefits for drug development by the early detection of nephrotoxicity (Burt et al., 2014, Benjamin et al., 2015). Biomarkers that translate from animal models to humans will allow for easier translation of interventions from preclinical to clinical, as has been demonstrated with the statin intervention studies in this thesis. Their use as outcome measures in clinical trials will enable smaller and shorter trials, such as PROteKT, which will again accelerate the translational process.

8.4 Prevention of aminoglycoside-induced nephrotoxicity

8.4.1 Novel models of aminoglycoside-induced nephrotoxicity

Two animal models of aminoglycoside-induced nephrotoxicity were utilized for the work presented in this thesis. The rat model of gentamicin-induced nephrotoxicity was already in existence, but for the first time in our practice, the urinary biomarker kim-1 was utilized as a measure of kidney injury in these animals. The measurement of kim-1 in rats is not novel in itself (Vaidya et al., 2010). However, its use demonstrates its potential utility as a translational biomarker, providing continuity from animal to human studies. Knowing that this biomarker can demonstrate the effectiveness of the statin intervention in rats gives additional confidence in using it as the primary outcome in a clinical trial in humans.

The guinea pig model provided a key translational bridge from rat to man. The similarity of guinea pig statin metabolism to man allowed the comparison of different statins to

establish which to take forward into human studies. Rosuvastatin was clearly superior to the more lipophilic simvastatin in its ability to inhibit gentamicin-induced nephrotoxicity. This model also allowed dose-ranging studies of rosuvastatin at concentrations that were translatable to humans. This allowed rosuvastatin 10mg, to be identified to take forward into the human study. This work could not have been done with the limitations of the rat model.

As part of this work, an *in vitro* model of aminoglycoside-induced nephrotoxicity has also been developed. Gentamicin-induced toxicity has been demonstrated previously in the ciPTEC model (Moghadasali et al., 2013), but here dose-dependent toxicity has also been shown with tobramycin and neomycin. This model will require further validation with additional assays of toxicity. Further independent characterization of the ciPTEC cell line is also required to verify proximal tubule cell characteristics and function (Wilmer et al., 2010). If this is confirmed, the cell line may prove a useful model for investigating the mechanisms of aminoglycoside-induced nephrotoxicity in more detail, and for developing novel therapeutic strategies.

8.4.2 Effect of statins on the anti-pseudomonal effects of aminoglycosides

An established method was used to investigate the impact of statins upon the MIC of tobramycin for *P. aeruginosa*. Neither rosuvastatin nor atorvastatin had any effect on the antipseudomonal effects of tobramycin. This again provides confidence in moving forward into human studies.

The LESB58 strain of *P. aeruginosa* produces a virulence factor called pyocyanin (Jeukens et al., 2014). When this strain is grown, the pyocyanin gives a green-blue colour to the broth. During the MIC experiments described in chapter 6 it was observed that when the LESB58 strain was dosed with statins, this green-bue colour change appeared to be reduced.

Preliminary data, measuring the colour change due to pyocyanin using a plate reader, suggested that production was significantly reduced ($p<0.001$) when this strain was dosed with a statin in addition to tobramycin compared to tobramycin alone. This effect was seen across variable concentrations of tobramycin and varying levels of atorvastatin and rosuvastatin between 0.1μM and 100μM, but was most pronounced at low levels of statin.

It has been shown that statins can have anti-inflammatory and anti-microbial effects (Hennessy et al., 2013, Mortensen et al., 2005, Almog et al., 2004). Statins have shown effects in the host that could modify the CF lung environment including a reduction in the levels of pro-inflammatory cytokines (Jouneau et al., 2011, Hennessy et al., 2014) and neutrophil activation (Lenglet et al., 2014). Statins can also have antimicrobial effects, particularly in terms of improved survival in statin-treated patients suffering from pneumonia (Schlienger et al., 2007) and sepsis (Almog et al., 2004). Furthermore, direct antimicrobial effects of high levels of statins have also been reported (Welsh et al., 2009, Motzkus-Feagans et al., 2012) along with a reduction in attachment and toxicity of *Streptococcus pneumoniae* under more physiologically relevant concentrations of statin (Rosch et al., 2010). A previous study used a limited strain panel of *P. aeruginosa* to show that there was a reduction in swarming motility in the presence of rosuvastatin, lovastatin and mevastatin, and biofilm formation in the presence of simvastatin (Hennessy et al., 2013). The current work adds to this the finding that atorvastatin and rosuvastatin can inhibit pyocyanin production.

This thesis has established that statins limit the nephrotoxic effects of aminoglycoside antibiotics and could extend the usefulness of these antibiotics. In addition, it has been shown that statins can inhibit production of the virulence factor pyocyanin by *P. aeruginosa*, and literature evidence suggests that statins have direct effects on respiratory

bacteria and the associated inflammatory response. Further work will be undertaken to understand these effects in more detail. Firstly, the mechanisms by which statins have a direct effect on the characteristics of clinically relevant strains of *P. aeruginosa* will be investigated in an *in vitro* model of biofilm formation that closely resembles biofilm formation in the CF lung. Secondly, an *in vivo* murine model will be used to determine how treatment with statins leads to alterations in the virulence and adaptation ability of *P. aeruginosa* and the host immune response. Thirdly, the effects of statins in combination with tobramycin on the entire respiratory bacterial population will be characterised in children with CF recruited to the PROteKT study, and biomarkers associated with *P. aeruginosa* virulence will be measured and compared between the intervention and control arms.

8.5 Concluding remarks

This thesis describes a translational journey, which has met its stated objectives: urinary KIM-1 has shown utility as a novel diagnostic tool for the early identification of aminoglycoside-induced nephrotoxicity in children; and the ability of statins to prevent aminoglycoside-induced nephrotoxicity has been demonstrated.

The next phase of this translational journey is the PROteKT study, a phase IIa randomised, controlled, clinical trial of rosuvastatin for the prevention of aminoglycoside-induced nephrotoxicity in children with CF. This study builds upon the twin objectives of the thesis, taking the statin hypothesis all the way from bench to bedside, and utilising change in urinary KIM-1 as its primary outcome measure.

The PROteKT study presents an exciting opportunity to develop an intervention that may protect the kidneys from the damaging effects of repeated exposure to aminoglycosides. Specifically, it aims to improve the long-term outlook for children with CF. In general, this

intervention could improve the risk-benefit balance of the aminoglycoside antibiotics for all patients. This would be an exciting and fulfilling culmination to this journey.

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10 Appendices

10.1 Gentamicin guideline, Liverpool Women's Hospital

10.2 Aminoglycoside guideline, Alder Hey Children's Hospital

10.3 McWilliam SJ *et al.* PLoS ONE, 2012

10.4 McWilliam SJ *et al.* Biomarkers in Medicine, 2014

10.5 Rodrigues *et al.* Bioanalysis, 2014