Diabetic Nephropathy:

Early Detection and Therapeutic Strategies

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A. Summary

The increasing global prevalence of diabetes poses a huge challenge to health services. The diagnosis is accompanied by a reduction in life expectancy, primarily due to cardiovascular disease which is inextricably linked to microvascular complications such as diabetic nephropathy (DN). Microalbuminuria (MA) is generally accepted as the primary clinical hallmark of DN, but despite widespread prescribing of agents blocking the renin angiotensin aldosterone system (RAAS) in these patients many continue to progress towards end-stage renal disease (ESRD). Clinical trials evaluating early initiation of RAAS blocking agents in untargeted, nonalbuminuric diabetic patients have shown potential for delaying disease progression but these effects are generally counterbalanced by side effects and adverse events associated with these therapies. Discovery of novel biomarkers to identify individuals at highest risk of DN who would stand to benefit most from targeted preclinical intervention would be a significant step towards implementation of personalised medicine in this population.

One technique which shows promise is proteomics, based on the concept of separation and quantification of peptides in a biological sample to produce a disease-specific pattern. A panel of 273 urinary peptides (CKD273) has been shown to have potential for identification of nonalbuminuric diabetic patients who are at risk of progression to overt DN. However, many such novel biomarkers are described in the literature and to date none have successfully made the transition from research studies to routine clinical practice. In order to be considered for clinical implementation novel biomarkers are required to be subject to a rigorous evaluation process. In brief there are several key steps beginning with proof-of-concept studies; progressing through validation in independent populations to demonstration of incremental value beyond the current guideline-endorsed tests; thereafter proof of clinical applicability in determining treatment strategies and cost-effectiveness are required. The work contained within this thesis is designed to address each of these aspects with regard to use of the CKD273 proteomic panel as a biomarker for early detection of DN.

The first challenge in biomarker evaluation requires demonstration of an unmet clinical need and availability of a suitable treatment option for individuals who are identified as “high risk”. Recent years have seen a resurgence of interest in the role of aldosterone in the initiation and progression of cardiorenal diseases. In fact, evidence suggests that aldosterone breakthrough can occur in patients established on angiotensin converting enzyme inhibitors (ACE-I) or angiotensin receptor blockers (ARB), suggesting a potential role for mineralocorticoid receptor antagonists in treatment and prevention of chronic kidney disease (CKD). In chapter 3 the results of a meta-analysis of studies evaluating effects of addition of MRA to ACE-I or ARB in patients with CKD are presented. This work confirms that this strategy is an effective means to lower blood pressure and minimise proteinuria in patients with CKD. A small reduction in glomerular filtration rate (GFR) associated with treatment likely reflects improvement of glomerular hyperfiltration and is associated with improved clinical outcomes. There is a threefold increase in relative risk of developing hyperkalaemia above predefine study limits with mean increase of 0.19mmol/L from baseline, hence the risk of hyperkalaemia is quantifiable and appropriate selection of patients may render this acceptable in the face of the potential benefits of treatment.

Chapter 4 illustrates a local pilot study where the CKD273 biomarker is validated for diagnosis of DN. This project included 45 individuals with type 2 diabetes (T2DM) fitting into 3 categories according to the traditional definition of DN: 15 were normoalbuminuric; 15 had microalbuminuria and 15 had overt macroalbuminuria. Urinary proteomic analysis confirmed that CKD273 classifier score did indeed increase across the study categories and receiver operating curve analysis confirmed that 0.343 was the near optimal threshold for diagnosis of DN; a finding that is consistent with other published literature.

Whilst its utility as an early marker of risk of progressive renal disease is debated, there is little doubt that MA serves as a very useful tool for highlighting increased cardiovascular risk. The work described within chapters 4 and 6 was designed in order to evaluate the specificity of CKD273 for identification of early renal disease rather than generalised subclinical vascular disease, through employing a number of clinical vascular phenotyping techniques and biomarker analyses. Results of these cross-sectional analyses showed that there was no

association between CKD273 classifier score and vascular phenotypes suggesting that CKD273 could indeed be a more specific renal biomarker, although such conclusions cannot be inferred from cross-sectional work.

Chapter 7 aimed to take the classifier from cross-sectional to prospective analyses for prediction of hard clinical endpoints. This study involved urinary proteomic analysis of stored baseline urine samples from a cohort of microalbuminuric T2DM patients with preserved renal excretory function at baseline who were followed up over a 4 year period. This cohort was of particular interest as the majority of patients who have MA do not progress to ESRD, meaning that biomarkers could be employed in this context to identify those at higher or lower risk of progressive disease. The overall number of events were low and survival analysis showed that the CKD273 classifier is not a predictor of decline in GFR in this cohort, a fact that is perhaps not surprising given that its role appears to be more that of a preclinical disease biomarker and these patients had evidence of established renal disease at baseline.

The ultimate test of a novel biomarker is evaluation in a prospective, biomarker-guided clinical trial where results of biomarker analysis are used to determine therapeutic intervention. The Proteomic Prediction and Renin Angiotensin Aldosterone System Inhibition Prevention of Early Diabetic Nephropathy in Type 2 Diabetic Patients with Normoalbuminuria (PRIORITY) trial, described in chapters 2 and 5 of this thesis aims to specifically address this requirement. This pioneering multicentre study is the first clinical trial to prospectively evaluate a proteomic biomarker and will serve to determine whether CK273 does indeed have the potential to make the leap from bench to bedside.

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F. Publications containing work undertaken in this thesis

1. Urinary proteomics for diagnosis and monitoring of diabetic nephropathy. **Currie G** and Delles C. *Current Diabetes Reports* 2016;16:104.

2. Effect of mineralocorticoid receptor antagonists on proteinuria and progression of chronic kidney disease: A systematic review and meta-analysis. **Currie G**, Taylor A, Fujita T, Ohtsu H, Lindhardt M, Rossing P, Boesby L, Edwards NC, Ferro C, Townend JN, Van den Meiracker AH, Saklayen MG, Oveisi S, Jardine AG, Delles C, Preiss DJ and Mark PB. *BMC Nephrology* 2016; 17:127

3. Chapter 14: Clinical cardiovascular proteomics. **Currie G**, Matt P and Delles C (2016). In Agnetti G, Lindsey M and Foster DB (Eds.). *Manual of Cardiovascular Proteomics*, Springer. ISBN 3319318268. (published July 2016)

4. Diabetic kidney disease: a case for precision medicine? **Currie G**, Mullen W and Delles C. *The Biochemist*, February 2016; p22-26.

5. Proteomic prediction and Renin angiotensin aldosterone system Inhibition prevention Of early diabetic nephRopathy in TYpe 2 diabetic patients with normoalbuminuria (PRIORITY): essential study design and rationale of a randomised clinical multicentre trial. Lindhardt M, Persson F, **Currie G**, Pontillo C, Beige J, Delles C, von der Leyen H, Mischak H, Navis G, Noutsou M, Ortiz A, Ruggenenti P, Rychlik I, Spasovski G and Rossing P. *BMJ Open* 2016; 6:e010310. doi:10.1136/bmjopen-2015010310

6. Biomarkers in diabetic nephropathy: Present and future. **Currie G**, McKay G, and Delles C. *World Journal of Diabetes* 2014; 15:763-776.

7. Proteinuria and its relation to cardiovascular disease. **Currie G** and Delles C. *International Journal of Nephrology and Renovascular Disease* 2013; 7:13-24

G. Published abstracts of conference proceedings

1. The CKD273 urinary proteomic classifier is not a marker of generalised vascular disease in type 2 diabetic patients with normal renal function. **Currie G**, Flynn J, Lindhardt M, Mischak H, Rossing P and Delles C. *Journal of Hypertension* 2016; 34: e14

2. Markers of subclinical cardiovascular and renal damage in patients with type 2 diabetes and normoalbuminuria: potential guides to personalised preventative therapy? **Currie G**, Flynn J, Lindhardt M, Rossing P and Delles C. *Diabetic Medicine* 2016; 33: 62-63

3. Meta-analysis of the effect of mineralocorticoid receptor antagonists on proteinuria and progression of chronic kidney disease. **Currie G**, Taylor A, Jardine A, Mark P and Delles C. *Hypertension* 2015; 66(S1): AP214.

4. Albuminuria within the normal range is associated with increased vascular stiffness in type 2 diabetes. **Currie G**, Flynn J and Delles C. *Journal of Human Hypertension* 2015; 29: 642

H. Presentations to learned societies of work undertaken in **this thesis**

Oral presentations

Currie G, Lindhardt M, Mischak H, Rossing P and Delles C. The CKD273 urinary proteomic classifier is not a marker of generalised vascular disease in type 2 diabetic patients with normal renal function. Presented at the 26th Meeting on Hypertension and Cardiovascular Protection, Paris, France, 2016.

Currie G, Lindhardt M, Mischak H, Rossing P and Delles C. The CKD273 urinary proteomic classifier for early diagnosis of nephropathy in type 2 diabetes: not simply an indicator of generalised vascular disease. Presented at the 29th Annual General Meeting of the European Diabetic Nephropathy Study Group, Pisa, Italy, 2016.

Currie G, Ravassa S, Mullen W, Mischak H, Lindhardt M, Rossing P and Delles C. Urinary proteomics and vascular phenotypes in diabetic nephropathy. Presented at the 28th Annual General Meeting of the European Diabetic Nephropathy Study Group, Copenhagen, Denmark, 2015.

Currie G. Urinary proteomics in type 2 diabetes. Presented to the Caledonian Endocrine Society, Dunkeld, 2014.

Currie G, Siwy J, Lindhardt M, Delles C, Jankowski J, Mischak H, Rossing P, PRIORITY investigators. Validation of a urinary proteomic classifier for diagnosis of diabetic nephropathy. Presented at Hypertension 2014, Athens, Greece, 2014.

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Poster presentations

Currie G, Flynn J, Lindhardt M, Mischak H, Rossing P and Delles C. Markers of subclinical cardiovascular and renal damage in patients with type 2 diabetes and normoalbuminuria: Guides to personalized preventative therapy? Presented at the Diabetes UK Professional Conference 2016, Glasgow, 2016.

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J. Author's declaration

This thesis was composed entirely by the author and has not previously been submitted for any other degree. The author was responsible for: study design, approvals and development; participant recruitment; study visits and related procedures; clinical vascular phenotyping; sample collection, preparation and storage; data collection and statistical analysis. A number of others also contributed to the conduct of this research. The work described in chapter 3 was a collaborative project with Dr Alison Taylor, and statistical support for this was provided by Dr David Preiss. Laboratory biomarker analyses within Glasgow were performed by Elaine Butler, Jim McCulloch and Ruth Mackenzie. Collagen biomarker analysis was performed at the University of Navarra, Pamplona by Dr Susana Ravassa. Urinary proteomic processing for studies described in chapters 4 and 7 was performed by Dr Bill Mullen and his team at the University of Glasgow; whilst the processing of samples for the main PRIORITY trial was performed by Professor Harald Mischak and colleagues at Mosaiques Diagnostics in Hannover. All proteomic scoring was performed at Mosaiques Diagnostics in Hannover. The work described within this thesis supervised by Professor Christian Delles, Professor John Petrie and Dr Marie Freel at the Institute of Cardiovascular and Medical Sciences, University of Glasgow.

K. Abbreviations

2-DE	2-dimensional gel electrophoresis
8-OHdG	8-oxo-7,8-dihydroguanosine
A1M	α-1 microglobulin
ACCORD	Action to Control Cardiovascular Risk in Diabetes
ACE	angiotensin converting enzyme
ACE-I	angiotensin converting enzyme inhibitor
ACR	albumin: creatinine ratio
ADVANCE	Action in Diabetes and Vascular disease: preterAx and diamicroN-MR Controlled Evaluation
AE	adverse event
AGA	α-1-acid glycoprotein
AGE	advanced glycation end-products
AIx	augmentation index
AIx75	augmentation index corrected for heart rate of 75 beats/minute
ALTITUDE	Aliskiren Trial in Type 2 Diabetes Using Cardiovascular and Renal Disease Endpoints
Ang II	angiotensin II
ANOVA	analysis of variance
ARB	angiotensin receptor blocker
ARTS-DN	Mineralocorticoid Receptor Antagonist Tolerability Study-Diabetic Nephropathy
ASCEND	A Randomised, Double Blind, Placebo Controlled, Parallel Group Study to Assess the Effect of the Endothelin Receptor Antagonist Avosentan on Time to Doubling of Serum Creatinine, End Stage Renal Disease or Death in Patients With Type 2 Diabetes Mellitus and Diabetic Nephropathy

ASCOT	Anglo-Scandinavian Cardiac Outcomes Trial
AT-1	angiotensin II type 1
AT-2	angiotensin II type 2
AUC	area under the receiver operating curve
AVOID	Aliskiren in the Evaluation of Proteinuria in Diabetes
BEACON	Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes Mellitus: the Occurrence of Renal Events
BENEDICT	Bergamo Nephrologic Diabetic Complications Trial
BMI	body mass index
CARS	cysteinyl-tRNA synthetase
CCB	calcium channel blocker
CE	capillary electrophoresis
cf-PWV	carotid-femoral pulse wave velocity
CHI	community health index
CITP	telopeptide of collagen type 1
CKD	chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CRP	C-reactive protein
CSO	Chief Scientists Office
CTGF	connective tissue growth factor
DCCT	Diabetes Control and Complications Trial
DEMAND	Developing Education on Microalbuminuria for Awareness of Renal and Cardiovascular Risk in Diabetes
DIRECT	Diabetic Retinopathy Candesartan Trials
DN	diabetic nephropathy
ECM	extracellular matrix
eCRF	electronic case report form

EDIC	Epidemiology of Diabetes Interventions and Complications
EF	ejection fraction
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
ELUCID	European Controlled Trial of Lisinopril in Insulin-Dependent Diabetes
EMPA-REG	(Empagliflozin) Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ESRD	end-stage renal disease
ET-1	endothelin 1
EudraCT	European Union Drug Regulating Authorities Clinical Trials
EURODIAB	European Concerted Action on the Epidemiology of Diabetes
FGF	fibroblast growth factor
FIND	Family Investigation of Nephropathy and Diabetes
FLEMENGHO	Flemish Study on Environment, Genes and Health Outcomes
FMD	flow-mediated dilation
FRMD3	FERM domain containing 3
GBM	glomerular basement membrane
GFR	glomerular filtration rate
GGT	gamma glutamyl transferase
GLP-1	glucagon like peptide-1
GLUT-1	glucose transporter-1
GoKinD	Genetics of Kidneys in Diabetes
HbA1c	glycated haemoglobin
HCTC	Hannover Clinical Trials Centre

HOPE	Heart Outcomes Prevention Evaluation
HSP70	heat shock protein 70
ICAM-1	intercellular adhesion molecule-1
IDF	international diabetes federation
IDNT	Irbesartan Diabetic Nephropathy Trial
IL	interleukin
IWRS	interactive web response system
KDIGO	Kidney Disease Improving Global Outcomes
KIM-1	kidney injury molecule-1
LC	liquid chromatography
LEADER	Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results
L-FABP	liver-type fatty acid binding protein
MA	microalbuminuria
MCP-1	monocyte chemoattractant protein-1
MDRD	modification of Diet in Renal Disease
micro-TOF	micro-time of flight
MMP	matrix metalloproteinase
MR	mineralocorticoid receptor
MRA	mineralocorticoid receptor antagonist
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NAG	N-acetyl-ß-D-glucosaminidase
NCI CTCAE	National Cancer Institute Common Terminology Criteria for Adverse Events
NEFRON	National Evaluation of the Frequency of Renal Impairment Co-existing with NIDDM
NEP	neutral endopeptidase

NF-κB	nuclear factor κB
NGAL	neutrophil gelatinase-associated lipocalin
NO	nitric oxide
Nrf2	nuclear 1 factor (erythroid-derived 2)–related factor 2
NT-proBNP	n-terminal pro-brain natriuretic peptide
ONTARGET	Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial
ORIENT	Olmesartan Reducing Incidence of End-stage Renal Disease in Diabetic Nephropathy
PAI-1	plasminogen activator inhibitor-1
PBMC	peripheral blood mononuclear cell
PERL	Preventing Early Renal Function Loss in Diabetes
PICP	procollagen type 1 carboxyterminal propeptide
PIS	participant information sheet
PKC	protein kinase C
PREVEND	Prevention of Renal and Vascular Endstage Disease
PRIORITY	Proteomic Prediction and Renin Angiotensin Aldosterone System Inhibition Prevention of Early Diabetic Nephropathy in Type 2 Diabetic Patients with Normoalbuminuria
PWV	pulse wave velocity
RAAS	renin angiotensin aldosterone system
RAGE	advanced glycation end-product receptor
RBP	retinol binding protein
RENAAL	Reduction of Endpoints in Non-insulin dependent diabetes with the Angiotensin Antagonist Losartan
RIACE	Renal Insufficiency and Cardiovascular Events
ROADMAP	Randomised Olmesartan and Diabetes Microalbuminuria Prevention

RR	risk ratio
RRI	renal resistive index
SAE	serious adverse event
SAS	statistical analysis software
SDRN	Scottish Diabetes Research Network
SELDI	surface-enhanced laser desorption/ionisation
SGLT2	sodium-glucose cotransporter-2
SONAR	Study of Diabetic Nephropathy with Atrasentan
SORBS1	sorbin and SH3 domain containing 1
SPRINT	Systolic Blood Pressure Intervention Trial
STNFR	soluble tumour necrosis factor receptor
SVM	support vector machine
T1DM	type 1 diabetes
T2DM	type 2 diabetes
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumour necrosis factor-α
TnT	high sensitivity cardiac troponin T
t-PA	tissue plasminogen activator
UACR	urine albumin: creatinine ratio
UAE	urine albumin excretion
UKPDS	UK Prospective Diabetes Study
UMOD	uromodulin
VA NEPHRON-D	Veterans Affairs Nephropathy in Diabetes
VEGF	vascular endothelial growth factor
v-WF	von Willebrand factor
β2M	β2 microglobulin

1. Introduction

1.1 Diabetes - a global healthcare emergency

The world prevalence of both type 1 (T1DM) and type 2 diabetes (T2DM) is increasing, with the rise in type 2 diabetes fuelled by global trends in urbanisation and modern lifestyle changes such as dietary choices and sedentary behaviour, which in turn lead to obesity. Figures from the 2015 International Diabetes Federation (IDF) Global Diabetes Atlas estimate that at present there are 415 million adults living with diabetes worldwide, a sobering 46% of these individuals are not yet diagnosed. By 2040 it is projected that 642 million people will be affected by diabetes unless preventative steps are taken, prompting the IDF to describe diabetes as "the largest global health emergency of the 21st century".

In 2015 diabetes caused 5 million deaths across the globe, more than the total combined mortality resulting from tuberculosis, HIV/AIDS and malaria [1]. Extremes of blood glucose account for only a small proportion of these deaths, and mortality in diabetes is more commonly attributable to complications of the disease including: cardiovascular disease; peripheral vascular disease; and kidney disease. Improvements in diabetes management over the past 50 years have led to reduction in complication rates and improved life expectancy for patients with diabetes. However, the increasing prevalence of diabetes is driving an increasing burden of morbidity and early mortality. As a result, diabetes and its complications place an enormous strain on healthcare services as well as affected individuals and their families. The sequelae of diabetes can in part be tackled by early diagnosis and intensified treatment strategies, a point which was highlighted at the 2015 G7 summit where the IDF urged all member nations to implement cost-effective policies to deal with the increasing burden of diabetes on healthcare services [1].

1.2 Diabetic nephropathy

Diabetic nephropathy (DN), traditionally defined as urine albumin excretion (UAE) > 300mg/24hrs and declining renal function in the absence of urinary tract infection or any other renal disease [2], remains the leading cause of end-stage renal disease (ESRD) necessitating transplantation or dialysis worldwide [3]. Despite an overall stabilisation of incidence rates in recent years [4] nearly one third of individuals with diabetes show some evidence of renal involvement during the course of their disease. DN accounts for approximately 20% of diabetes-related deaths directly, as well as indirect effects which arise as a consequence of the significantly increased cardiovascular risk associated with the condition [5]. In the Finnish Diabetic Nephropathy (FinnDiane) cohort of patients with T1DM, DN was associated with a 3.6-fold increase in mortality in comparison to the general population over a 7 year follow-up period [6]. The UK Prospective Diabetes Study (UKPDS) demonstrated that risk of death increased 3.4-fold in T2DM patients with DN, and 14-fold in patients with DN and ESRD over 10 years of follow-up [7]. The association between DN and mortality is a graded relationship, increasing as the disease progresses [6, 7].

1.2.1 Classical 5-stage natural history of DN

Mogensen et al [8] first defined what are traditionally thought of as the 5 stages of DN in 1983, derived mainly but not exclusively from studies in patients with T1DM. This classification is described below and summarised in Table 1-1. The first 2 stages cannot be detected by conventional blood or urine tests and therefore evolve in clinical silence over a number of years.

Stage	Description	Onset/Duration	Histology	GFR	UAE	BP
1	Glomerular hyperfiltration	Often at diagnosis; may persist for many years	Increased glomerular size; nephron hyperplasia	Increased	Normal; may rise transiently with exercise	Normal
2	Mesangial expansion	Detectable by 2 years after diagnosis; progresses over several years	Thickened GBM; mesangial matrix expansion	Increased	Normal	Normal
3	Microalbuminuria (“incipient DN”)	Present after 10-15 years; approximately 80% progress to overt DN	As above	Normal/increased	30-300mg/24 hours	Incipient increase
4	Macroalbuminuria (“overt DN”)	Onset after 15-20 years; progress to ESRD inevitable	Kimmelstiel-Wilson lesions; diffuse glomerulosclerosis; arteriolar hyalinosis	Declines by 10ml/min/1.73m² per year	>300mg/24 hours	Elevated
5	ESRD	Outcome after 25-30 years	Glomerular closure	Less than 15ml/min/1.73m²	May decline due to nephron closure	Elevated

Table 1-1. The traditional 5-stage classification of diabetic nephropathy.

GBM, glomerular basement membrane; GFR, glomerular filtration rate; UAE, urine albumin excretion; BP, blood pressure; DN, diabetic nephropathy; ESRD, end-stage renal disease. Adapted from Mogensen et al, Diabetes 1983; 32: 64-78.

The first stage is characterised by a relative improvement in kidney function, termed “glomerular hyperfiltration”. This is generally accepted as an increase in glomerular filtration rate (GFR) of > 2 standard deviations above the mean but the threshold for classification has ranged between 125 and 140ml/min/1.73m^2 in different studies [9-11]. Hyperfiltration can be demonstrated at the time of clinical diagnosis of diabetes in some individuals, and has been attributed to both increased glomerular capillary plasma flow resulting from altered resistance in the efferent and afferent arterioles as well as the effect of increased resorption of glucose at the proximal tubule [12, 13]; a theory supported by the fact that these changes are at least partially reversible by insulin therapy [14, 15]. Histologically this process is accompanied by increased glomerular size and nephron hyperplasia; as well as a measurable increase in overall kidney size [8]. The pathogenic significance of hyperfiltration in relation to the evolution of DN remains under scrutiny. In a meta-analysis of 10 cohort studies including 780 patients with T1DM, hyperfiltration was associated with an increased risk of progressive disease [16], however these findings are more challenging to corroborate in T2DM due to the insidious onset of the disease and the co-existence of non-diabetic renal disease in some patients.

The hallmark of the second stage of DN is the development of significant structural changes within the renal parenchyma in the absence of any measurable clinical decline in renal function. The characteristic pathological abnormalities include thickening of the glomerular basement membrane (GBM) followed by alterations in the composition of the mesangium [8] as a consequence of extracellular matrix (ECM) accumulation. Expansion of the mesangium distorts the glomerular capillaries thereby diminishing the filtration surface [17]. Hyalinosis of the efferent arteriole is another pathological feature which is relatively specific for DN and can be used to differentiate the condition from nephropathy of alternative aetiology at biopsy. This term refers to intramural collections of plasma proteins and lipids within the renal capillaries and arterioles [17]. Distortion of the GBM and mesangium can be considerably advanced by the time clinical signs of DN are detectable, and the presence of these histological alterations in T1DM has been shown to be predictive of progressive disease in 30-40% of patients [18, 19].

Following the clinically silent haemodynamic and morphological changes that precede any measurable decline in renal filtration or excretory function, the primary manifestation of stage 3 of Mogensen's DN classification is development of microalbuminuria (MA). This parameter remains the earliest and most commonly used indicator of DN in current clinical practice. The diagnostic thresholds traditionally used to define MA are: UAE of 30-300mg over the course of a 24-hour urine collection; or spot albumin: creatinine ratio (ACR) of 2.5-30 mg/mmol for males, 3.5-30mg/mmol for females in 2 of 3 first morning collections. There is good agreement between these methods [20] but spot collections are generally more convenient for patients in day to day clinical practice. Mogensen termed this stage "incipient DN" as although renal function remains within normal limits, progression of UAE over several years appears to be paralleled by an upward trend in blood pressure, and studies at the time suggested that approximately 80% of patients who developed MA were likely to progress further in subsequent years [21, 22]. It was also noted at this stage that patients experienced transient exercise-induced amplification of UAE, returning to baseline levels during recovery. Progression of the structural and microcirculatory changes in the glomeruli described above are very likely to be involved in this phenomenon [8]. "Incipient DN" is essentially the interphase between preserved renal function and the later decline that is characteristic of more advanced disease, highlighting risk of progression and therefore also the need for clinical intervention to slow the development of DN.

Stage 4 of the traditional classification system is the point of transition to overt DN, with its key clinical feature being persistent albuminuria (UAE>300mg/24hrs or >30mg/mmol in spot ACR). Histopathologically, advanced DN is characterised by nodular glomerulosclerosis, also termed "Kimmelstiel-Wilson lesions". These are composed of accumulated mesangial matrix, lipid particles and other cellular debris and their presence is generally associated with longer duration of diabetes as well as other unfavourable clinical features [23]. The changes seen in diffuse glomerulosclerosis represent the endpoint of multiple mechanisms resulting in excessive accumulation of matrix proteins within the mesangium, and in advanced disease are often followed by interstitial fibrosis and tubular atrophy [24]. These changes are accompanied clinically not only by albuminuria but also often by hypertension. It is at this time point that GFR begins to fall; in

untreated patients the approximate rate of decline is 10ml/min/1.73m^2 per year [8] and is the final path to ESRD (stage 5).

1.2.2 Proposed pathological classification of DN

Although pathological staging systems exist for many renal conditions this has not traditionally been the case for DN, despite the existence of a number of hallmark histological features as described above. In 2010 a uniform classification system to encompass both T1DM and T2DM was proposed by the Renal Pathological Society with the aim of categorising prognostic severity [17] (Table 1-2). Appropriate standards suggested for full evaluation of renal tissue in order to identify these key features include: light microscopy with staining; immunofluorescence; and electron microscopy.

a. Glomerular lesions in DN

Class	Description	Required Criteria
I	Mild, non-specific changes and GBM thickening	No evidence of changes associated with stages II-IV; GBM>395nm in females, >430nm in males
IIa	Mesangial expansion (mild)	<25% total mesangium involved
IIb	Mesangial expansion (severe)	>25% total mesangium involved
III	Nodular glomerulosclerosis (Kimmelstiel-Wilson lesions)	At least 1 Kimmelstiel-Wilson lesion
IV	Advanced glomerulosclerosis	Lesions from class I-III may be present; sclerosis in >50% of glomeruli

b. Interstitial and vascular lesions in DN

Lesion	Required Criteria	Score
Interstitial lesions (IFTA)	Absent	0
	<25%	1
	26-50%	2
	>50%	3
Inflammatory infiltrates	Absent	0
	IFTA-associated	1
	IFTA-independent	2
Arteriolar hyalinosis	Absent	0
	At least 1 area	1
	More than 1 area	2
Arteriosclerosis in large vessels	No intimal abnormality	0
	<thickness of media	1
	>thickness of media	2

Table 1-2. Proposed pathological classification of diabetic nephropathy.

DN, diabetic nephropathy; GBM, glomerular basement membrane; IFTA, interstitial fibrosis and tubular atrophy. Adapted from Tervaert et al, JASN 2010; 21: 556-563.

1.2.2.1 Glomerular lesions

The 4 classes of glomerular lesions seen in DN are briefly summarised below.

- Class I: These specimens show either no specific changes or GBM thickening defined as >395nm in females and >430nm in males.
- Class II: This stage is characterised by mesangial expansion, which can be further defined as mild or severe depending on the total volume of mesangium involved. Class IIa refers to "mild" mesangial expansion affecting less than 25% of the total mesangium observed at biopsy, while class IIb indicates "severe" mesangial expansion which is seen in more than 25%.
- Class III: A specimen containing at least one Kimmelstiel-Wilson lesion with no more than 50% global glomerulosclerosis is designated as class III.
- Class IV: A class IV biopsy specimen displays more than 50% global glomerulosclerosis with supportive evidence that this is attributable to DN, i.e. other lesions described for class I-III or indicative features from the clinical history.

1.2.2.2 Vascular lesions

- As described previously, efferent arteriolar hyalinosis is highly specific for DN, whereas hyalinosis of the afferent arteriole occurs in a variety of other renal pathologies. However, most studies do not distinguish between these subtypes and refer simply to "arteriolar hyalinosis". Biopsy specimens are allocated a score of between 0 and 2 depending on the presence and extent of this feature.
- Non-specific arteriosclerosis of larger vessels can also be observed in DN biopsy specimens. This can also be associated with other conditions such as hypertension. Samples are again scored in the range 0-2, depending on the presence and extent of vascular intimal thickening.

1.2.2.3 Tubulointerstitial lesions

- Inflammatory infiltrates comprising macrophages and lymphocytes are a widely recognised feature of DN. Specimens are scored 0 if this feature is not detected; 1 if it is only observed around atrophic tubules; and 2 if other areas are also involved.
- Interstitial fibrosis and tubular atrophy tend to follow initial glomerular changes in DN. These are scored in combination according to the total percentage of tubules and interstitium affected. A score of 0 is awarded if this feature is absent; 1 is indicative of less than 25%; 2 if between 26 and 50%; and 3 if more than 50% of the biopsy is affected.

This classification system does not discriminate between T1DM and T2DM. Recently a number of histopathological differences between nephropathy in T1DM and T2DM have been described, including earlier appearance of atubular glomeruli and increased glomerular volume in T2DM compared to T1DM [25, 26]. Furthermore, there are some limitations in terms of clinical application. First, there is little published evidence as yet to corroborate these histopathological disease stages with clinical outcomes, therefore no comment on prognostic severity can be made in relation to staging at biopsy. In a retrospective study using renal biopsy samples from 396 patients with T2DM and DN the severity of glomerular lesions; degree of interstitial inflammation; and tubular atrophy were all predictive of renal outcomes independent of other clinical parameters. However promising these results, it should be borne in mind that this was retrospective work and the effect of different pharmacological strategies was not assessed [27]. Second, it is not widespread routine clinical practice to perform a renal biopsy in all patients with diabetes and renal dysfunction, far less those in the earlier stages of disease with MA in the context of normal GFR. Therefore such a system could not be implemented at present for routine diagnosis of DN, rather in cases where there is significant diagnostic doubt and exclusion of other renal pathologies is required.

1.2.3 The evolving natural history of DN

The classical progressive 5-stage model of DN first described in 1983 was developed primarily from studies in patients with T1DM. Disentangling the natural history of T2DM is more challenging due to the insidious onset of the disease, the confounding influence of the effects of ageing on the kidney and vasculature and the not-infrequent co-existence of multiple comorbid conditions, including the presence of non-diabetic renal disease in some patients. In recent years a number of features of the traditional 5-stage progression of DN have been challenged.

1.2.3.1 MA is not indicative of inevitable disease progression

At the time the original 5-stage classification of DN was developed, studies suggested that the majority of MA positive patients, i.e. those in disease stage 3, were likely to progress to macroalbuminuria over the next 5-15 years [22, 28, 29]. Prospective work in the subsequent 2 decades has confirmed that progression rates are significantly lower than this. Furthermore regression to normoalbuminuria is not infrequent and in many cases outweighs progression to macroalbuminuria.

In a 10-year observational study of 939 patients with T1DM, Rossing and colleagues reported that only 28% of those who were MA-positive at baseline progressed to overt DN during follow up while 16% regressed to normoalbuminuria [30]. The Joslin Study of the Natural History of Microalbuminuria reported similar results; 33% of their MA-positive patients progressed to overt DN over an 8 year follow up period while 40% regressed to normoalbuminuria [31]. In the European Concerted Action on the Epidemiology of Diabetes (EURODIAB) study, of 352 patients who were MA positive at baseline only 13.9% progressed to macroalbuminuria and 35.5% reverted to normoalbuminuria during a 7.5 year follow up period [32].

In the early studies the predictive power of MA was significantly lower in T2DM due to the substantial cardiovascular mortality rates among these patients, thereby limiting progression to ESRD. In a 1984 retrospective analysis by Mogensen and colleagues the 10 year mortality rate among a cohort of patients with T2DM was 77.6%, mainly due to cardiovascular events, while only 22.4% of the cohort progressed to macroalbuminuria as a result [21]. More recently in the Steno-2 study where 151 subjects were microalbuminuric at baseline, 31.1% progressed to macroalbuminuria and 30.5% regressed to normoalbuminuria during the 7.8 year follow up period [33]. Similarly a 2005 Japanese study with 8 years follow-up found that 17% of patients who were microalbuminuric at inclusion progressed to macroalbuminuria while 21% reverted to normoalbuminuria [34]. The near-universal use of drugs blocking the renin-angiotensin aldosterone system (RAAS) over the past 2 decades must be considered as a factor in the evolution of our understanding of DN. Some studies have certainly described an effect of RAAS blockade [33, 35] whilst others have seen changes independent of drug therapy [31, 34].

1.2.3.2 Early renal function decline

Although early studies defined MA as a step towards inevitable development of advanced kidney disease it is now widely accepted that decline in GFR is not conditional on progression through micro- then macroalbuminuria, and several authors have described “early renal function decline” in patients with diabetes. Of the 480 patients with T1DM followed up in the Pittsburgh Epidemiology of Diabetes Complications Study, 9.3% of those who were noted to be MA-positive at baseline already had impaired renal function [36]. The 1st Joslin Kidney Study on the Natural History of Microalbuminuria in Type 1 Diabetes focused solely on prospective follow up of patients who were MA positive and reported that although rate of progression to advanced kidney disease was high, almost 50% of cases did not develop overt albuminuria in advance of decline in GFR, and in the subgroup who did develop albuminuria GFR had already begun to decline before UAE reached the diagnostic threshold [37]. Further analysis of GFR decline in this cohort using the linear slope of serum cystatin C concentrations

demonstrated that the process of renal function deterioration began during the MA stage in a third of the cohort [38]. More recently the early GFR slope has been shown to be more strongly associated with time to ESRD than traditional risk predictors such as glycated haemoglobin (HbA1c), systolic blood pressure or UAE [39].

The prevalence and predictive value of early renal function decline is less well-studied in T2DM. This is because time of onset of diabetes is often difficult to determine and the disease can go unrecognised for many years, therefore complications are often present at diagnosis. In a longitudinal study of 195 Pima Indians with T2DM, 42% of those who were MA-positive at baseline had simultaneous evidence of reduced GFR, and the cumulative incidence of ESRD after 10 years was almost 3-times greater in the subgroup who showed evidence of early renal function decline. Despite this progression to ESRD remained commoner in those patients who developed macroalbuminuria, and although the hazard ratio for subsequent development of ESRD was 4.78 time higher in patients with early renal function decline after adjustment for traditional factors such as age and HbA1c, this association was attenuated after adjustment for UAE [40]. This work echoed the observation in T1DM that early renal function decline can precede macroalbuminuria in a proportion of patients.

Taken together these data suggest the emergence of an alternative model of the natural history of DN whereby the onset of MA signals a progressive decline in renal function in a subgroup of patients that occurs independently of, or in parallel to, progression to macroalbuminuria. Specific correlates of early GFR loss include age and HbA1c [37, 41] as well as inflammatory markers such as soluble tumour necrosis factor receptors (STNFR) and high-normal serum uric acid levels [41], leading some authors to hypothesise that this phenotype is associated with inflammation and tubular injury rather than the glomerular damage highlighted by elevated UAE [42]. MA and early renal function decline could therefore represent two separate phenotypes with distinct underlying aetiologies. Supportive evidence for this model comes from the description of a further disease subtype, non-albuminuric DN.

1.2.3.3 Non-albuminuric DN

While studies have shown that early loss of GFR can occur in microalbuminuric patients predominantly although not exclusively with T1DM, many reports have now described development of renal dysfunction at an even earlier stage when UAE is still within normal limits, termed "non-albuminuric DN". This pathway has been described in studies of T1DM and to a greater extent in T2DM.

In the multicentre Developing Education on Microalbuminuria for Awareness of renal and cardiovascular risk in Diabetes (DEMAND) study of almost 25,000 patients with T2DM, 40% of those who had estimated GFR (eGFR) below 60 ml/min/1.73m^2 were normoalbuminuric [43]. By the same token of 1,132 participants in UKPDS who developed renal impairment over the 15 year follow up period, 50% remained normoalbuminuric [44]. Similar rates of non-albuminuric DN were found in the National Evaluation of the Frequency of Renal Impairment co-existing with NIDDM (NEFRON) and Renal Insufficiency and Cardiovascular Events (RIACE) cohort, where 55% and 56% of participants with renal impairment respectively were normoalbuminuric [45, 46]. Lower rates of non-albuminuric DN were found in T1DM from the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) study, in which only 29% of those who developed renal impairment during follow up were normoalbuminuric [47].

The theory that non-albuminuric DN represents a distinct disease phenotype driven by alternative pathogenic mechanisms is reinforced by a number of additional points. First, data primarily generated from studies in T2DM suggests that the independent determinants of reduced eGFR and increased UAE are different [44, 48] as shown in Figure 1-1.

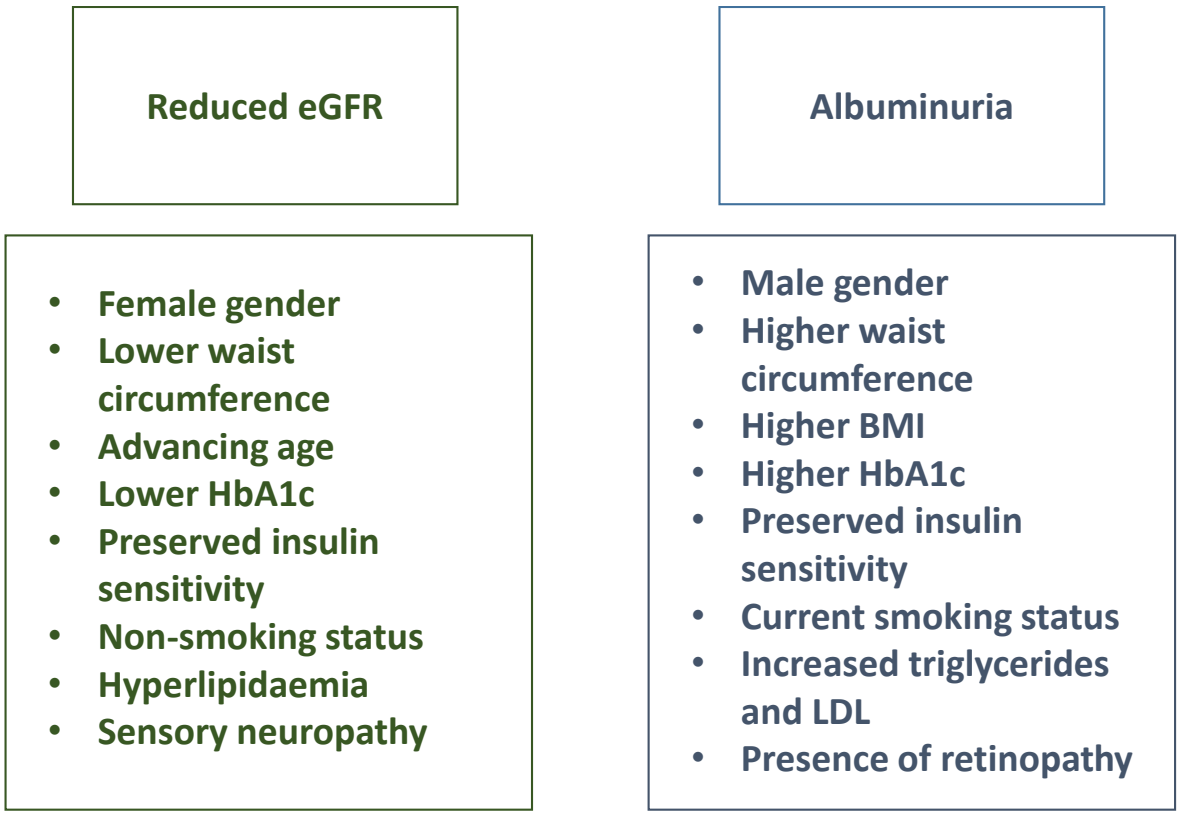

Figure 1-1. Independent correlates of reduced eGFR and albuminuria

eGFR, estimated glomerular filtration rate; HbA1c, glycated haemoglobin; BMI, body mass index; LDL, low density lipoprotein.

Second, the association of this phenotype with the presence of retinopathy is weaker than that generally seen in albuminuric DN. For example in the RIACE cohort most patients with non-albuminuric DN did not have diabetic retinopathy, in fact 43% of participants with eGFR below 60 ml/min/1.73m^2 had neither retinopathy nor albuminuria. Conversely, chronic kidney disease (CKD) was present in almost 60% of those with diagnosis of advanced retinopathy [46, 49]. Thirdly, it has been proposed that early senescence of the diabetic kidney encompassing processes such as vascular disease, fibrosis and cholesterol emboli may contribute to the development of non-albuminuric DN [50], rather than the classical diabetic glomerulosclerosis described previously. This is evident at a histopathological level in renal biopsies from patients with T2DM, renal dysfunction and varying degrees of UAE. In one study only around half of the normoalbuminuric patients displayed typical glomerulopathy as described earlier and all had evidence of arteriosclerosis; while tissue from patients with micro- or macroalbuminuria demonstrated classic histological appearances associated

with DN [51]. The weaker association with retinopathy and lack of association with HbA1c have also been purported to indicate that that prevailing pathology underlying non-albuminuric DN is macrovascular rather than microvascular disease [46].

The evidence set out above further supports the concept that a rise in UAE and decrease in GFR do not necessarily go hand in hand in the natural history of DN. In fact they could be viewed as complementary manifestations rather than obligatory diagnostic features of the disease as illustrated in Figure 1-2. It should be borne in mind that both are associated with increased risk of acute kidney injury, repeated episodes of which can also underlie progression of CKD [52]. The modern history of MA has evolved into one of frequent remissions/regressions rather than the previously held belief that it represented inevitable progression to DN.

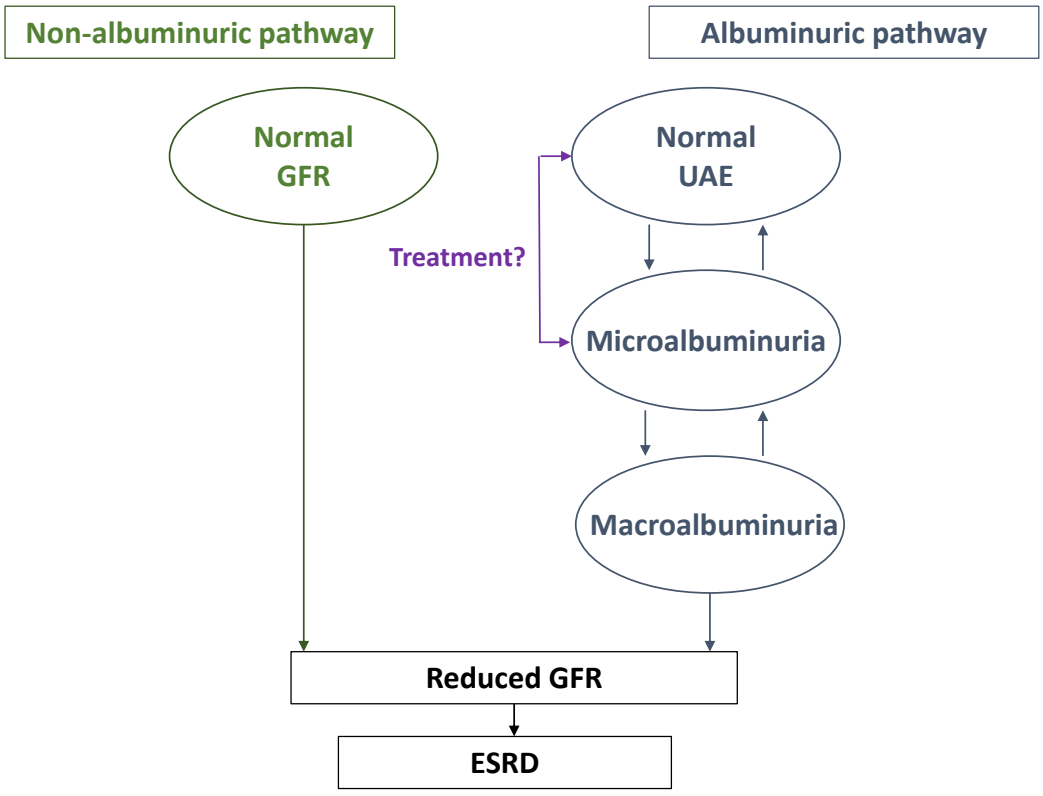

Figure 1-2. Albuminuric and non-albuminuric pathways to DN.

GFR, glomerular filtration rate; UAE, urine albumin excretion; ESRD, end-stage renal disease.

1.3 Pathogenesis of DN

Although hyperglycaemia is a crucial factor in the evolution of DN many additional factors have been implicated. The interaction between these pathways is complex and as such the pathogenesis of DN remains incompletely understood.

1.3.1 The glomerular filtration barrier

In order to understand how these mechanisms contribute to the development of DN it is important to appreciate the structure and functions of the glomerular filtration barrier. Successful urine production in the kidney by filtration of blood requires the presence of an intact glomerular filtration barrier. This structure facilitates selective ultrafiltration of blood as it is freely permeable to water, small solutes and low molecular weight proteins but generally precludes the passage of large, or negatively charged, plasma proteins. The glomerular filtration barrier is comprised of 3 layers as illustrated in Figure 1-3: the fenestrated endothelium; the GBM; and the podocytes [53].

Glomerular endothelial cells are characterised by the presence of fenestrations, transcellular holes that occupy up to 40% of the cell surface. This adaptation results in efficient filtration function by rendering the glomerular endothelium highly permeable to water. However they are also large enough to allow free passage of larger molecules such as albumin which require reabsorption by the tubules [54]. The glomerular endothelial cells are covered by glycocalyx, a layer of proteoglycans and glycoproteins with specific molecular and charge characteristics that regulates endothelial permeability and glomerular filtration [55].

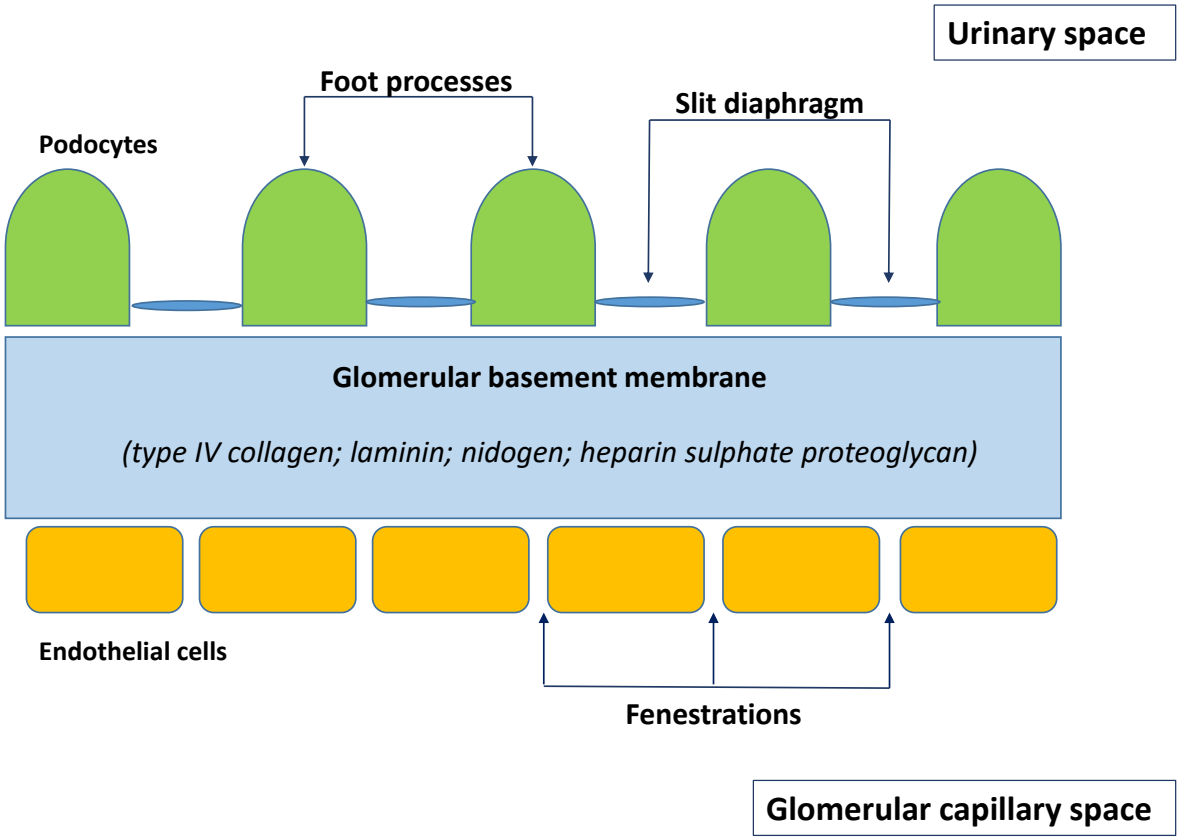

Figure 1-3. Main components of the glomerular filtration barrier.

Adapted from Mora-Fernandez et al, J Physiol 2014; 592: 3997-4012.

The GBM separates the urinary space from the vasculature and is composed of a layer of ECM proteins situated between the glomerular endothelium and the podocytes. Its main components are: type IV collagen; laminin; nidogen; and heparan sulphate proteoglycan, with type IV collagen being the most abundant. The GBM offers structural support to the glomerular capillaries and contributes to permselectivity as its components form an anionic charge barrier to diffusion [56].

Podocytes cover the urinary aspect of the GBM. These are glomerular visceral epithelial cells which have a network of cellular extensions commonly referred to as “foot processes”. The podocyte foot processes interact at intercellular junctions termed “slit diaphragms”. These further enhance the filtration barrier

and are involved in several signalling pathways [53]. One of the major components of the slit diaphragms is nephrin, a transmembrane glycoprotein which bridges adjacent foot processes thereby acting as the “pore” of the slit diaphragm. Any injurious stimulus to the podocytes can result in effacement and loss of the slit diaphragm, leading to dysfunction of the glomerular filtration barrier and proteinuria [57].

1.3.2 Cellular effect of hyperglycaemia

The DCCT and UKPDS established a key role for hyperglycaemia in the development of diabetic microvascular complications [58, 59]. The effects of hyperglycaemia at a cellular level and the complex inter-related mechanisms subsequently modulated by these effects are discussed below. Notably, it has been shown that DN is not attributable to hyperglycaemia alone. Transplant studies in which kidneys from non-diabetic donors were transplanted into diabetic patients have shown that nephropathy can develop in the recipient irrespective of the level of glycaemic control [60]. It is widely acknowledged that hyperglycaemia is a single element in what is a far more complex interaction involving a network of pathophysiological mechanisms (Figure 1-4).

The damaging effects of hyperglycaemia are primarily seen in vascular endothelial cells; mesangial cells and capillaries in the glomerulus; and Schwann cells of the peripheral nerves. Glucose-related damage occurs in only a few particular cell types despite the fact that all cells of the body are exposed to elevated glucose levels in diabetes. This is because the majority of cells are able to rapidly respond to hyperglycaemia by reducing glucose transport into the cell, thereby maintaining constant intracellular glucose concentrations. In contrast, the cell types most frequently affected in hyperglycaemic conditions do not have the capability to respond to fluctuating extracellular glucose levels rapidly or efficiently, resulting in continued transport of glucose into the cells [61, 62]. Exposure of these cells to hyperglycaemia and subsequent inability to modulate glucose transport triggers a number of processes including: activation of protein kinase C (PKC); enhanced flux of polyols; generation of advanced

glycation end-products (AGEs) and reactive oxygen species. The end-result is unregulated cellular energy production in the context of excessive glucose availability.

PKC plays a central role in the pathogenesis of DN. This enzyme is activated by diacylglycerol, a product of glucose metabolism by glycolysis, as well as AGEs and the polyol pathway [57]. Several PKC isoforms are expressed in the kidney. PKC activation affects a number of transcription factors, cytokines, and functional enzymes which are central to DN development including: endothelial nitric oxide synthase (eNOS); endothelin-1 (ET-1); vascular endothelial growth factor (VEGF); matrix-metalloproteinase-2 (MMP-2); transforming growth factor-ß (TGF-ß); connective tissue growth factor (CTGF); and nuclear factor-κB (NF-κB). The end result is altered microvascular flow and angiogenesis, increased capillary permeability and increased ECM protein production [63].

Excess glucose is also channelled via the **polyol pathway**. Under basal conditions the enzyme aldose reductase metabolises toxic aldehydes to inactive alcohols, but in hyperglycaemic conditions it acts to reduce glucose to sorbitol. In a high glucose environment almost a third of circulating glucose is diverted down this pathway. As aldose reductase activity is dependent on nicotinamide adenine dinucleotide phosphate (NADPH), increased glucose flux via the polyol pathway leads to depletion of NADPH. As NADPH is an essential cofactor for regeneration of reduced glutathione, metabolism of glucose via the polyol pathway dysregulates glutathione and glutathione peroxidase activity resulting in decreased nitric oxide (NO) availability and oxidative stress [62].

Nonenzymatic reduction of sugars with free amino groups of proteins, nucleic acids and lipids results in the formation of **AGEs**. Again, under basal conditions AGE production is low but their concentrations are increased in hyperglycaemic conditions. After binding to the AGE receptor (RAGE), intracellular actions of AGEs include activation of PKC and transcription factors such as NF-κB which modulate expression of growth factors and cytokines, in particular TGF-ß, which plays a pivotal role in development of glomerulosclerosis and fibrosis [64]. At an extracellular level, AGE form covalent crosslinks between proteins, thereby altering the composition and function of vessel walls and basement membranes.

As a result of modification by AGEs these matrix components are rendered less susceptible to enzymatic degradation by matrix metalloproteinases (MMPs) and are therefore able to accumulate in an unregulated manner, contributing to GBM thickening and mesangial expansion. Glycation of proteoglycans alters their electronegativity and AGEs can thereby modify filtration across the basement membrane [57].

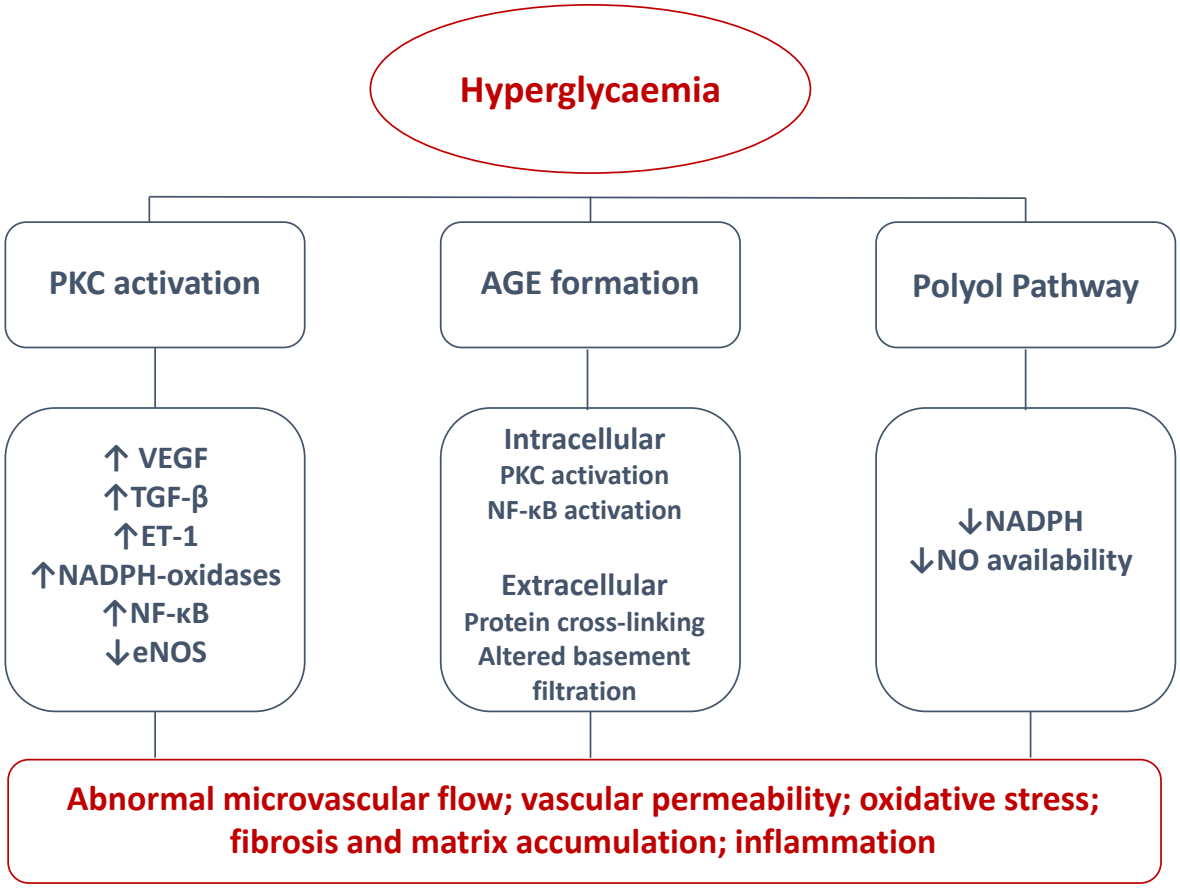

Figure 1-4. Cellular effects of hyperglycaemia.

PKC, protein kinase C; AGE, advanced glycation end-products; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor β; ET-1, endothelin-1; NADPH, nicotinamide adenine dinucleotide phosphate; NF-ĸB, nuclear factor kappa B; eNOS, endothelial nitric oxide synthase; NO, nitric oxide.

1.3.3 Collagen turnover

The most prominent histological feature of DN is excess accumulation of ECM in the mesangium and GBM, resulting from an imbalance between production and degradation of its components. Thickening of the GBM is accompanied by glomerular hyperfiltration and alterations in hydrostatic pressure which are thought to lead to development of MA. Changes in the mesangial matrix and tubulointerstitium appear to be more closely related to decline in renal function as impingement of expanded matrix on glomerular capillaries reduces available filtration surface and narrows the vessel lumen [65].

These histological appearances are accompanied by accumulation of ECM components including collagen types I, III and IV, proteoglycans and fibronectin resulting from activation of fibroblasts. A number of stimuli have been shown to potentiate fibroblast activity including infiltration of inflammatory mediators such as TGF-β, fibroblast growth factor (FGF), leucocytes and macrophages. The RAAS plays a pivotal role in many aspects of the pathogenesis of DN, and ECM synthesis by renal fibroblasts can also be triggered through angiotensin II (Ang II) mediated activation of the angiotensin type 1 receptor [66]. Environmental stimuli such as hyperglycaemia and hypoxia are also thought to play a role [67]. In fact, hyperglycaemia can also mediate these pro-fibrotic effects through its action on other renal cell types. For example, exposure to high glucose levels upregulates TFG-β expression in glomerular epithelial cells [68].

Since ECM also undergoes metabolic turnover through enzymatic degradation by MMPs it follows that any inhibitory effects on this process may also promote ECM accumulation. MMP production by mesangial cells accounts for the majority of ECM turnover in the kidney [69]. Their activity is regulated by tissue inhibitors of metalloproteinases (TIMPs) and both have been implicated in the pathogenesis of DN. Studies have also suggested a role for pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and TGF-β as well as AGEs in regulation of MMP activity [70, 71]. Similarly, in addition to their role in ECM turnover, MMPs have also been shown to stimulate the release of cytokines and growth factors associated with renal fibrosis; further evidence that many of the mechanisms underlying DN are intertwined.

In rodent models of T2DM, expression of MMP-9 is increased in the kidneys of animals that develop DN [72]. Increased urinary MMP-9 concentration has been demonstrated in humans with T2DM and nephropathy, and levels increase with degree of UAE [73]. Notably, plasma MMP-9 concentration has been shown to correlate with degree of urinary podocyte excretion in patients with DN, suggesting that dysregulation of ECM turnover may have adverse effects on podocyte integrity [74]. Evidence surrounding the role of MMP-2 in DN is conflicting. It has been shown that MMP-2 induces activation of renal tubular myofibroblasts, a key step in the development of renal disease [75]. In addition, expression and activity of MMP-2 appear reduced in advanced human DN. However, in rodent models of streptozotocin-induced DN renal expression and activity of MMP-2 are increased in the early stages of disease, and MMP-2 knockout mice progress to advanced DN more rapidly [76] suggesting a potential protective role.

In addition to their role in MMP activity, high glucose concentrations and AGEs have also been shown to alter the availability of TIMPs through enhancing CTGF expression which subsequently triggers increase in TIMP-1 availability from human mesangial cells [77]. Data focussing on the role of TIMPs in DN are inconsistent. For example, circulating TIMP-1 concentrations have been shown to be reduced in humans with T2DM and nephropathy in comparison to non-diabetic renal disease, and levels are correlated with worsening degrees of glomerular lesions [78]. However in patients with T1DM from the EURODIAB study, higher plasma TIMP-1 levels were associated with higher degrees of albuminuria [79]. TIMP-1 has also been shown to have pro-inflammatory effects through up-regulation of intercellular adhesion molecule-1 (ICAM-1) [80] as well as modulating cell proliferation, apoptosis and angiogenesis [81]; additional functions which could contribute to the development of renal fibrosis and further evidence of the complex interrelationships between multiple pathogenic pathways in the development of DN.

Although the evidence surrounding availability of MMPs and TIMPs in DN is conflicting, it highlights complex alterations in matrix turnover as a key pathway to disease development. Of course altered collagen turnover is also key to the evolution of cardiovascular disease, where biomarkers such as the telopeptide of

collagen type 1 (CITP) as an indicator of collagen breakdown, and procollagen type 1 carboxyterminal propeptide (PICP) as an indicator of collagen production have been shown to be differentially regulated in myocardial disease [82]. The conflicting evidence described above may be a reflection of the fact that collagen turnover is differentially regulated in the vasculature and renal parenchyma during the development of DN.

1.3.4 Endothelial dysfunction and inflammation

The endothelium is a unicellular layer of squamous cells lining the internal wall of the vasculature. Once considered to act simply as a semi-permeable membrane between the interstitium and the vascular wall; it is now recognised that the endothelium is a tissue capable of a wide range of biological functions including: adhesion and migration of inflammatory cells; fibrinolysis; and angiogenesis [83]. To carry out these functions the endothelium produces ECM components; NO, ET-1; Ang II; tissue plasminogen activator (t-PA); plasminogen activator inhibitor-1 (PAI-1); von-Willebrand factor (vWF); as well as various cytokines and adhesion molecules [84]. The endothelium thereby serves as a key locus for maintenance of vascular function by balancing the counter-regulatory pathways controlling vasoconstriction; inflammation; oxidative stress; cell proliferation; and thrombosis – mechanisms which have been implicated in the pathogenesis of many diabetes complications including DN. Any injurious stimulus to the endothelium can result in endothelial dysfunction, thereby upsetting the delicate balance between these processes.

Endothelial dysfunction is first evident when stressed tissues release chemokines such as monocyte chemoattractant protein-1 (MCP-1). These factors trigger increased expression of adhesion molecules such as ICAM-1 and E-selectin as well as attracting inflammatory cells to the stressed site. Factors such as reactive oxygen species, Ang II, AGEs and reduced bioavailability of NO can induce inflammatory cell proliferation and activation of genes within these cells leading to production of cytokines including TNF-α and interleukin (IL)-1 as well as IL-6 - an important regulator of one of the most clinically relevant markers of

inflammation, C-reactive protein (CRP). The injured endothelial cells increase their expression of PAI-1 and release pro-coagulant factors, thereby contributing to the evolution of impaired vasoreactivity and a pro-coagulant state [85].

Accumulating evidence points towards the enzyme eNOS as a key mediator of many of the mechanisms described above. NO generation has an inhibitory effect on platelet aggregation and adhesion of inflammatory cells to the vascular endothelium [86] and regulation of vascular smooth muscle tone and endothelial permeability is in part controlled by the eNOS-NO system [87]. Similarly there has been considerable interest in the role of ET-1, a peptide produced primarily by the endothelium that potentiates vascular injury through stimulation of growth factors and cytokines, in the progression of diabetes complications and renal disease in particular. The kidneys are exquisitely sensitive to ET-1 as components of the ET system are present in glomerular cells, microvasculature and the tubules [88] and ET-1 is therefore capable of promoting intrarenal inflammation, fibrosis and ultimately glomerulosclerosis.

1.3.4.1 Clinical assessment of endothelial function

The heterogeneous functions of the vascular endothelium render clinical assessment of endothelial function particularly challenging, as no single test can provide a comprehensive physiological overview of the entire vascular tree. Although a number of the factors mentioned above can be measured in serum or plasma and have therefore been proposed as clinical markers of endothelial dysfunction [89], the preferred clinical method for assessment of endothelial function in humans is endothelium-dependent vasorelaxation [90]. The majority of techniques to assess endothelium-dependent vasorelaxation focus primarily on regulation of vascular tone as a surrogate measure of the NO-mediated vasodilator response and the effect of shear stress. Originally developed in coronary vessels many are now conducted in the forearm and digital circulation in order to permit more practical, non-invasive, repeatable studies. In particular brachial artery flow-mediated dilatation (FMD) is currently accepted

as the gold standard for non-invasive assessment of peripheral vasoreactivity [90].

1.3.4.2 Endothelial dysfunction in diabetes

High glucose conditions have been shown to blunt the NO response in human endothelial cells [91], an effect which may account for the many cellular effects of hyperglycaemia as well as triggering disequilibrium at a microvascular level. AGEs, which are generated as a result of cellular exposure to hyperglycaemia, have also been shown to have an NO-scavenging effect and thereby may also contribute to compromised endothelial function in diabetes [92]. It has also been demonstrated that ET-1 synthesis is enhanced by hyperglycaemia [93]. Endothelial progenitor cells (EPCs) gather at sites of injury and contribute to re-endothelialisation of damaged vessels. Circulating EPC levels have also been shown to be reduced in both number and function in patients with diabetes [94]. In view of the above it is perhaps not surprising that impaired endothelial function, as indicated by abnormal endothelium-dependent vasodilation and increased plasma levels of biomarkers (such as vWF and adhesion molecules), have been demonstrated in patients with both T1DM and T2DM [95, 96]. It is important to note that as with the general complications of diabetes, hyperglycaemia is only one factor in the development of endothelial dysfunction and others such as genetic determinants and environmental influences may also play a key role in determining which patients progress to more aggressive angiopathy.

1.3.4.3 Endothelial dysfunction in diabetic nephropathy

In 1989 Deckert and colleagues published the “Steno Hypothesis”. They proposed that albuminuria is in fact indicative of a state of global systemic microvascular dysfunction and therefore a possible unifying link between the

increased UAE observed in DN and the burden of vascular disease commonly associated with the condition [97].

There is considerable evidence implicating pro-inflammatory and adhesion molecules in DN development. In cross sectional studies in T1DM and T2DM levels of biomarkers such as CRP and adhesion molecules are increased in patients with DN [98, 99]. In addition brachial artery FMD is significantly lower in DN and correlates with degree of proteinuria [100]. Prospective studies have also shown that composite scores of endothelial dysfunction biomarkers predict progression of renal disease in patients with DN independently of major risk factors such as hypertension and poor glycaemic control [101, 102].

At an earlier stage in the traditionally accepted disease process, markers of inflammation and endothelial dysfunction are strongly associated not only with progression of UAE but also with risk of death in patients with diabetes and MA [103]. Clinical assessment of endothelium-dependent vasodilation has also confirmed impaired brachial FMD in patients with MA compared to normoalbuminuric controls [104]. This finding was extended to the general population in the Hoorn Study, where FMD was shown to be impaired in patients with MA irrespective of whether they had a history of diabetes [105], further supportive evidence for the Steno Hypothesis that MA is indicative of generalised endothelial dysfunction. It is important to note, however, that not all microalbuminuric patients have evidence of endothelial dysfunction. It has been hypothesised that those MA positive patients who do display some imbalance at endothelial level are more likely to develop aggressive complications [106].

Endothelial dysfunction appears to be present in some patients with diabetes at an even earlier stage in the disease process, before development of clinically evident complications. For example, circulating markers of endothelial dysfunction such as CRP and E-selectin are elevated while EPC counts are reduced in some young people with T1DM without clinical evidence of microvascular disease or MA. Brachial FMD is also impaired in this patient group in comparison to non-diabetic controls [107]. Similarly, endothelial dysfunction indicated by elevated plasma concentrations of biomarkers and impaired

endothelium dependent vasodilation has been demonstrated in early and otherwise uncomplicated T2DM [96, 103].

As endothelial dysfunction appears to precede MA in some patients with diabetes it is tempting to postulate that MA and hence DN are direct consequences of underlying disequilibrium at an endothelial level. In the DCCT baseline E-selectin levels were predictive of later development of DN [108]. It has been proposed that endothelial dysfunction could trigger MA through direct effects on glomerular pressure and permeability. In addition the paracrine functions of the endothelium could indirectly influence mesangial cells and podocytes, however the molecular pathways underlying this relationship remain to be fully dissected [109]. Similarly, few have evaluated the role of endothelial dysfunction in the evolution of non-albuminuric DN.

1.3.5 The renin angiotensin aldosterone system

The RAAS is another critical element underlying the development of CKD. The classical RAAS cascade begins with secretion of prorenin from juxtaglomerular cells in response to a reduction in blood volume. This is then converted to renin which activates production of angiotensin I from angiotensinogen. Ang II is one of the key effector hormones of the RAAS, and is produced as a result of the action of angiotensin converting enzyme (ACE) on angiotensin I. The immediate effects of Ang II to support the circulation in the event of intravascular volume depletion include: vasoconstriction; potentiation of aldosterone secretion and subsequent salt retention; as well as increased myocardial contractility and therefore cardiac output. Despite its physiological role, Ang II has a number of deleterious effects on the vasculature in the longer term, effecting a slow structural remodelling of the cardiovascular system through processes such as hyperplasia of vascular smooth muscle, sensitisation of the vessels to low concentrations of vasoconstrictors and excessive deposition of ECM components [110]. These effects are mainly mediated via the Ang II type 1 (AT_1) receptor and explain the amelioration of cardiovascular diseases seen independently of reduction in blood pressure in treatment with RAAS blocking agents. Conversely

stimulation of the Ang II type 2 (AT_2) receptor generally opposes these actions, promoting vasodilation through production of NO in the microcirculation [111].

Aldosterone is secreted by the zona glomerulosa of the adrenal cortex in response to Ang II and potassium, and acts at the mineralocorticoid receptor (MR) located in the collecting duct of the kidney to increase sodium absorption and potassium loss, thereby mediating volume status and blood pressure. Recently there has been a resurgence of interest in the non-genomic actions of aldosterone. In addition to the renal collecting duct, the MR is also expressed on vascular smooth muscle cells, fibroblasts and cardiac myocytes. Binding of aldosterone to the MR in these sites results in pro-fibrotic and pro-inflammatory effects, stimulation of cytokine release and cell proliferation thereby triggering development of vasoconstriction and cardiovascular disease [112, 113].

1.3.5.1 The RAAS in DN

The RAAS has long been implicated in the pathophysiology of DN, in particular since the first studies showing significant benefits of ACE-inhibitors (ACE-I) on progression of renal disease. The circulating RAAS appears to be suppressed in patients with diabetes, where plasma renin activity and Ang II are generally normal or reduced. Notably RAAS components are also located in the kidney and studies have demonstrated intrarenal generation of Ang II, leading to the suggestion that paracrine actions of the intrarenal RAAS or enhanced renal sensitivity to Ang II are important in DN [114, 115].

In rat mesangial cells exposure to hyperglycaemia has been shown to trigger release of Ang II and TGF-β [116]. Similarly, in animal models of diabetes renin mRNA and protein expression are increased in the juxtaglomerular and tubular cells in association with increased Ang II production [117], while down-regulation of the protective AT_2 receptor is consistently reported [118]. Furthermore, renal concentrations of Ang II have been shown to progressively increase in these animals in conjunction with reduction in availability of NO metabolites, effects which are ameliorated by treatment with an AT_1 receptor blocker [119]. Ang II

produced by mesangial cells mediates ECM deposition via TGF-β, another mechanism which appears to be inhibited by AT_1 receptor blockade in both human and animal studies [116, 120]. The above evidence supports a model of downregulation of AT_2 receptors through which the AT_1 receptor is left unopposed with rising Ang II levels leading to hypertension, mesangial cell contraction, enhanced deposition of ECM and release of growth factors such as TGF-β.

As detailed above, aldosterone has emerged as a key mediator of accelerated vascular and renal damage in recent years. Broadly speaking its effects can be considered in two distinct categories. First, the classical picture of increased sodium reabsorption results in functional alterations in renal haemodynamics. For example, in animal models of mineralocorticoid-induced hypertension increased glomerular flow is evident in association with vasodilation of the afferent and efferent arterioles [121]. In human subjects with primary hyperaldosteronism pressure-natriuresis curves shift to the right, indicating that higher blood pressure is required in order to maintain sodium excretion in the face of the increased tubular reabsorption triggered by aldosterone excess [122]. Taken together, the classical effects of aldosterone act to drive increased perfusion pressures through an intrarenal vasodilatory response, which in turn causes glomerular hyperfiltration to counteract the effects of aldosterone on sodium reabsorption at the distal tubule.

Second, the non-genomic effects of aldosterone can effect histological damage and structural changes in renal vessels, although to an extent some of these changes can also be attributed to functional changes in blood flow within the vessels. It is now widely accepted that aldosterone stimulates collagen production and modulates fibroblast differentiation and activity, as well as promoting myocardial and vascular hypertrophy and intrarenal vascular remodelling [123]. In animal models, aldosterone has been shown to induce vascular damage independently of effects on volume status and blood pressure. This is thought to result from multiple mechanisms; these include endothelial dysfunction, upregulation of AT_1 receptors, increased production of TGF-β and reactive oxygen species and alterations in the properties of vascular smooth muscle cells, the end result being altered ECM and vessel wall composition

within the kidney [123]. For example, action of aldosterone on fibroblasts leads to increased expression of type I collagen mRNA and stimulates type IV collagen deposition in rodent mesangial cells [124]. In addition, aldosterone-dependent podocyte apoptosis has been demonstrated in rat models of diabetes [125]. Aldosterone has also been shown to enhance PAI-1 expression in smooth muscle cells which in turn results in excess circulating TGF-ß thereby promoting fibrosis [126] as well as mediating NF-κB dependent inflammation [127]. Through promotion of vascular smooth muscle cell hyperplasia, upregulation of AT_1 receptors and inhibition of norepinephrine reuptake aldosterone advances vasoconstriction [128]. In terms of clinical data fewer studies are available. In patients with primary hyperaldosteronism there is a clear association between aldosterone and UAE [129], and baseline GFR has been noted to be higher in these patients in comparison to matched controls with essential hypertension, reflecting hyperfiltration due to elevated intraglomerular hydrostatic pressure [130].

The majority of clinical evidence for the role of aldosterone in progression of DN comes from clinical studies of RAAS blockade with ACE-I or angiotensin receptor blockers (ARBs) where the phenomenon of “aldosterone breakthrough” has been described. This term essentially refers to incomplete suppression of plasma aldosterone in patients treated with ACE-I or ARB. Studies have shown that in a proportion of patients commenced on these therapies plasma aldosterone level returns to pre-treatment level or even higher [131]. Reported incidence of aldosterone breakthrough varies between 20 and 50% of patients in published studies [132]. It appears that those patients who do demonstrate aldosterone breakthrough have worse clinical outcomes. For example, in hypertensive T2DM patients treated with ARB those who showed aldosterone breakthrough experienced a progressive increase in UAE after 6 months of therapy compared to the group whose aldosterone levels remained controlled. The effect was attenuated following addition of MR antagonist (MRA) [132]. Similarly, in T1DM patients with hypertension and DN those who exhibited aldosterone breakthrough experienced enhanced decline in GFR [133]. Few have investigated the determinants of this phenomenon but in one post-hoc analysis factors independently associated with aldosterone breakthrough at one year were greater magnitude of short-term reductions in systolic blood pressure,

eGFR and sodium intake, suggesting that intensive blood pressure lowering with sodium restriction may be a triggering factor [134]. Although data are relatively limited and the longer term implications are unknown, given what is known about the deleterious non-genomic effects of aldosterone [135] the breakthrough phenomenon could have important clinical consequences, and may provide at least partial explanation for the relentless progression of DN in some patients despite treatment with ACE-I or ARB.

1.3.6 Haemodynamic changes

Systemic hypertension is an important factor in progression of renal disease in diabetes. Of equal importance however are the intrarenal haemodynamic changes resulting from the processes of inflammation, matrix accumulation and vascular remodelling described above which ultimately contribute to albumin leakage from the glomerular capillaries and decreased resistance in the glomerular arterioles. This allows transmission of systemic pressure to the glomerular capillaries and development of hyperfiltration; the earliest stage of DN [136]. Animal studies confirm the presence of increased intraglomerular pressure as a result of preferential vasodilation of the afferent arteriole [137]. The resultant increased mechanical strain to which these vessels are subjected induces overexpression of glucose transporter-1 (GLUT-1) thereby triggering a self-perpetuating cycle of increased glucose uptake, further activation of the polyol pathway, PKC and generation of AGEs [138].

As previously described, ischaemic atherosclerotic changes can also be present in the renal microvasculature in DN. Renal resistive index (RRI) as measured by duplex ultrasonography is an integrated measure of vascular compliance and downstream impedance. RRI is a useful method for quantification of alterations in the renal parenchymal circulation and is closely related to renal arteriosclerosis [139]. Clinical studies have shown increased RRI in patients with DN compared to control subjects [140]. Notably, higher RRI has been shown to predict progression of renal disease in diabetic patients with MA [141] and in fact normoalbuminuric patients with diabetes have higher RRI than control

subjects, reflecting the fact that these haemodynamic alterations occur early in the disease process [142].

1.3.7 A final common pathway?

The pathogenesis of DN is complex and remains only partially understood. The mechanistic pathways described above do not function in isolation, but rather loss of glomerular autoregulation and the metabolic effects of hyperglycaemia interact as shown in Figure 1-5. Hyperglycaemia, often in association with systemic hypertension leads to glomerular hypertension via a number of mechanisms. Glomerular hypertension results in injury to the microvasculature and thereby triggers a sequence of reactions at the level of the endothelium and mesangium. Vascular stress results in over-expression of GLUT-1 which potentiates further glucose entry into cells and in turn leads to PKC activation, increased production of TGF-ß and AGEs, reduced NO bioavailability as well as collagen deposition. The reduced bioavailability of NO increases Ang II activity at the AT_1 receptor, further potentiating the cycle of glomerular hypertension, expression of TGF-ß, ECM deposition and damage to the renal microvasculature and parenchyma [143].

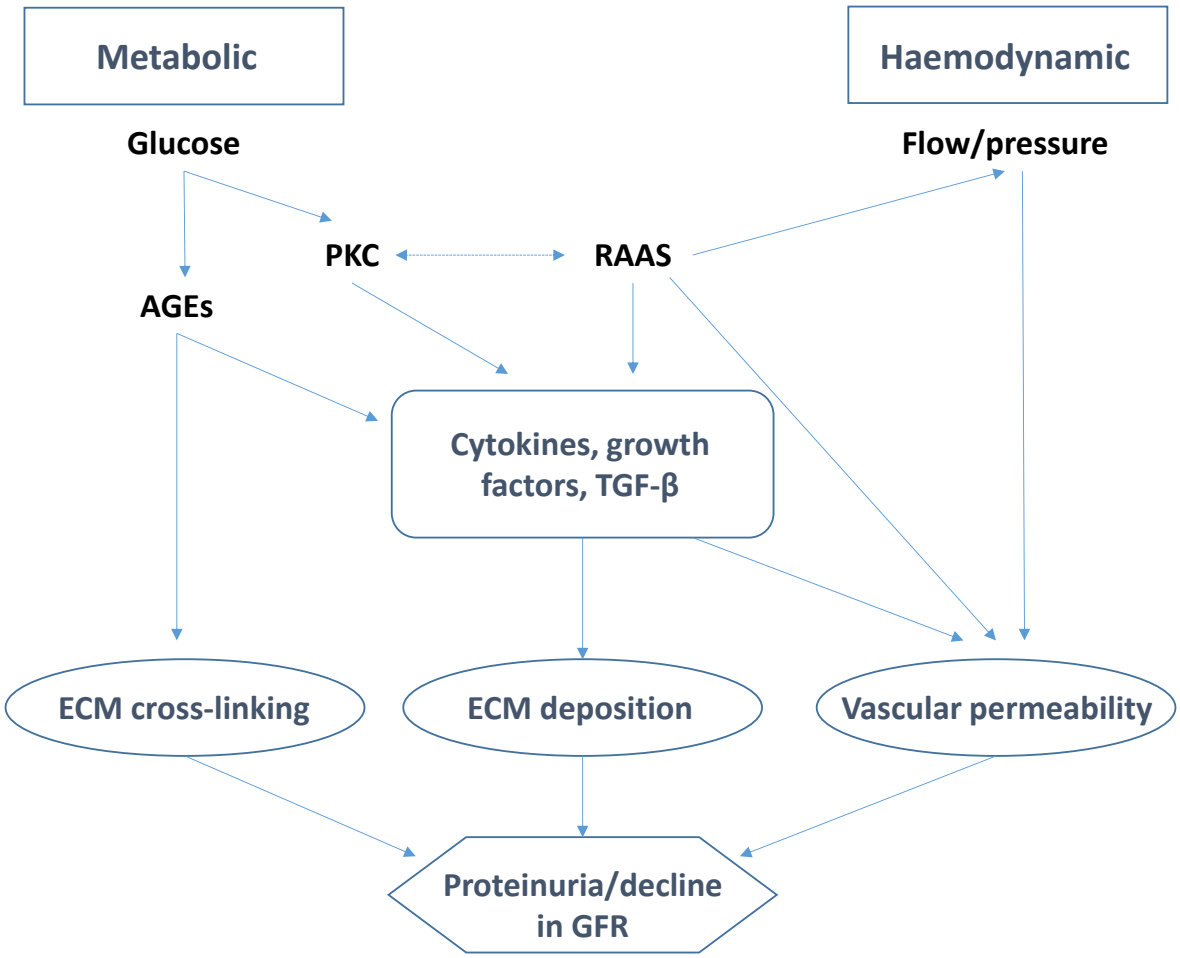

Figure 1-5. Interaction of metabolic and haemodynamic pathways in DN.

AGEs, advanced glycation end-products; PKC, protein kinase C; RAAS, renin angiotensin aldosterone system; TGF-β, transforming growth factor β; ECM, extracellular matrix; GFR, glomerular filtration rate. Adapted from Cooper, Lancet 1998; 352: 213-19.

1.4 Biomarkers for prediction of DN

The Kidney Disease Improving Global Outcomes (KDIGO) clinical practice guidelines highlight the importance of prompt identification and intervention in CKD, irrespective of aetiology [144]. Development of ESRD requiring dialysis or transplantation is among the most feared consequences of CKD, however symptoms experienced by patients tend to result from the more advanced stages of disease and are therefore a late indicator. The earlier stages of CKD are often clinically silent and identified incidentally during assessment of other conditions. Failure to recognise any downward trend in renal function in a timely manner leads to relentless progression towards ESRD as well as development of complications of CKD including cardiovascular morbidity and mortality; acute kidney injury; hypertension; anaemia and disorders of bone metabolism. In contrast, early detection of CKD allows introduction of interventions and risk factor management to minimise these risks where possible; a strategy that is beneficial to patients, clinical services and healthcare budgets. In current clinical practice diagnosis and prognosis of CKD is based on two key biomarkers: eGFR and albuminuria. Although the gold standard tool for confirmation of DN is renal biopsy the majority of diagnoses are made on clinical grounds according to the traditional definition of DN which requires the presence of albuminuria and/or reduced GFR. Current guidelines recommend monitoring of both these parameters on a regular basis, as both have been shown to be independently associated with progressive renal and cardiovascular disease [145].

1.4.1 Glomerular filtration rate

GFR is the preferred index of renal function for diagnosis and classification of CKD (Table 1-3). The normal GFR in healthy adults is approximately 125 ml/min/1.73m^2 although this declines with age [144]. The gold standard method for determination of GFR is measured clearance of exogenous filtration markers such as inulin, iothalamate and ^{51}Cr-EDTA [146]. These techniques are time-consuming and require experienced personnel, therefore their clinical use is

often limited to tertiary referral centres and situations where a precise measure of GFR is required, such as assessment of potential kidney donors [144]. The routine assessment of GFR in clinical practice relies on formulae that generate estimates based on serum creatinine. Until recently the Modification of Diet in Renal Disease (MDRD) formula was most commonly used for estimation of GFR in clinical laboratories. However, this tool was developed in patients with pre-existing CKD, meaning that results can be imprecise at general population level and GFR at higher levels (i.e. >60ml/min/1.73m^2) is underestimated [147]. The MDRD formula has since been superseded in many institutions by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) tool, which offers increased accuracy at higher levels of eGFR [148] and thereby reduces the rate of false-positive diagnosis of stage 3 CKD [149]. Irrespective of the formula used, in clinical practice eGFR levels of less than 60ml/min/1.73m^2 are reported numerically to the requesting clinician, but results higher than this threshold are not generally formally quantified. This is based on the rationale that GFR of less than 60ml/min/1.73m^2 has been shown to confer a greater risk of progression to ESRD [150].

CKD category	Description	eGFR (ml/min/1.73m^2)
G1	Normal or increased GFR	>90
G2	Mildly decreased	60-89
G3a	Mild-moderately decreased	45-59
G3b	Moderately-severely decreased	30-44
G4	Severely decreased	15-29
G5	Renal failure	<15

Table 1-3. CKD classification based on estimated glomerular filtration rate (eGFR categories).

CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate. GFR, glomerular filtration rate. Adapted from KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease, Kidney International Supplements, 2013; volume 3.

The traditional definition of DN relies in-part on detection of reduced GFR, however this method is less than ideal for identification of the early stages of disease for a number of reasons.

- Although clinically useful in day-to-day practice, the P30 (an estimated performance measure, expressed as the percentage likelihood that the eGFR is within 30% of the measured value) for most formulae used for estimating GFR is between 80 and 90%, meaning that eGFR has at most a 90% chance of being within 30% of the actual measured GFR [151].
- The existing equations for estimation of GFR are thought to be less precise at higher levels, a fact that is of some concern in DN as the earliest stages of disease are characterised by a relative increase in GFR as previously described. The link between hyperfiltration, progressive renal function decline and albuminuria remains to be fully elucidated, however this potentially critical stage in the evolution of DN remains undetected by routine clinical parameters.
- Both the MDRD and CKD-EPI formulae have been shown to be less accurate in patients with diabetes in comparison to healthy control populations [152].
- As creatinine is derived from muscle, reliance on creatinine-based methods for determination of eGFR will be subject to significant variability, particularly in individuals who are significantly over- or underweight [153].

1.4.2 Albuminuria

Diagnosis and classification of CKD now relies on combined quantification of both GFR and level of UAE, as the presence of both these factors identifies individuals at particularly high risk of progression to ESRD [144, 150]. Although often transient in nature, the presence of persistent albuminuria has marked clinical significance as an early indicator of underlying renal pathology. Levels of albumin excretion are quantified as normal; moderately increased or severely increased (Table 1-4), and levels above 30mg/g (or 3mg/mmol) have been shown

to be associated with subsequent risk of mortality and CKD progression across both general and high-risk populations [154, 155]. One such high-risk group includes patients with diabetes.

Albuminuria category	Description	UACR
A1	Normal - mildly increased	<30mg/g <3mg/mmol
A2	Moderately increased	30-300mg/g 3-30mg/mmol
A3	Severely increased	>300mg/g >30mg/mmol

Table 1-4. CKD classification based on albuminuria categories.

UACR, urine albumin: creatinine ratio. Adapted from KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease, Kidney International Supplements, 2013; volume 3.

The development of assays for detection of MA in the 1960s revolutionised diabetes management, and almost 60 years later this marker continues to be monitored in routine practice as the earliest index of DN. Traditionally UAE has been graded using the following categories in patients with diabetes: "normoalbuminuria" (UACR <30mg/g or <3mg/mmol); "microalbuminuria" (UACR 30-300mg/g or 3-30mg/mmol); and "macroalbuminuria" (UACR >300mg/g or >30mg/mmol). If a positive result is obtained repeat confirmatory testing should be performed, with two out of three positive subsequent measurements considered to imply persistence.

Long term follow up studies published in the 1980s, prior to the introduction of RAAS blocking agents and therefore not influenced by their effect on UAE, demonstrated that MA was highly predictive of progression to overt DN in both type 1 and type 2 diabetes [21, 22, 28]. In a recent meta-analysis, drug-induced reduction in albuminuria of 30% translated into a 25% reduction in risk of progressive ESRD irrespective of choice of agent [156]. In addition MA has been

demonstrated to provide important prognostic information on cardiovascular morbidity and mortality in diabetic patients with and without nephropathy [145, 157-159] as well as at general population level [160]. Moreover, the presence of MA has been shown to indicate increased risk of all-cause mortality in both patients with T1DM and T2DM in comparison to those who remain normoalbuminuric [161, 162]. Consequently, UAE has evolved into both a key prognostic marker and therapeutic target in the management of patients with diabetes.

Although undoubtedly an essential tool for stratification of risk and monitoring of treatment effects in current clinical practice, there are a number of limitations associated with use of UAE as an early biomarker of DN.

- Our understanding of the natural history of DN has evolved as described earlier in this chapter. We now know that significantly fewer patients with detectable MA progress to overt DN than previously thought [30-32] and it is widely accepted that decline in GFR in patients with diabetes is not conditional on progression through the traditionally defined grades of UAE, as evidenced by the description of disease subtypes such as early GFR decline [36, 37, 40] and non-albuminuric DN [43, 44]. Similarly, the presence of MA is no longer viewed as a definitive and irreversible stage of DN. Studies have shown that remission of MA to normoalbuminuria occurs frequently in patients with diabetes [31, 33, 35, 163].
- Although traditionally accepted as the earliest clinical indicator of DN, by the time MA reaches the diagnostic threshold of 30mg/g or 3mg/mmol significant histological changes have already developed within the GBM and mesangial matrix in relative clinical silence [38, 164]. These are often preceded by alterations in glomerular perfusion pressure and its associated injurious effects on the renal microvasculature. Rather than being an early indicator of disease, clinical MA is effectively identifying individuals who may have already advanced through the initial stages of DN development.
- It is clear from recent literature that the relationship between UAE, renal and cardiovascular outcomes is a continuum starting from levels well within the limits of what is currently considered to be the “normal” range

[145, 154, 165, 166]. In this regard the thresholds defined in clinical guidelines are somewhat arbitrary and there has been a trend towards reporting UAE as a continuous variable alongside CKD stage to define risk [151].

- Measurement of UAE is not standardised and results are variably reported as 24-hour excretion rates or spot collections corrected for urinary creatinine. In addition day-to-day variability within individuals is significant and UAE can be influenced by a number of patient-specific factors such as blood pressure; dietary protein intake; exercise; fever; and presence of urinary tract infection [151].
- Independent prediction of cardiovascular morbidity and mortality as well as progression to ESRD by MA renders it an important clinical tool. However, at present there is no clear means by which to differentiate MA linked to cardiovascular disease from MA relating to renal disease; moreover albumin excretion is influenced by the multitude of patient-specific factors listed above. It could therefore be argued that any fluctuation in UAE may reflect modification of a different disease process and is not specific to the evolution of renal disease.

The pathogenesis and clinical presentation of DN are far more varied than previously defined in the traditional 5-stage classification and as a result there is currently no precise formula for diagnosis of the condition. The increasing prevalence of diabetes and the invasive nature of the procedure means that it is impractical to perform a renal biopsy on all patients with elevated UAE or reduced eGFR and clinicians therefore rely on laboratory measures interpreted in the context of patient characteristics to reach a diagnosis.

While eGFR and UAE undoubtedly provide important information on prognosis their utility as tools for early identification of DN is limited and there is an unmet clinical need for specific novel biomarkers to identify patients in the earlier stages of DN as well as to categorise individuals in whom there is diagnostic uncertainty. In recent years there has been a surge of publications describing novel biomarkers for DN. A number of individual molecules have been described as illustrated in Figure 1-6, most of them proteins and polypeptides,

each of which typically captures a specific mechanistic process underlying DN development.

1.4.3 Cystatin C

Cystatin C is a low molecular weight plasma protein that is freely filtered at glomerular level and subsequently catabolised and reabsorbed in the tubules to such an extent that under normal physiological conditions it does not return to the blood in its intact form [167]. Serum levels are closely correlated with measured GFR and unlike creatinine are not affected by gender, age or body mass [168]. Cystatin C has been shown to be an accurate marker of renal function, even at the low levels found when GFR remains within the currently accepted "normal" range [169] and increases in serum concentration have been shown to predict decline in GFR independently of UAE [170]. In patients with diabetes, serum cystatin C increases progressively with declining GFR, and elevated levels can be detected even when GFR is within the supra-normal range while anti-hypertensive medications may render MA undetectable as can be seen in the early stages of DN [171]. Whilst it shows promise as a tool for early identification of GFR decline and formulae for estimation of GFR based on Cystatin C are more accurate at higher levels of GFR, they have not yet been shown to improve precision of monitoring early GFR decline in patients with diabetes [169].

1.4.4 Markers of glomerular dysfunction

As described above, renal damage in diabetes is in part characterised by alterations in GBM structure and permeability. A number of urinary biomarkers have been described for identification of glomerular damage in patients with diabetes.

1.4.4.1 Transferrin

Transferrin is a plasma protein with slightly greater molecular weight than albumin. As it is less ionic and therefore less easily repelled by GBM, it is more readily filtered at the glomerulus [172] and thereby excreted earlier and in greater quantities than albumin. Early clinical studies in DN used transferrin rather than albumin to assess progression of DN [173, 174] and transferrinuria has been shown to rise in parallel with UAE [175]. The presence of transferrinuria has been demonstrated in patients with diabetes and glomerular lesions, even in the normoalbuminuric stage, and the degree of excretion correlates with the degree of underlying interstitial fibrosis and tubular atrophy at a histological level [176]. Furthermore, increased baseline urinary transferrin has been shown to independently predict development of MA over 2-5 years follow up [172, 175]. Although these findings suggest that measurement of urinary transferrin could be a useful tool for early detection of DN it is not a specific indicator and is also elevated in a number of other renal diseases [177].

1.4.4.2 Type IV collagen

Type IV collagen is a major constituent of the mesangial matrix as well as glomerular and tubular basement membranes. Its high molecular weight means that under normal conditions it cannot be filtered at glomerular level and excretion is therefore thought to be indicative of the rate of matrix turnover in kidney disease [175]. Studies have shown an association between urinary type IV collagen excretion and risk of progressive decline in renal function in patients with type 2 diabetes and proteinuria [178] as well as in normoalbuminuric patients [179]. No clear association has been demonstrated with progressive UAE. The ratio of type IV collagen to albumin appears to be increased to a greater degree in DN as opposed to other glomerular diseases, indicating a potential role differentiating DN from non-diabetic renal disease [180].

1.4.4.3 Caeruloplasmin

Caeruloplasmin is an acute phase protein which plays a key role in copper metabolism. Urinary caeruloplasmin: creatinine ratio is elevated in patients with DN and excretion appears to rise in parallel with UAE [181]. In a small prospective study higher urinary caeruloplasmin levels predicted progression to clinical MA in normoalbuminuric patients with type 2 diabetes and excretion has been shown to regress following intervention to improve glycaemic control or addition of ARB treatment [175].

1.4.4.4 Podocytes and related proteins

Recent years have seen a heightened interest in urinary excretion of podocytes or podocyte-specific proteins as early markers of glomerular pathology. It has been reported that morphologically the number of podocytes present per glomerulus is a strong predictor of progressive renal disease in Pima Indians with MA [182]. Urinary podocyte excretion has been shown in diabetic patients with both MA and overt albuminuria and is thought to result in disruption of the glomerular filtration barrier. Reduced podocyte numbers have not been seen in normoalbuminuric patients [74]. Few authors have focussed on podocyte alterations in Caucasian patients to date, but small studies have confirmed increased podocyte loss in T2DM patients with DN in correlation with foot process widening and increasing proteinuria [183, 184].

Nephrin is a transmembrane protein expressed in podocytes and its urinary level is regarded as an indicator of podocyte damage. Urinary nephrin excretion has been shown to correlate with degree of UAE and decline in GFR [185]. Several studies have shown elevated urinary nephrin levels in normoalbuminuric patients with diabetes [186-188] although to date none have confirmed that this finding is predictive of trends in UAE or GFR.

Podocalyxin is another podocyte-associated protein which can be quantified in urine. Urinary podocalyxin has been shown to be elevated in patients with

diabetes, increasing progressively from normo- to micro- and macroalbuminuria, and in addition excretion appears to be correlated with clinical risk factors such as HbA1c [189]. As with nephrin, no prospective studies have confirmed the clinical predictive potential of podocalyxin.

1.4.5 Markers of tubular dysfunction

Low molecular weight plasma proteins can be excreted in the urine in increased quantities either as a result of abnormal tubular reabsorption or secretion by tubular epithelial cells. Similarly detection of a variety of enzymes in the urine is another sensitive indicator of tubular dysfunction. These cannot be filtered at the glomerulus, thus their presence in the urine is indicative of release from damaged tubular epithelial cells.

1.4.5.1 Neutrophil gelatinase-associated lipocalin (NGAL)

This molecule belongs to the lipocalin family; a group of proteins which regulate the binding and transporting of small hydrophobic molecules as well as immune regulation and apoptosis. Stored primarily in the neutrophil granules it is also expressed in the kidney where it acts as a mediator of tubular cell proliferation and undergoes near complete tubular reabsorption in healthy conditions. NGAL is considered a sensitive marker of acute kidney injury [190] but recent studies have also explored its role in DN.

Associations between urinary NGAL excretion and features of diabetes and the metabolic syndrome such as obesity, hyperglycaemia and insulin resistance have been reported [191]. In addition, urinary NGAL excretion has been shown to increase from normo- to micro- and macroalbuminuria in patients with type 1 or type 2 diabetes and to parallel eGFR in diabetic patients with glomerular hyperfiltration, suggesting utility as a diagnostic marker in early DN [192, 193]. Higher urinary NGAL was a predictor of subsequent decline in eGFR in a cohort

of T2DM patients with overt proteinuria [194] but to date there is no evidence that it can perform adequately as a prospective indicator of DN risk in normoalbuminuric patients.

1.4.5.2 Kidney injury molecule-1 (KIM-1)

KIM-1 is a membrane protein expressed on proximal tubular cells that is involved in phagocytosis of damaged cells. Undetectable in the urine in healthy conditions, it appears to be a sensitive and specific biomarker of proximal tubular injury as well as for early detection of acute kidney injury [195]. To date the evidence for its use as a biomarker of DN is conflicting. A small prospective study demonstrated association between baseline urinary KIM-1 and subsequent GFR decline in a cohort of patients with DN; however this lost significance following adjustment for traditional risk markers [195] and similar results have been reported in T2DM [196]. In one study, however, low baseline urinary levels of KIM-1 were associated with regression of MA in T1DM [197] and more recently KIM-1 was included in a panel of 14 serum biomarkers significantly associated with rapid GFR decline over a 3 year follow up period in a small cohort of T2DM patients with CKD 3 [198]. Most recently in a large study of 1500 patients with T1DM baseline KIM-1 was not found to add any incremental prognostic benefit to measurement of UAE for DN prediction over 6 years of follow-up [199].

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

NAG is a high-molecular weight lysosomal enzyme secreted by the proximal tubule in response to kidney injury, and urinary excretion is increased in a variety of renal diseases. Elevated urinary NAG excretion has been demonstrated in patients with diabetes compared to healthy controls, even in normoalbuminuric groups [194]. Higher baseline NAG excretion as well as upward trends over time independently predicted onset of MA in samples from

the DCCT [200] and similarly lower levels at baseline were associated with regression of MA in another study [197]. Significant increases in urinary NAG excretion have also been reported with other microvascular diabetes complications, leading some authors to attribute NAG similar diagnostic utility to MA [201].

1.4.5.4 α_1-microglobulin (A1M)

A1M is a low molecular weight glycoprotein with the result that it is freely filtered at the glomerulus and reabsorbed in the proximal tubule. Damage to tubular cells therefore results in increased urinary excretion of A1M. Elevated urinary A1M excretion has been demonstrated in patients with diabetes compared to control subjects and correlate with HbA1c and diabetes duration [202]. In cross sectional studies urinary A1M has been shown to increase with degree of UAE and increased excretion has also been detected in normoalbuminuric patients, leading to speculation that it may provide information in addition to UAE for early DN detection [203]. Prospective work exploring the potential of A1M as an early predictor of DN is lacking.

1.4.5.5 β_2-microglobulin (β_2M)

β_2M is another low molecular weight protein which can be detected in increased quantities in the urine of patients with tubular injury [204]. Elevated urinary excretion has been associated with macrovascular complications and hypertension in patients with diabetes [205] but results focussing on β_2M as a biomarker of DN are conflicting. Small studies have suggested an association with other validated biomarkers such as cystatin C [206] but the relative instability of urinary β_2M in response to variations in temperature or pH limit its clinical applicability and no direct comparisons with UAE have been published.

1.4.5.6 Retinol-binding protein (RBP)

RBP is primarily synthesised in hepatocytes but is also expressed in other insulin sensitive tissues such as adipose tissue and muscle. As with A1M its low molecular weight mean that following glomerular filtration it is almost completely reabsorbed at the tubule and thus its excretion in urine is indicative of tubular dysfunction. Increased urinary RBP has been reported in diabetic patients with MA compared to normoalbuminuric subjects [202]. Associations between urinary RBP and both micro- and macrovascular diabetes related complications have been reported [205]. In one recent cross-sectional study serum RBP levels progressively increased from normo- to micro and macroalbuminuria and were found to be more specific for DN than NGAL or UAE [207]. Again, no larger scale prospective work has confirmed the proposed predictive potential of RBP.

1.4.5.7 γ-Glutamyltransferase (GGT)

GGT is predominantly considered to be a marker of hepatobiliary function but is also present in the proximal tubules and urinary excretion has been investigated as an indicator of tubular dysfunction. Detection of increased urinary GGT has been reported in patients with T2DM and levels correlated with traditional markers of renal function as well as glycaemic control [208]. More recently elevated urinary GGT excretion was demonstrated in patients with T2DM and glomerular hyperfiltration [209].

1.4.5.8 Liver-type fatty acid binding protein (L-FABP)

L-FABP is mainly expressed in the liver and proximal tubules. Acting as an intracellular carrier protein, L-FABP is produced in response to tubular injury and its presence in the urine is thought to be indicative of the same [210]. A cross-sectional study in patients with T1DM showed significantly higher urinary L-FABP excretion in diabetic patients compared to control subjects. Furthermore,

levels increased with degree of UAE and regressed upon treatment with ACE-I [211]. In T2DM, L-FABP was not found to be predictive of GFR decline over 2 years of follow-up [212].

1.4.5.9 Uromodulin (UMOD)

Also known as Tamm-Horsfall protein, UMOD is a glycoprotein produced in the ascending limb of the proximal tubule and the distal convoluted tubule. Its physiological functions remain incompletely understood but research has highlighted a potential role in the pathogenesis of CKD [213]. There is ongoing debate as to whether UMOD acts primarily in the initiation and progression of DN, or plays a more protective role. Certainly in the later stages of fulminant nephropathy urinary excretion of UMOD appears to be reduced [214-216]. However, the picture is complicated by the fact that in the earlier stages of disease excretion is increased. Studies have shown elevated baseline UMOD excretion in normoalbuminuric patients with T1DM who later progressed to develop MA, and in subjects with diabetes and normal GFR [217, 218]. However, no consistent correlation between UMOD excretion and UAE has been demonstrated to date.

1.4.6 Markers of inflammation

Accumulating evidence points towards a mechanistic role for inflammation in the pathogenesis of DN [97, 98, 101, 103]. Studies have shown increased subclinical inflammation and endothelial dysfunction in diabetic patients who progress to overt DN years before the development of disease that is detectable by traditional clinical means [108]. As such, a number of mediators of these processes have attracted attention as potential early biomarkers of DN.

1.4.6.1 TNF-α

TNF-α is an inflammatory cytokine which plays a key role in mediating tissue damage. As well as production by infiltrating inflammatory cells in kidney disease, TNF-α can also be produced locally within renal endothelial, tubular and glomerular epithelial cells. Through promotion of inflammation, extracellular matrix accumulation, production of reactive oxygen species and glomerular barrier damage TNF-α exerts a focal role in the pathogenesis of kidney injury and progression of albuminuria [219, 220]. Serum and urinary levels of TNF-α are increased in patients with diabetes compared to healthy controls [221] and urinary excretion appears to increase progressively with nephropathy severity in patients with T2DM and micro- or macroalbuminuria as opposed to normoalbuminuric subjects [222].

The effects of TNF-α are mediated by TNF receptors 1 and 2 which although membrane-bound can also be detected in serum in soluble form (STNFR 1 and 2). Serum levels of STNFR correlate with GFR in patients with diabetes [223] and accumulating evidence highlights the potential importance of these receptors as early biomarkers of DN. For example, patients with higher baseline STNFR levels have been shown to be significantly more likely to progress to CKD stage 3 and later ESRD over 12 years of follow-up, both in T1 and T2DM [224, 225]. This association appears to be stronger in patients without proteinuria, raising the possibility that the STNFRs could indeed have to potential to provide prognostic information above UAE status in the normoalbuminuric population. Until recently, little was known about the underlying histological lesions developing in human kidneys when these markers are elevated. This relationship was recently examined in a cohort of Pima Indians with T2DM where an inverse correlation was seen between STNFR levels and glomerular filtration surface, and STNFR correlated positively with GBM width and percentage of glomerular sclerosis [226]. These results corroborate the roles of inflammation and TNF signalling in the pathogenesis of DN. However, it is unclear whether the measurement of serum SNTFR abundance is indicative of renal or rather systemic receptor activation.

1.4.6.2 IL-6

Inflammatory cytokines such as IL-6 have been shown to be powerful independent predictors of cardiovascular morbidity and mortality [227]. Inflammation has also been shown to predict future development of CKD. In a large population based cohort study IL-6 was among a number of inflammatory markers that was not only associated with prevalent CKD at baseline, but also with development of CKD over a 15 year follow up period [228]. Serum IL-6 is elevated in patients with diabetes in comparison to healthy control subjects, and has been shown to be directly correlated with GBM thickening in a cohort of patients with T2DM [229]. Peripheral blood mononuclear cells (PBMCs) from patients with diabetes appear to display a more inflammatory phenotype with increased mRNA expression of IL-6 in individuals with micro- or macroalbuminuria, and in one study UAE was an independent predictor of PBMC IL-6 mRNA expression [221]. In a cohort of patients with T2DM and MA, a collection of inflammatory biomarkers (CRP, IL-6 and fibrinogen) and in particular baseline IL-6, independently predicted progression to onset of clinical DN [102]. To date there is no prospective evidence confirming any benefit above UAE in terms of early identification of DN risk in nonalbuminuric patients.

1.4.6.3 MCP-1

MCP-1 is a proinflammatory chemokine which has been shown to induce cytokine production as well as monocyte recruitment. In the kidney MCP-1 is synthesised in tubular epithelial cells and mesangial cells through a NF-κB dependent mechanism. The resultant recruitment of inflammatory cells into the interstitium culminates in the fibrosis, inflammation and tubular atrophy that is characteristic of DN [230]. Elevated urinary MCP-1 levels have been described in patients with DN [231]. Furthermore, excretion appears to parallel progression through the clinical stages of disease and correlate with UAE [232, 233]. These results support a role of MCP-1 in evolution of DN, however prognostic utility beyond MA is yet to be proven.

1.4.6.4 α-1-acid glycoprotein (AGA)

AGA is produced mainly in the liver and released in response to stimulation by inflammatory cytokines including TNF-α and IL-6. Increased levels are associated with a number of cardiovascular conditions including diabetes [234]. Urinary AGA excretion correlates with UAE in patients with diabetes and the two appear to increase in parallel with each other [235, 236]. More recently in a urinary proteomic study, AGA was shown to predict progression to MA over 6 years of follow up in a cohort of T1DM patients without any evidence of nephropathy at baseline [217].

1.4.7 Markers of oxidative stress

As described earlier in this chapter, oxidative stress is thought to be a key factor underlying development of diabetes complications and a number of products of oxidative damage can be measured in blood and urine. Several of these show some promise as biomarkers of DN.

1.4.7.1 8-oxo-7,8-dihydroguanosine (8-OHdG)

8-OHdG is a by-product of oxidative DNA damage. It is renally excreted without further degradation and therefore has been proposed as a useful urinary marker of oxidative stress [237]. Increased urinary excretion of 8-OHdG has been described in patients with diabetes compared to healthy control subjects, and appears to parallel increases in UAE [238, 239]. Treatment with ARBs has also been shown to reduce urinary 8-OHdG excretion in patients with DN and hypertension [240]. Furthermore, in a study of 500 T2DM with normo- or microalbuminuria those with higher urinary 8-OHdG at baseline were more likely to progress to DN, and 8-OHdG predicted disease progression more strongly than other traditional risk factors including blood pressure and HbA1c [239], hinting at potential as an early biomarker of risk of progressive disease. However, it

remains to be seen whether 8-OHdG offers any additional prognostic information beyond markers currently used in clinical practice.

1.4.7.2 Pentosidine

AGEs are the result of cellular exposure to hyperglycaemia and have been implicated in the pathogenesis of DN as described previously. Pentosidine is a structural component of AGEs which is filtered at the glomerulus and subsequently catabolised at the proximal tubule. While serum levels reflect oxidative stress, urinary pentosidine is thought to signify a degree of tubular dysfunction in addition. Increased urinary excretion has been demonstrated in patients with diabetes compared to healthy controls, while higher urinary and plasma levels are seen in overt DN [241-243] as well as in patients with MA [244]. At a histological level pentosidine has been shown to accumulate in mesangial and nodular lesions in DN [245]. Moreover, in a nested case-control study from the DCCT urinary pentosidine excretion was found to be predictive of subsequent progression to macroalbuminuria on univariate analysis, although significance was reduced in multivariate modelling [200].

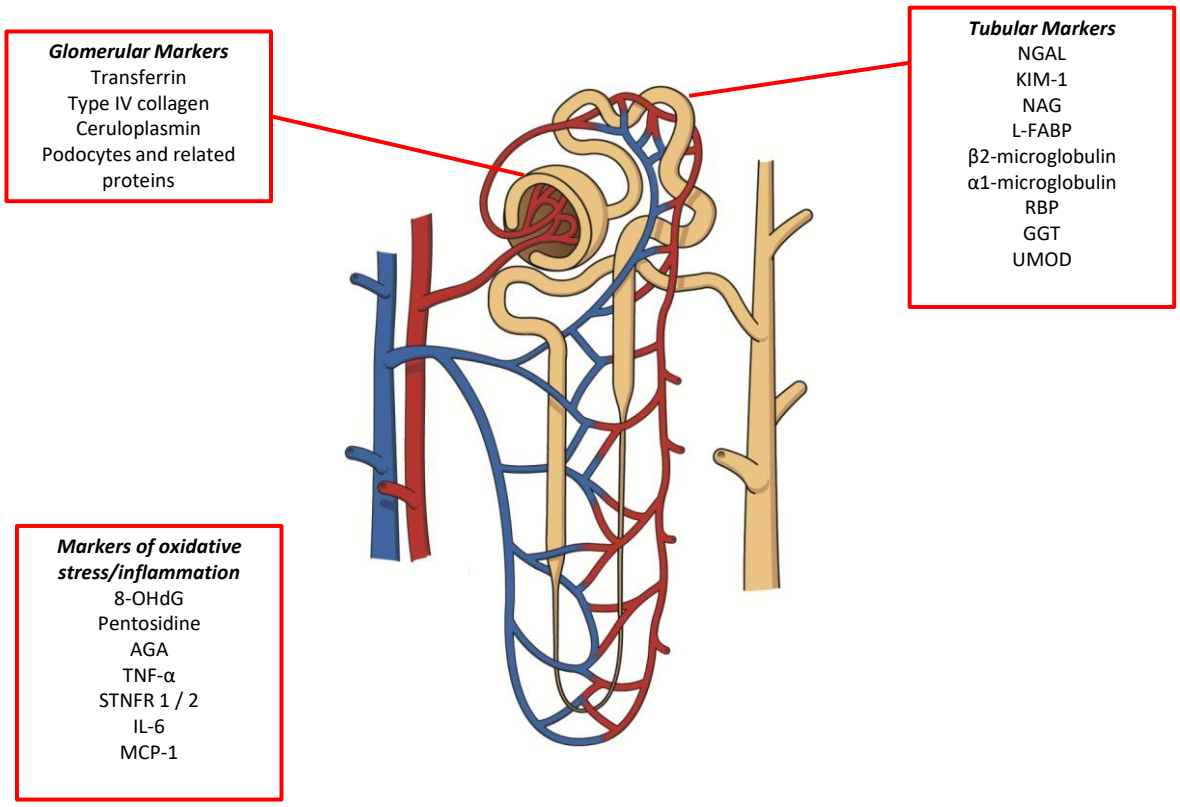

Figure 1-6. Biomarkers evaluated in DN.

DN, diabetic nephropathy; NGAL, neutrophil gelatinase associated lipocalin; KIM-1, kidney injury molecule 1; NAG, N-acetyl-b-d-glucosaminidase; L-FABP, liver-type fatty acid binding protein; RBP, retinol binding protein; GGT, gamma glutamyl transferase; UMOD, uromodulin; 8-OHdG, 8-oxo-7, 8-dihydro-2'-deoxyguanosine; AGA, alpha-1-acid glycoprotein; STNFR 1 / 2, soluble TNF-α receptors 1 and 2; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1. Adapted from Currie, World Journal of Diabetes 2014; 5: 763-776. Image courtesy of Medical Illustration Department, Glasgow Royal Infirmary, Glasgow.

1.4.8 Genetic markers

Strong familial clustering of DN has previously been shown [246, 247], leading to attempts to identify associated genetic variants. This brings a number of challenges and results to date have largely been disappointing as the complex underlying pathophysiology is likely to be determined by numerous variants, potentially acting additively or synergistically. In addition it is difficult to account for the significant influence of environmental factors.

Family Investigation of Nephropathy and Diabetes (FIND) and Genetics of Kidneys in Diabetes (GoKinD) are examples of groups using genome wide association scans to study DN susceptibility. The multicentre FIND consortium used family based linkage analyses in diverse ethnic groups to identify genetic loci that are associated with nephropathy in patients with T2DM [248]. A preliminary genome scan found links between multiple loci (e.g. 7q21.3; 10p15; 14q23.1) and DN status as well as GFR phenotype in African-American and European-American populations [249]. The GoKinD group focus on genetic association studies of DN in T1DM [250] and have linked candidate loci near the FERM domain containing 3 (*FRMD3)* gene, which is involved in cytoskeletal integrity, to DN [251]. In addition this work identified sorbin and SH3 domain containing 1 (*SORBS1)* gene polymorphisms which were significantly associated with DN, although these were not confirmed in a further analysis using additional cohorts [252]. A meta-analysis of genetic association studies in DN published in 2011 found 21 different variants associated with DN. Two of these were in the *FRMD3* gene, others included cysteinyl-tRNA synthetase (*CARS*), *VEGF* and *ACE* [253]. Further studies are required to confirm these associations and to explain the pathways through which they influence development of DN.

1.4.9 Summary

Translation of a biomarker into clinical practice requires extensive validation in large studies to confirm accuracy, reproducibility, sensitivity and specificity. These stages are discussed in more detail in section 4.1. Despite extensive

research into biomarkers with potential for early prediction of DN, no single candidate has emerged that unequivocally outperforms UAE in these regards. Although some show promise, in particular the STNFRs, none have yet been confirmed to provide additional benefit beyond the traditional parameters used in day to day clinical practice. As described earlier in this chapter, there are multiple pathophysiological processes contributing to development of DN. Furthermore, the interaction between these mechanisms remains incompletely understood meaning it may be impossible for a single biomarker to accurately predict progression of such a complex condition. The next logical step in the search for early identifiers of at-risk individuals may be use of panels of multiple biomarkers, thereby simultaneously capturing several pathophysiological mechanisms to improve disease prediction. High-throughput tools such as proteomic and metabolomic profiling are emerging as key strategies for biomarker discovery and these methods will be discussed below.

1.5 Metabolomics

The human metabolome is the product of interactions between the genome, transcriptome and proteome. Metabolomics has recently emerged as a technique with potential for biomarker discovery and essentially involves measurement of intermediates and end-products of cellular metabolism in a biological sample, thereby characterising physiological and pathological changes that result from different disease processes. Metabolomic analysis can be performed on any biological material including serum; plasma; tissue or urine; and in recent years a number of studies have explored the application of untargeted metabolomic profiling in chronic kidney disease [254] although to date relatively few focussed on DN in particular.

In a small cross-sectional study of plasma samples from non-diabetic subjects with CKD stage 2-4 major differences in arginine metabolism, carboxylate anion transport and coagulation pathways were identified with CKD progression [255]. Small cross-sectional studies using serum samples from patients with T2DM have identified a number of differentially-regulated metabolites including products of

amino acid and lipid metabolism as well as indicators of altered protein methylation such as symmetric dimethylarginine, to distinguish patients with overt DN from normoalbuminuric subjects [256, 257]. Application of metabolomic analysis to stored baseline samples from the Prevention of Renal and Vascular Endstage Disease (PREVEND) trial highlighted differences in urine hexose and glutamine between individuals who transitioned in albuminuria status over a median 2.9 years follow up period. Addition of these metabolites to a predictive model including traditional risk markers such as UAE and eGFR appeared to improve accuracy of risk estimation for CKD progression [258]. Whilst these results are interesting, metabolomic research is still in its infancy and prospective projects assessing the predictive power of these tools to identify individuals at risk of DN are lacking. The complexity of the metabolome is probably the key factor limiting clinical applicability of these techniques at present.

1.6 Proteomics

Proteomics is defined as "the knowledge of the structure, function and expression of all proteins in the biochemical or biological context of organisms" [259] and involves large-scale evaluation of the protein and peptide content of a biological sample. Whilst genomics describes the potential to develop disease and may play a key role in risk prediction, each gene in the human genome can be subject to differential translation and post-translational modifications to encode numerous different proteins. Proteomics therefore indicates the actual state of the organism at a given time point that results from interplay between genetic and environmental factors, thereby acting to identify the point at which an individual's genetic risk progresses to clinical disease. Proteomic analysis of biological samples can highlight differentially expressed proteins to distinguish particular disease processes; offer potential for early disease detection prior to onset of characteristic symptoms or standard biochemical abnormalities; and offer "hypothesis-generating" capabilities by providing deeper mechanistic insights into the pathogenesis of complex conditions. As a result, the number of published papers describing the proteomic signature of specific conditions has

grown exponentially in recent years and a draft map of the human proteome encompassing more than 80% of protein-coding genes was published in 2014 [260] with ongoing initiatives such as the Human Proteome Organisation now comprehensively exploring this on a much larger scale [261].

1.6.1 Sample selection and preparation

Proteomic analysis can be conducted on any biological sample or tissue: cultured cells; bodily fluids i.e. blood, urine or cerebrospinal fluid. Large scale work is generally limited to blood or urine as obtaining tissue samples often requires invasive procedures.

Plasma and serum are often the biofluids of choice as blood is in direct contact with organs. However, these sample types are prone to proteolytic degradation which can make accurate analysis challenging; for example increased proteolytic activity is observed in serum or plasma samples immediately following phlebotomy and clotting [262]. In addition, the plasma proteome is highly complex due to albumin-binding of a number of proteins as well as the variable concentrations of plasma proteins in a sample, ranging from picomolar to millimolar.

For these reasons urine is an attractive sample type for proteomic research. Urine contains proteins originating from numerous biochemical processes within the body; it can be collected non-invasively; is generally available in large quantities; and undergoes the majority of its proteolytic degradation during passage through the urinary tract prior to voiding, making it more stable for accurate analysis of peptide composition and meaning that less pre-analytical preparation is required. In addition, the urinary proteome remains stable for several hours when stored at room temperature and even after freezing at -20°C for several years [263, 264].

Ideally pre-analytical handling of samples for proteomic processing should be minimised to avoid interference or artefact. However, a number of abundant

molecules such as albumin, salt and lipids can affect the reproducibility and comparability of results, hence most protocols require a degree of sample preparation to remove these. Pre-analytical fractionation reduces the complexity of samples allowing efficient and reproducible analysis. The steps taken to minimise variability in sample processing for the purpose of this specific research project will be discussed in more detail in chapter 2.

1.6.2 Proteomic platforms

In general terms proteomics first requires separation of proteins and peptides from other sample components as well as from each other; followed by subsequent identification and analysis of their individual abundance using mass spectrometry. Many different techniques are available for proteomic analysis. The advantages and disadvantages of each have been extensively reviewed [263, 264] and choice of platform is generally dependent on the sample type to be analysed; the number of samples to be processed; available equipment and expertise. A detailed description of each of these platforms is beyond the scope of this thesis but they will be summarised briefly in the following paragraphs.

1.6.2.1 2-dimensional gel electrophoresis (2-DE)

This protocol separates the proteins contained in a sample based on their isoelectric point and molecular weight. Following treatment with in-gel proteolytic digestion the gel spots are extracted and mass spectrometry (MS) is employed to identify and quantify peptide fragments. Although 2-DE enables high resolution protein separation the lack of automaticity means that large scale simultaneous analysis of high numbers of proteins is not feasible. Furthermore its utility for high resolution separation of the low-molecular weight proteome is limited [265].

1.6.2.2 Surface-enhanced laser desorption/ionisation (SELDI) – mass spectrometry

SELDI-based proteomic techniques are based on selecting a small well-defined fraction of the sample proteome characterised by factors such as pH and salt content. The process uses hydrophilic surfaces which selectively adsorb proteins allowing unbound molecules to be removed. Subsequent addition of a matrix to the surface results in crystallisation and separation of proteins by binding to the matrix, reducing sample complexity. The reproducibility of these peptide binding techniques is variable and much of the sample proteome is removed prior to MS analysis, making comparisons of different datasets unreliable and resulting in loss of a significant quantity of proteome information [266].

1.6.2.3 Liquid chromatography (LC) – mass spectrometry

LC is a powerful tool for peptide separation. Peptides are separated using a chromatographic column, based on their elution through a liquid phase from a solid or stationary phase as a result of different characteristics such as charge or hydrophobicity. The eluting peptides are ionised before being separated and quantified using MS. The main limitations with this technique are the moderate reproducibility of what can be a complex separation process as well as the length of time taken to run a single sample [264].

1.6.2.4 Capillary electrophoresis (CE) – mass spectrometry

Capillary electrophoresis separates peptides in a sample in a single step by passage through a buffer-filled capillary. From the end of the capillary the separated peptides are delivered into a mass-spectrometer in a nano-ion spray. The advantages of CE-MS include the robust and repeatable single-step separation of peptides, which is particularly beneficial when analysing a large number of samples that may contain interfering compounds, and the fact that CE capillaries are relatively inexpensive in comparison to LC columns. The

limited loading capacity in comparison to LC-MS is a disadvantage, and as with LC-MS this technique is reliable only for evaluation of the low molecular weight urinary proteome, proteins with molecular weight greater than 20kDa must be removed from the sample by ultrafiltration prior to analysis [267].

In order to allow direct comparison between disease cases and controls, specific software tools are required. A commonly used classification of samples is based on support vector machine modelling by generation of high dimensional polypeptide models reliant on peptides which display significant differences between cases and controls. Each peptide then allegorises one dimension in an n-dimensional space [268]. The end result of this processing is a single numerical score or "classification factor" for the purposes of diagnosis of disease.

The proteomic data described in this thesis were generated using CE-MS technology which will be described in detail in chapter 2.

1.6.3 The clinical perspective

Application of proteomic technologies in clinical research is attractive for a number of reasons.

- Non-targeted techniques offer a means to disentangle the complex cellular processes contributing to disease development in an unbiased manner.
- Deeper understanding of the integrated cellular perturbations which precede the development of clinically detectable disease brings potential to identify novel therapeutic targets.
- Proteomic techniques essentially offer a "multi-marker" approach to disease detection, capturing alterations in multiple pathophysiological mechanisms simultaneously. This could offer improved and more accurate disease detection at a much earlier stage.

- Tools to identify individuals in the very early stages of disease may inform earlier intervention for “at risk” individuals, or targeting of therapies towards those who stand to gain most benefit rather than broadly exposing a large, unselected population to the risks associated with many pharmacological agents.

Despite the potential advantages that translation of these approaches into routine clinical practice could bring, proteomic research remains largely in the preclinical stages. The following paragraphs will illustrate the current state of clinical proteomic research in cardiovascular and renal disease.

1.6.3.1 Atherosclerosis

Despite being an almost ubiquitous precursor to numerous cardiovascular and renal conditions there are relatively few published data from human studies applying proteomics in atherosclerotic disease. A number of studies have used cultured cells to build insights into the mechanisms underlying early development of atherosclerosis. However, one key limitation of this approach is that the culture process itself can alter the cellular proteome meaning that probing the precise proteome of these cells within the vessel lumen is not possible. A more reliable approach may be focussing on plaque proteomics. Reduced secretion of heat shock protein 27 from ruptured plaques has been described, as well as a correlation with reduced plasma levels in patients with atherosclerotic disease [269]. Osteopontin is also among the more extensively investigated biomarkers highlighted by proteomic studies in atherosclerotic plaques [270], however this work remains in its early stages and proteomic tools for prediction of atherosclerotic disease are lacking.

1.6.3.2 Hypertension

Hypertension remains one of the most important challenges to health services. With the exception of rarer cases of secondary hypertension, the multitude of interrelated processes underpinning essential hypertension remain poorly understood and use of untargeted proteomic approaches seems an attractive means to identify as yet unknown mechanisms. Despite this, clinical studies in human subjects are lacking with the majority of work to date focussing on tissue from animal models such as Dahl salt-sensitive rats and rodents exposed to aortic constriction to induce pressure overload. Studies have shown differential expression of proteins associated with oxidative stress, glycolysis and collagen turnover in ventricular homogenates from these animals [271, 272]. Furthermore, these peptide patterns are altered following treatment suggesting potential utility of proteomic biomarkers for gauging response to treatment [273]. Processes such as hypertension and atherosclerosis are often the clinically silent precursors to overt cardiovascular diseases such as heart failure and coronary artery disease, both of which have been the focus of proteomic research in recent years.

1.6.3.3 Stroke

Prompt and accurate diagnosis of stroke is important to guide therapeutic intervention, particularly in view of the limited time frame for administration of thrombolytic therapy. Use of proteomic biomarkers to accurately confirm presence of cerebral ischaemia in cases of diagnostic doubt is an attractive prospect which remains minimally investigated in human subjects. One recent study used CE-MS analysis of urine samples obtained from patients within 24 hours of diagnosis of acute stroke for development of a diagnostic proteomic signature. A panel of 35 differentially expressed urinary peptides including uromodulin and various collagen fragments was able to distinguish stroke cases from controls with high specificity [274].

1.6.3.4 Heart Failure

Using 2D-E coupled to MS for analysis of myocardial tissue from heart failure patients a number of altered protein patterns have been described. Heat shock protein 70 (HSP70), belonging to the stress protein family, is among the most altered and has been found to be increased 1.64-fold in failing myocardium across different aetiologies in one study [275]. This highlights again the potential of discovery-based proteomic strategies in identifying novel mechanisms and biomarkers although tissue work should be interpreted with caution given the potential effects of pre-analytical processing on the proteome.

A number of studies have described alterations in the human plasma proteome for detection of left ventricular remodelling post-myocardial infarction; a clinically silent process which is strongly predictive of subsequent heart failure. In plasma samples from patients with sequential echo data available post-translational variants of α1-chain of haptoglobin were elevated in those who displayed higher degrees of remodelling. Importantly, this marker is not readily detectable by Western blot or enzyme-linked immunosorbent assay (ELISA) and traditional biomarkers such as creatine kinase did not differentiate between patients with high versus low remodelling [276]. Analysis of the "deep" plasma proteome has also highlighted down-regulation of the N-terminal human albumin fragment in patients with excessive remodelling as a potential tool for identification of these patients at an earlier stage [277]. Urinary proteomic studies using CE-MS processing have compiled a panel of 85 peptides for identification of patients with asymptomatic diastolic dysfunction, the majority of which were differentially regulated collagen fragments [278].

1.6.3.5 Coronary Artery Disease

Coronary artery disease remains one of the leading causes of mortality worldwide, with traditional parameters of limited use in determining risk on an individual patient basis. Identification of novel biomarkers highlighting those with early stage or asymptomatic disease may allow clinicians to direct

aggressive therapies towards those who are at highest risk and a number of studies focussing on proteomics for detection of coronary artery disease have been published.

Proteins shown to be up-regulated from plasma studies in coronary artery disease patients include complement components, proteolytic enzymes such as cathepsin S, and coagulation cascade components such as fibrinogen [279, 280]. Alterations in the plasma proteome continue to develop months after an acute ischaemic event raising the question of the influence of pharmacotherapy and potential utility of proteomic techniques for monitoring response to treatment [281].

Although the majority of clinical proteomic studies published to date have been cross-sectional, a panel of urinary peptides has recently been described for diagnosis of coronary artery disease which has also shown potential for prediction of later coronary events. The CAD238 panel was developed using CE-MS analysis of spot urine samples from individuals with angiography-proven coronary artery disease compared to healthy control subjects, identifying coronary artery disease in a blinded test cohort with a high degree of sensitivity and specificity [282]. Application of this biomarker panel to stored baseline urine samples collected from Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) participants showed it to be predictive of later cardiac events over five years in patients without known coronary disease at baseline [283]. CAD238 has recently been shown to correlate with measures of coronary artery disease severity [284] but it remains to be seen whether CAD238 can outperform other cardiovascular biomarkers or inform therapeutic decision-making.

1.6.3.6 Chronic kidney disease

As discussed in section 1.4, CKD is a strong independent predictor of cardiovascular morbidity and mortality [285, 286] and risk is increased even in the earliest, pre-clinical stages of disease which are not detectable by traditional clinical parameters [287]. Consequently, there is an unmet clinical

need for novel disease biomarkers to allow early, targeted intervention to be guided towards the patients who stand to benefit most from treatment. A multitude of biomarkers have been evaluated in this context, but none have transitioned into clinical practice.

Proteomics is an attractive tool in CKD, as a panel of multiple biomarkers has the potential to capture early changes in the multiple interrelated mechanisms implicated in CKD development and provide an integrated overview of an individual's disease status. For example, untargeted plasma proteome analysis using LC-MS in samples from patients with CKD recently highlighted differential regulation of peptides associated with a number of molecular mechanisms implicated in CKD development such as coagulation, inflammation and endothelial dysfunction. In addition increased expression of lysosome C and leucine-rich-alpha-2 glycoprotein, mediators thought to be involved in vascular and cardiac disease, was noted; highlighting the close interrelationship between renal and cardiovascular disease [288].

Urine has proved to be a particularly useful sample type for proteomic studies in CKD. In 2010, Good et al performed CE-MS analysis of urine samples from 230 patients with CKD of mixed aetiology and 379 healthy control subjects. This work resulted in identification of a panel of 273 differentially expressed urinary peptides that differentiated CKD from controls with high sensitivity and specificity in a blinded test cohort [268]. This classifier was termed CKD273, and to date remains the proteomic classifier that has been most rigorously evaluated in independent studies, with a classifier score above 0.343 defined as the threshold for CKD [268]. Component peptides include mainly fragments of collagen types I and III (approx. 70%), albumin fragments (3%), as well as uromodulin (4%), α-1 antitrypsin (6%), fibrinogen-α chain (2%), with others such as $ß_2M$, clusterin, osteopontin, cystatin B and antithrombin III making up the remaining 15% [268]. There is some overlap with other cardiovascular disease panels such as CAD238, potentially reflecting multiple common mechanistic pathways linking CKD and cardiovascular disease.

In addition to potential diagnostic utility, the predictive power of CKD273 has been evaluated in a number of studies. In 76 patients attending renal outpatient

services with CKD of any aetiology, baseline CKD273 score was predictive of death or requirement for dialysis over mean 3.6 years follow-up [289]. Data from a recent cross-sectional study using urine samples from 522 patients with CKD showed that baseline CKD273 score identified those who displayed rapid GFR decline (>5% per year) over 4 years of follow up with higher sensitivity and specificity than UAE. Addition of CKD273 to a model including traditional parameters such as eGFR and UAE significantly improved prediction of adverse outcomes [290].

Findings in patients with pre-existing CKD cannot be readily extrapolated to the general population. However, a recent study of 621 samples from patients participating in the population-based Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) study, CKD273 predicted subsequent increase in serum creatinine and decline in eGFR over 4.8 years follow up [291]. Similarly, it has been shown that the classifier performs best as a predictor of progressive CKD in patients with early stage disease where eGFR remains above 70ml/min/1.73m^2 [292]. In the studies described above, CKD273 correlated with UAE and eGFR, leading some to suggest that it has value as a composite single marker of renal excretory function and glomerular filtration.

1.6.3.7 CKD273 in diabetic nephropathy

As the complex interrelationships between the multiple mechanistic pathways underlying DN development remains incompletely understood, a proteomic approach is an appropriate strategy both for delineating these mechanisms further and for identifying early disease-specific biomarkers. Multi-marker proteomic approaches are advancing from the realms of discovery and validation into prospective clinical studies in DN. The availability of stored sequential urine samples from large diabetes cohorts with long follow-up periods has allowed researchers to investigate the urinary proteome not only of patients with a clinical diagnosis of DN, but also to delve into earlier changes in normoalbuminuric patients, many years before the development of overt DN. CE-MS analysis has been primarily employed for DN biomarker discovery and

several disease-specific proteomic panels have been described [293-295]. Although initially developed in samples from patients with CKD of multiple diverse aetiologies, the CKD273 panel has shown particular promise in DN.

When applied to urine samples from a longitudinal cohort of patients with both T1 and T2DM who were normoalbuminuric at baseline, the CKD273 classifier identified individuals who later progressed to macroalbuminuria up to 5 years before its development, and was shown to perform better than MA in this regard (receiver operating characteristic plot analysis gave area under the receiver operating curve (AUC) for CKD273 0.93; 0.67 for MA), with patients reaching the diagnostic CKD273 threshold on average 1.5 years prior to development of MA. This relationship remained statistically significant following adjustment for traditional parameters such as age, eGFR at baseline, blood pressure and HbA1c [295]. Further to this, CE-MS analysis of urine samples from participants in the PREVEND study showed that baseline CKD273 score accurately identified individuals who "transitioned" in UAE status over 3 years follow up; either from normo-to microalbuminuria, or from micro-to macroalbuminuria [296]. Recently, urinary proteomic analysis of stored baseline urine samples from participants in the Diabetic Retinopathy Candesartan Trials (DIRECT) showed that CKD273 independently predicted development of MA over a 4 year follow up period [297]. It appears therefore that changes in the urinary proteome have the potential to distinguish normoalbuminuric patients at risk of progressive renal disease, even in the absence of any other clinical indicator of renal dysfunction.

In addition to its potential predictive power, the CKD273 classifier has been shown to be stable despite long term storage over up to 35 months [295] and to accurately identify DN with high consistency in a validation study undertaken across multiple centres, irrespective of confounding factors such as age and gender [298]. Application of the classifier to samples from hypertensive T2DM patients with MA randomised to receive the ARB Irbesartan or placebo over 2 years showed that the peptide pattern evolved towards a "healthier" profile after ARB treatment while no change was observed in those treated with placebo [299]. This indicates that urinary proteomics could theoretically be employed to determine the impact of therapeutic interventions in DN. While

the results to date are interesting, it should be borne in mind that they have been generated through retrospective analyses of small heterogeneous cohorts, often with limited follow-up and comparative studies with alternative DN biomarkers are lacking. Furthermore, the technology required for CE-MS analysis is expensive and not widely available. There preliminary data require substantiation by way of prospective testing in large studies with adequate follow up recording clinically meaningful endpoints, along with a full assessment of cost-effectiveness in clinical practice.

1.7 Treatment of diabetic nephropathy

Mortality rates have consistently been shown to be elevated in patients with diabetes complicated by nephropathy in comparison to those with uncomplicated disease. The goals of treatment for these patients are to prevent progression of UAE and decline in GFR leading to ESRD. Much of the excess mortality results from cardiovascular disease, and both UAE and eGFR are known to be independent and additive predictors of cardiovascular morbidity and mortality as well as all-cause mortality [300]. Current guidelines recommend that clinicians should consider the presence of diabetes, CKD or both in combination as a coronary risk equivalent, thus employing the same multifactorial preventative strategies to manage cardiovascular risk is critical both in patients with MA and overt DN [151, 301-303]. Treatment strategies in patients with DN will be discussed below. While each of these will be discussed individually, studies have shown that intensive risk factor control addressing blood pressure, glycaemic control, cardiovascular risk, diet and lifestyle is key to risk reduction.

1.7.1 Control of blood pressure and albuminuria

The prevalence of hypertension is increased in patients with DN and control of blood pressure is a key factor in DN management. Optimal blood pressure

targets in the general diabetic population have been debated over recent years. The majority of randomised trials support a beneficial impact of blood pressure control on cardiovascular complications of diabetes, and these are summarised in Table 1-5. While target blood pressures varied considerably between these trials a reduction in renal endpoints as well as cardiovascular events were generally seen in association with improved control. The exception was the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial, in which particularly tight control (target systolic pressure of below 120mmHg) was associated with increased rates of adverse events such as electrolyte disturbance and symptomatic hypotension and no significant benefit on cardiovascular outcomes [304]. Combination of these data in a recent meta-analysis showed that a 10mmHg reduction in systolic blood pressure nevertheless conferred 13% reduction in all-cause mortality and 11% reduction in cardiovascular events including myocardial infarction, stroke, heart failure and revascularisation in T2DM [305].

It should be borne in mind, however, that while retrospective studies have explored tight blood pressure control in CKD, there have been few specific prospective studies evaluating the impact of different blood pressure goals on progression of DN. In fact, much of the evidence supporting tight blood pressure goals in DN is extrapolated from post-hoc analyses of studies where comparison between different blood pressure targets was not a prespecified endpoint. As such the evidence is inconsistent [306]. Post-hoc analyses of early randomised controlled trials of ARB treatment in DN have suggested that systolic pressures of above 140mmHg were associated with higher risk of progression to the primary renal endpoint [307, 308]. In view of the lack of prospective studies there is little consensus across guidelines; with some suggesting that clinicians should aim for a blood pressure of less than 140/90mmHg to slow progression of DN, whereas others advocate a target of 130/80mmHg in certain circumstances [144, 302, 309-311].

Trial Name	BP in conventional arm (mmHg)	BP in intensive arm (mmHg)	Impact on cardiovascular endpoints	Impact on microvascular endpoints
UKPDS	154/87	144/82	32% reduction in diabetes-related deaths	37% reduction in microvascular endpoints, particularly progression of retinopathy. Trend towards reduction in progression of DN
STENO-2	146/78	131/73	29% reduction in risk of cardiovascular events	Reduced incidence of DN and progressive retinopathy
ADVANCE	140/75	136/74	14% reduction in all-cause mortality 18% reduction in cardiovascular death	9% reduction in major microvascular or macrovascular events (separate reductions not independently significant)
ACCORD	134/71	119/64	No improvement	No reduction in advanced microvascular complications, but delayed onset of macroalbuminuria and 21% reduction in development of MA

Table 1-5. Summary of achieved BP in trials of intensive multifactorial therapy in diabetes.

Of note, BP control was a component of multifactorial intervention in all trials. UKPDS, UK Prospective Diabetes Study; ACCORD, Action to Control Cardiovascular Risk in Diabetes; ADVANCE, Action in Diabetes and Vascular disease: preterAx and diamicroN-MR Controlled Evaluation; BP, blood pressure; DN, diabetic nephropathy; MA, microalbuminuria.

1.7.1.1 ACE-inhibitors and angiotensin receptor blockers

As described earlier in this chapter, activity of the RAAS is a critical pathway in DN development. It is clear from a multitude of landmark clinical trials that ACE-I and ARBs have renoprotective qualities independent of their effect on blood pressure in DN [312-316] and these agents should therefore be considered as first-line treatment in patients with diabetes and hypertension, MA or advanced DN [144, 302, 309].

Beyond their antihypertensive effects these agents also have beneficial effects on UAE. Although evidence confirming a link between reduction of UAE and corresponding impact on cardiovascular endpoints is lacking, minimising UAE is associated with slowed progression of DN. This was first evidenced by the Captopril Trial [312] and later confirmed in the Reduction of Endpoints in Non-insulin dependent diabetes with the Angiotensin Antagonist Losartan (RENAAL) trial, where 35% reduction in proteinuria led to a 16% reduction in renal endpoint rates [315]. Similar results were shown in the Irbesartan Diabetic Nephropathy Trial (IDNT) [314]. A recent meta-analysis evaluating the impact of minimising UAE on renal endpoints found that reducing albuminuria by 30% conferred a 25% reduction in risk of progression to ESRD [156]. This effect was independent of drug class and highlights the potential long term benefits of this strategy in patients with DN.

The use of ACE-I and ARB has significantly improved the outlook for patients with DN, but many still progress to ESRD and there remains an unmet need for therapeutic strategies to delay DN progression or prevent its development altogether. A multitude of studies have evaluated alternative or enhanced RAAS-blocking strategies, some of which will be summarised below.

1.7.1.2 Dual RAAS blockade

In general, the term dual RAAS blockade is used to refer to studies of combined ACE-I and ARB therapy rather than other RAAS blocking agents such as direct

renin inhibitors or MRAs. In view of the impressive results from trials of ACE-I or ARB monotherapy in DN it was assumed that combination of these agents may serve to intensify the anti-albuminuric effect.

The Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial (ONTARGET) evaluated the effect of telmisartan, ramipril or both in combination on renal endpoints in a large cohort of 25000 patients with atherosclerotic disease or diabetes (37% of participants) and evidence of end-organ damage over a period of 56 months. The combined primary endpoint of death, dialysis or doubling of creatinine was similar in the ramipril and telmisartan groups, but incidence increased significantly in the dual therapy arm. The same effect was seen in the higher renal risk cohort including patients with DN; diabetes and hypertension and eGFR below 60ml/min/1.73m^2. Despite the higher number of renal events with combination therapy, this intervention was associated with beneficial effects on risk of developing MA or overt albuminuria [317]. A post-hoc analysis investigating whether individuals with lower baseline eGFR or higher UAE would gain more benefit from this treatment did not yield any more positive results with more renal events, hyperkalaemia and symptomatic hypotension associated with dual RAAS blockade [318].

The Olmesartan Reducing Incidence of End-stage Renal Disease in Diabetic Nephropathy (ORIENT) trial investigated the effects of dual RAAS blockade on a cohort of 577 patients with T2DM and DN. While additional olmesartan treatment did reduce blood pressure and albuminuria, there was no difference in the primary renal outcome between arms of the study and discontinuation of therapy due to hyperkalaemia was more common in patients on dual therapy. These effects were overshadowed by a significantly higher number of cardiovascular deaths in the intervention arm [319].

Most recently the Veterans Affairs Nephropathy in Diabetes (VA NEPHRON-D) randomised 1400 patients with T2DM, reduced eGFR and albuminuria to combined therapy with lisinopril and losartan in comparison to losartan monotherapy. While combination therapy was associated with slower decline in eGFR and reduced ESRD events at 6-12 months, this effect was not sustained over longer follow up. Again, this trial was terminated prematurely in view of

safety concerns resulting from increased numbers of patients reaching the primary endpoint (death, decline in eGFR or dialysis) in the group treated with dual therapy. In addition, combination therapy was associated with higher rates of hyperkalaemia and acute kidney injury [320]. In response to these results dual RAAS blockade is not endorsed by clinical guidelines as a therapeutic strategy in DN.

1.7.1.3 Renin inhibitors

In recent years the addition of direct renin inhibitors to ACE-I or ARB in patients with DN has been evaluated. The open-label Aliskiren in the Evaluation of Proteinuria in Diabetes (AVOID) trial randomised 600 T2DM patients with hypertension and albuminuria to receive either the direct renin inhibitor aliskiren or placebo in addition to ARB therapy for 6 months and found that aliskiren treatment reduced UAE by 20% in comparison to placebo, with very minimal differences in blood pressure or decline in GFR between the groups [321]. Following this promising result the Aliskiren Trial in Type 2 Diabetes Using Cardiovascular and Renal Disease Endpoints (ALTITUDE) trial was designed. This study randomised over 8000 patients with T2DM and renal or cardiovascular disease to receive aliskiren or placebo in addition to ACE-I or ARB. The trial was stopped after a mean follow up of 33 months due to increased cardiovascular events in the intervention group. There was no significant difference in renal endpoints between aliskiren or placebo treated patients [322]. As a result combination therapy with aliskiren and other RAAS blocking agents is not recommended in T2DM following an announcement from the European Medical Association in February 2012.

1.7.1.4 Mineralocorticoid receptor antagonists

Despite the widespread use of ACE-I and ARB many patients continue to progress to overt DN and ESRD. There has been a resurgence of interest in the non-

genomic effects of aldosterone beyond its effects on blood pressure and electrolyte balance [112, 135]. In addition, as described earlier in this chapter, evidence suggests that a subgroup of patients treated with ACE-I or ARB display "aldosterone breakthrough" and in retrospective analyses this cohort tend to have more rapidly progressive disease [132-134]. As a result the incorporation of MRA with ACE-I or ARB remains an area of interest in DN.

This combination has been explored in a number of studies which in general show beneficial effects on blood pressure and albuminuria in patients with DN [323-330] as well as CKD of alternative aetiology [331-341]. Trials to date have been relatively short, and the majority are small and underpowered to detect significant differences in meaningful clinical renal or cardiovascular endpoints. In addition, use of MRA in patients with CKD raises safety concerns due to increased risk of hyperkalaemia and acute kidney injury and this strategy is not currently recommended in clinical guidelines. The introduction of novel potassium binding agents and nonsteroidal MRAs may however herald a paradigm shift in this debate. A comprehensive meta-analysis of trials evaluating use of MRA in addition to conventional RAAS blocking agents in CKD will be presented in chapter 3.

1.7.2 Glycaemic control

Landmark trials such as UKPDS and DCCT demonstrated that improved glycaemic control significantly reduced incidence and progression of microvascular complications of diabetes, including DN. In fact, the DCCT showed a decline in microvascular complications with HbA1c reduction as values approached the non-diabetic range [58, 59]. Based on these promising results, two larger studies were developed to test the hypothesis that near-normalisation of glucose in patients with T2DM would lead to even more significant reductions in microvascular and macrovascular complications; ACCORD and ADVANCE (Action in Diabetes and Vascular disease: preterAx and diamicroN-MR Controlled Evaluation) [316, 342]. Both trials demonstrated beneficial effects of intensive glucose lowering on UAE but not on the later stages of DN, and neither showed a

significant benefit in terms of cardiovascular outcomes. In fact, the more stringent glycaemic target in ACCORD (HbA1c<6.5%, or 48mmol/mol) was proposed as a factor underlying increased mortality rates in the intensive treatment group. These findings are summarised in Table 1-6. It is important to note that the evidence of benefit from improved glycaemic control in DN is almost exclusively based on impact on UAE, there is little evidence of benefit on other renal outcomes such as doubling of serum creatinine or decline in GFR.

Trial Name	HbA1c in conventional arm	HbA1c in intensive arm	Impact on CVD	Impact on UAE
DCCT	8.5% (75mmol/mol)	7.1% (53mmol/mol)	Trend towards reduction in risk of all macrovascular events Trend towards reduction in risk of cardiac events	60% reduction in risk of developing sustained MA 54% reduction in risk of developing clinical DN
UKPDS	7.9% (63mmol/mol)	7.0% (53mmol/mol)	Trend towards reduction in MI	33% reduction in development of sustained MA
ADVANCE	7.3% (56mmol/mol)	6.5% (48mmol/mol)	No significant reduction in macrovascular outcomes	90% reduction in new onset MA 30% reduction in progression to macroalbuminuria
ACCORD	7.6% (60mmol/mol)	6.3% (45mmol/mol)	Trend towards reduction in MI, stroke and cardiovascular death	21% reduction in new onset MA 32% reduction in progression to macroalbuminuria
VADT	8.4% (68mmol/mol)	6.9% (52mmol/mol)	No significant reduction in macrovascular outcomes	32% reduction in new onset MA 37% reduction on progression to macroalbuminuria

Table 1-6. Summary of HbA1c and resultant outcomes in trials of intensive therapy in diabetes.

Of note, glycaemic control was a component of multifactorial intervention in the majority of trials. UKPDS, UK Prospective Diabetes Study; ACCORD, Action to Control Cardiovascular Risk in Diabetes; ADVANCE, Action in Diabetes and Vascular disease: preterAx and diamicroN-MR Controlled Evaluation; VADT, Vetarans Affairs Diabetes Trial; HbA1c, glycated haemoblogin; CVD, cardiovascular disease; UAE, urine albumin excretion; DN, diabetic nephropathy; MI, myocardial infarction; MA, microalbuminuria.

Although none of these studies focussed specifically on patients with DN, most guidelines advocate an HbA1c target of <7% (53mmol/mol) to minimise progression of microvascular complications [144, 309]. These are subject to interpretation based on patient characteristics, for example in older individuals with significant comorbidities and limited life expectancy or patients at risk of hypoglycaemia a target of >7% (53mmol/mol) may be more appropriate, while young patients without pre-existing cardiovascular disease may be more able to achieve a lower target with minimal adverse effects.

1.7.2.1 Renal endpoints in recent outcome studies of glucose-lowering medications

Several eagerly anticipated outcome studies have evaluated the impact of novel pharmacological approaches to T2DM treatment on macro- and microvascular outcomes. Two key recently published studies have shown particularly encouraging results.

The (Empagliflozin) Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG) study demonstrated that the sodium-glucose cotransporter-2 (SGLT2) inhibitor empagliflozin produces significant reductions in cardiovascular mortality in patients with T2DM [343]. At a mechanistic level this agent has been shown to reduce intraglomerular pressure and thereby minimise hyperfiltration in patients with T1DM [344, 345]. A secondary aim of the EMPA-REG trial was to determine the impact of therapy on microvascular outcomes, particularly the progression of CKD. Results showed that treatment with empagliflozin reduced the relative risk of doubling of serum creatinine, requirement of RRT and progression to macroalbuminuria in this patient group [346]. The nonglycaemic effects of SGLT2 inhibitors include natriuresis, modulation of glomerular haemodynamics and uric acid lowering [347], all of which could contribute to the renal outcomes seen in EMPA-REG. The trial population included patients with T2DM at high cardiovascular risk and a proportion had established kidney disease (eGFR 30-59ml/min/1.73m^2 or

albuminuria) at baseline, therefore these promising results cannot be immediately generalised to all T2DM patients.

The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial evaluated the impact of the glucagon-like peptide-1 (GLP-1) receptor agonist Liraglutide on cardiovascular endpoints in patients with T2DM. Experimental studies have shown that GLP-1 receptor agonists may protect the vascular endothelium by ameliorating oxidative stress and inflammation, thereby potentially acting to minimise glomerulosclerosis and albuminuria [348]. As well as showing significant benefit in terms of cardiovascular outcomes, liraglutide treatment was associated with a reduction in new onset of macroalbuminuria over a 3.5 to 5 year period [349]. Beyond the beneficial impact on glycaemic control, blood pressure and bodyweight, effects on proximal tubular sodium reabsorption leading to natriuresis have also been highlighted as potential mechanisms underlying the renal outcomes reported in LEADER [350].

1.7.3 Lipid-lowering

DN is accompanied by changes in lipid metabolism as CKD progresses. Clinical trials in patients with CKD who are not dialysis dependent suggest that statins alone, or in combination with ezetimibe, reduce incidence of cardiovascular morbidity and mortality compared to placebo. In fact, the absolute benefit from statin treatment tends to be higher in patients with diabetes compared to the general population, most likely due to the inherent increased cardiovascular risk associated with the condition. The clinical benefit of treatment in patients receiving dialysis is less certain [351]. In view of the significant cardiovascular burden associated with DN, statins are recommended in all diabetic patients with nephropathy [352] and there are meta-analysis data supporting their safety in this population [351]. While guidance suggests that statin therapy should not be initiated in patients who are receiving dialysis, these medications should not necessarily be discontinued [353].

1.7.4 Dietary modifications

Guidelines recommend reducing sodium intake to less than 2g per day. The rationale behind this is based on proposed effects on blood pressure and cardiovascular disease. Whether this is true in patients with diabetes remains unclear [353, 354]. However, low sodium intake has been shown to enhance the effects of ACE-I and ARB on blood pressure and UAE [355].

The relationship between dietary protein intake and DN has long been debated. Certainly kidney disease confers metabolic abnormalities that can complicate identification of optimal protein intake, such as loss of muscle mass and susceptibility to malnutrition. Small studies in patients with T1DM and DN suggested that low protein diet (0.6-0.89g/kg/day) leads to slower rate of GFR decline and reduction in relative risk of ESRD [356], however other studies and meta-analyses have failed to show a significant benefit on renal function in diabetes [357]. At present guidelines recommend an intake of 0.8g protein/kg/day for patients with overt DN who are not on dialysis [144, 309].

1.7.5 Novel treatment strategies

There have been no notable advances in the management of DN since the publication of studies such as RENAAL and IDNT [314, 315]. In fact, the majority of recent attempts to develop new therapies have failed to translate into clinical practice due to lack of efficacy, unacceptable side effects or unacceptable cardiovascular effects. Some key studies evaluating alternative pharmacological interventions in DN will be summarised below.

1.7.5.1 Endothelin antagonists

ET-1 has been highlighted as a mediator of progressive renal disease through its effects on the endothelin type A receptor. Short-term proof of concept studies

evaluating the addition of endothelin antagonists to ACE-I or ARB in DN showed that these agents significantly reduced proteinuria and blood pressure [358], however subsequent clinical trials have been less convincing. In 2010 A Randomised, Double Blind, Placebo Controlled, Parallel Group Study to Assess the Effect of the Endothelin Receptor Antagonist Avosentan on Time to Doubling of Serum Creatinine, End Stage Renal Disease or Death in Patients With Type 2 Diabetes Mellitus and Diabetic Nephropathy (ASCEND) was published. Patients with T2DM and overt nephropathy were randomised to receive the ET antagonist avosentan or placebo in addition to RAAS blockade for 6 months. Although the significant antiproteinuric effect of treatment was confirmed, the study was terminated prematurely due to serious safety concerns after increased rates of death, congestive cardiac failure and pulmonary oedema were observed with avosentan treatment [359]. Subsequent to this the safety of lower doses of a more selective ET-antagonist, atrasentan, was evaluated in a similar patient cohort and shown once again to significantly reduce proteinuria. Although there was more weight gain in the intervention group, rates of cardiac failure and oedema were not different [360]. A definitive conclusion to the endothelin story may come from the multicentre Study of Diabetic Nephropathy with Atrasentan (SONAR) trial, which is due to complete in 2018.

1.7.5.2 Nuclear 1 factor (erythroid-derived 2)-related factor 2

Oxidative stress is another key mediator of CKD progression. Animal studies have shown that this mechanism is associated with impaired nuclear 1 factor (erythroid-derived 2)–related factor 2 (Nrf2) receptor activity. Bardoxolone is a potent activator of the Nrf2 pathway, and its clinical utility in treatment of DN was evaluated in the Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes Mellitus: the Occurrence of Renal Events (BEACON) trial. This study was also terminated prematurely after an increased number of heart failure related deaths or hospitalisations were noted in the intervention group [361].

1.7.5.3 Neutral endopeptidase inhibition

Inhibitors of neutral endopeptidase (NEP) have been shown to augment the natriuretic and blood pressure–lowering effects of natriuretic peptides. Daglutril is a combined endothelin converting enzyme and NEP inhibitor which was recently evaluated in combination with losartan in a small cohort of patients with T2DM and albuminuria. Over a short treatment period daglutril did lead to reductions in blood pressure, but did not significantly affect UAE in comparison to placebo [362]. The combined NEP and angiotensin inhibitor sacubitril/valsartan has recently entered routine clinical practice in management of patients with heart failure. Studies focusing on renoprotective utility of this agent have been conflicting and the overall effect of NEP inhibition on renal haemodynamics is unclear. Its short term effects on eGFR, blood pressure and UAE in patients with CKD is currently being assessed in a multicentre study of 400 participants, at least 20% of whom have DN, and results are anticipated in 2017 [363].

1.7.5.4 Pentoxifylline

As described earlier, inflammation appears to be another key mechanism in the initiation and progression of DN, and inflammatory biomarkers such as the STNFRs have shown promise as tools for early disease detection. Pentoxifylline is a nonspecific phosphodiesterase inhibitor which is often used clinically in the treatment of peripheral vascular disease. The anti-inflammatory and anti-proliferative actions of the drug have led to beneficial effects in animal models of progressive renal disease and beneficial effects on protein excretion in diabetic nephropathy have been noted [364]. In a small, open-label randomised trial, administration of pentoxifylline in addition to RAAS blocking agents led to significant reductions in UAE and reduced decline in GFR in patients with T2DM and DN over 2 years of treatment [365]. More definitive results from larger scale, multicentre studies are required to confirm this promising finding.

1.7.5.5 Uric acid lowering

Longitudinal studies have shown that baseline serum uric acid levels are associated with later development of albuminuria or GFR decline in patients with diabetes [366] [367], lending support to the hypothesis that uric acid lowering could potentially be a useful strategy in delaying the progression of DN. In small prospective studies including patients with CKD both with and without hyperuricaemia at baseline, allopurinol treatment has been shown to slow disease progression and reduce the incidence of cardiovascular events over a 1 to 2 year follow up period [368, 369]. This strategy will be evaluated for prevention of DN in patients with T1DM in a prospective multi-centre double blind placebo-controlled trial designed by the Preventing Early Renal Function Loss in Diabetes (PERL) consortium [370].

1.8 Prevention of diabetic nephropathy

MA is predictive of development of progression to overt DN and ESRD, and highlights risk of cardiovascular morbidity and mortality. This linear association is evident at levels of UAE well within what is traditionally accepted as “normal” range. Not only are RAAS blocking medications key tools for blood pressure reduction, they have also been shown to have effects on UAE independent of blood pressure lowering. Use of these agents in established DN is almost universal practice, however whether the benefits of treatment extend into the earlier, pre-clinical stages of disease in patients with lower cardiovascular or renal risk burden remains unclear. A number of studies have tested the hypothesis that early initiation of RAAS blockade is a potential approach for prevention of MA, and thereby indirectly for prevention of the excess associated renal and cardiovascular risk.

1.8.1 ACE-Inhibitors

The European Controlled Trial of Lisinopril in Insulin-Dependent Diabetes (EUCLID) first demonstrated a large reduction in risk of development of MA with ACE-I in normoalbuminuric patients with T1DM, although this did not reach statistical significance [371]. Subsequently the Heart Outcomes Prevention Evaluation (HOPE) study assessed the impact of ACE-I on later cardiovascular events in patients at cardiovascular risk and included a subgroup of more than 3000 patients with diabetes, resulting in a large sub-study focussing on MA and cardio-renal outcomes in this population. While treatment with ACE-I significantly reduced the risk of cardiovascular and renal outcomes a 9% reduction in relative risk of developing MA was also noted in patients who were normoalbuminuric at study inclusion, although the study was underpowered to evaluate differences in particular subgroups [372]. The Bergamo Nephrologic Diabetic Complications Trial (BENEDICT) was later designed to determine whether ACE-I and calcium channel blocker (CCB) could prevent onset of MA. The investigators randomly assigned 1200 normoalbuminuric T2DM patients with hypertension to receive either ACE-I; CCB; both in combination; or placebo over a 3 year treatment period. Results showed that intervention with combined ACE-I and CCB, as well as ACE-I alone, led to a 44% reduction in the incidence of MA [373]. The lack of any enhancing effect of additional CCB suggested that this relationship was indeed independent of degree of blood pressure reduction achieved, although patients with higher baseline blood pressure seemed to benefit more from treatment [374]. More recently, the ADVANCE investigators observed a 21% reduction in incidence of MA in a cohort of patients with T2DM and additional cardiovascular risk factors treated with ACE-I in conjunction with tight glycaemic targets [316]. Once again it should be noted that baseline blood pressure in the ADVANCE trial was above optimal levels. It appears, therefore, that ACE-I can blunt or prevent early increases in UAE in patients whose blood pressure is suboptimal.

1.8.2 Angiotensin receptor blockers

Primarily designed to evaluate the use of the ARB candesartan for prevention of retinopathy events in normoalbuminuric, normotensive patients with diabetes, DIRECT study included incidence of MA as a prespecified endpoint. In the renal outcomes analysis, treatment with ARB did not affect the incidence of MA over a follow up period of 4.7 years. It was postulated that these findings could be explained by the relatively young age and low vascular disease burden of the study cohort, meaning that a significantly longer duration of follow-up would be required to establish if early initiation of treatment would be clinically beneficial [375]. The Randomised Olmesartan and Diabetes Microalbuminuria Prevention (ROADMAP) trial evaluated the impact of early initiation of the ARB olmesartan in a large cohort of normoalbuminuric T2DM patients, treated to a tighter blood pressure target of less than 130/80mmHg. Over a mean follow up period of 3.2 years treatment with olmesartan was associated with 23% increase in time to onset of MA in comparison to placebo, an effect which retained significance following adjustment for blood pressure. As seen in the BENEDICT and ADVANCE cohorts, those with higher baseline blood pressure tended to have a greater benefit, possibly a reflection of greater RAAS activity translating into increased treatment effect [376]. However, the apparently beneficial renal effects seen in ROADMAP were overshadowed by a 5-fold (statistically significant) increase in incidence of fatal cardiovascular events in patients on active treatment.

1.8.3 Summary

The majority of the trials described above were included in a 2012 meta-analysis on the effects of ACE-I or ARB therapy on renal outcomes in diabetes, with studies comparing ACE-I or ARB monotherapy to other active antihypertensive drugs or placebo included in the final analysis. Results showed that while these agents were efficacious in reducing the risk of established clinical endpoints such as doubling of serum creatinine; progression to macroalbuminuria; and reducing risk of ESRD in the context of no significant difference in blood pressure

reduction against comparator drugs, there was no clear beneficial effect on development of MA itself [377]. Included studies were certainly heterogeneous at baseline with differences in eGFR; UAE; and baseline blood pressure which may go some way towards accounting for this finding. The data are conflicting however, as a recent systematic review found a 16% reduction in relative risk of developing MA with preventative ACE-I or ARB therapy [378]. Nonetheless, current clinical guidelines do not support the use of early "preventative" therapy in normoalbuminuric, normotensive patients with diabetes. A number of issues remain to be addressed however.

- The first point to consider is that these studies of "early intervention" were carried out in unselected populations. This is important as it is now widely accepted that rates of progression to overt DN are far lower than described in the early literature. It would seem therefore that universal prescribing of these agents has the potential to create a significant burden of adverse effects in many patients who do not derive any clear clinical benefit. Use of predictive biomarkers to identify those who are at highest risk of progressive disease to target individualised preventative therapy may lead to significant improvements in outcomes.
- Second, although RAAS blockade with ACE-I and ARB has revolutionised management of diabetes and its complications and these agents are almost universally prescribed to patients with hypertension or MA, many patients continue to progress to ESRD. In view of progress in understanding of the complex pathogenesis of DN, it is feasible that alternative therapeutic strategies could also be of use in this regard and time will tell whether the novel therapies currently under evaluation will be the next "game changer" in DN prevention.
- Finally in order to translate findings into clinical practice the focus is almost universally on established clinical endpoints such as progression to ESRD; doubling of creatinine; or death. These events may occur with some regularity in trials involving patients with CKD, but in order to build similar meaningful data in lower risk patients who are at an early stage in the DN pathway very large sample sizes with far longer follow periods would be needed. There is therefore an argument for the use of surrogate markers in such "low risk" groups. UAE is one such surrogate,

although its utility is debated. It could be argued that the strong association with renal and cardiovascular outcomes as well as the proposed clinical impact of reduction in UAE make this an ideal surrogate endpoint for clinical trials. On the other hand, UAE is highly variable and is not specific to DN; moreover not all treatments that lower UAE reduce the risk of ESRD. Perhaps alternative biomarkers are needed not only for accurate risk stratification of patients, but also to inform recruitment into clinical trials, meaning that smaller study populations and shorter term trials could be used to determine efficacy of treatments. Whether this strategy would provide adequate information regarding patient safety is unclear.

1.9 The PRIORITY trial

Although an innumerable number of biomarkers have been described in the literature for early diagnosis of DN, as detailed earlier in this chapter, none have yet been shown to outperform MA. In order to make the transition from research tool into clinical practice such novel markers must be rigorously evaluated using a number of key steps [379] including:

- validation in prospective studies;
- proof of incremental predictive value beyond the currently established standard biomarkers;
- utility in informing clinical decision-making;
- demonstration of potential for improving clinical outcomes through evaluation in a randomised clinical trial.

To date, the CKD273 biomarker has been proven to identify DN with a high degree of sensitivity and specificity, as well as to predict renal endpoints including progression to ESRD. The next logical step is to assess whether it can outperform MA as a predictor of DN and consequently inform therapeutic decision-making, for example targeting of early intensified RAAS blockade towards higher risk normoalbuminuric patients with diabetes. The Proteomic

Prediction and Renin Angiotensin Aldosterone System Inhibition Prevention of Early Diabetic Nephropathy in Type 2 Diabetic Patients with Normoalbuminuria (PRIORITY) trial was specifically designed to address these key questions [380].

PRIORITY is a multicentre study funded by a European Union Seventh Framework Programme Grant. Part-observational study and part-biomarker directed randomised controlled trial, PRIORITY aims to determine whether the CKD273 urinary proteomic classifier can accurately predict development of MA in normoalbuminuric diabetic individuals as well as to evaluate whether early enhanced RAAS blockade with addition of MRA can reduce risk of progression to MA in “high risk” individuals. Much of the work contained within this thesis was generated in parallel to the conduct of the PRIORITY trial, which will be described in greater detail in chapter 2.

1.10 Aims of this thesis

This thesis will focus primarily on the CKD273 urinary proteomic biomarker and its utility as an early indicator of DN. The complementary studies described will take the biomarker from validation to prospective evaluation in a clinical trial and are based on the following hypotheses:

- CKD273 has potential as a tool for early, preclinical detection of DN in normoalbuminuric patients with type 2 diabetes.
- CKD273 is a specific biomarker of early renal disease and not simply related to underlying vascular disease.
- CKD273 may be useful to identify patients with elevated UAE who are at risk of progressive GFR decline.
- Spironolactone is a safe and effective treatment option for slowing progression of CKD.

Specific aims of this thesis are therefore:

- To validate the CKD273 urinary proteomic panel for diagnosis of DN in a local test-cohort;
- To explore clinical utility beyond the current guideline-endorsed standard tools for DN diagnosis, in particular to demonstrate the specificity of CKD273 as a renal biomarker;
- To determine whether CKD273 is a useful indicator of risk of progressive disease in patients with evidence of early-stage established kidney disease;
- To evaluate the safety and effectiveness of MRAs as a therapeutic strategy in patients with established CKD, thereby demonstrating the availability of a potentially suitable treatment option for early stage “high risk” patients;
- The above concepts will then be used to inform the development of a biomarker-guided prospective clinical trial as the ultimate test of the clinical potential of a novel biomarker.

2. Methods

2.1 Summary

The work contained within this thesis was conducted in parallel with the multicentre PRIORITY trial. Several complementary projects designed and implemented alongside the main clinical trial are summarised below.

- To determine the safety and effectiveness of enhanced RAAS blockade with MRAs in patients with CKD, a meta-analysis of randomised controlled trials of addition of MRA to ACE-I or ARB was performed focussing on effects on blood pressure; GFR; proteinuria and potassium (chapter 3).
- In advance of the commencement of the main trial a pilot study was carried out in order to refine study procedures and inform the setup of both the PRIORITY trial and additional sub-studies locally (chapter 4).
- In order to explore the relationship between the CKD273 classifier and subclinical vascular and renal disease in more detail a number of additional vascular phenotyping and biomarker sub-studies were implemented (chapter 6) in the local PRIORITY study cohort (chapter 5).
- To assess the utility of the CKD273 proteomic panel for prediction of renal and cardiovascular endpoints in patients with more advanced kidney disease, proteomic analysis was performed using stored baseline urine samples from a cohort of microalbuminuric T2DM patients with 6 years of follow up data available (chapter 7).

This chapter will focus primarily on the design and methodology of the PRIORITY trial. Specific clinical and laboratory techniques employed in the complementary projects summarised above will be described in more detail in the relevant results chapters.

2.2 The PRIORITY trial

This multicentre randomised controlled trial and observational study was designed to address two key objectives:

1) To confirm that urinary proteomics can predict development of MA (as a surrogate marker for the development of overt DN) in a cohort of 2000 normoalbuminuric T2DM patients.
2) To investigate whether early initiation of preventive therapy with spironolactone 25mg once daily reduces risk of transition to MA in those patients deemed by urinary proteomic analysis to be at high risk of progression to DN.

The chief investigator of the PRIORITY trial is Professor Peter Rossing, lead clinician at the Steno Diabetes Research Centre in Gentofte, Denmark. The protocol was developed in discussion with all study partners as well as an external advisory board and safety aspects are monitored by an independent data monitoring committee.

2.2.1 Funding and ethical approval

The PRIORITY trial was funded by a European Commission Research and Innovation 7th Framework Programme Grant (PRIORITY; 279277) and was registered with the European Union Drug Regulating Authorities Clinical Trials (EudraCT) database (ref 2012-000452-34). The study was approved by the Medicines and Healthcare Regulatory Authority (ref 42739/0001/001-0001) on 8th October 2013. Local approval was obtained from the West of Scotland Research Ethics Committee 1 (ref 13/WS/0284) on 13th December 2013, and from NHS Greater Glasgow and Clyde Research and Development (R&D) (ref GN12DI096) on 28th February 2014. Invitation letters, participant information sheet, consent form, alert card and information letter to the GP can be found in Appendices 1 to 5.

2.2.2 Study population and selection of participants

Investigators at 17 European centres aim to recruit a total of 2000 T2DM patients between February 2014 and October 2016, with Glasgow proposing to contribute 500 participants. Individuals with T2DM aged between 18 and 75 years with eGFR ≥ 45ml/min/1.73m^2 and normoalbuminuria defined as UACR < 30mg/g in two of three consecutive morning urine samples were deemed eligible for inclusion dependent on the criteria shown in Tables 2-1 and 2-2.

1. Written informed consent

2. Age ≥18 years and <75 years

3. Diagnosed with T2DM according to WHO criteria

4. Normoalbuminuria (UACR <30 mg/g in at least 2 of 3 samples from 'run in' period)

5. eGFR >45 mL/min/1.73 m^2 at screening visit

6. Willing and able to comply with the protocol for the duration of the study

7. Female without childbearing potential at the screening visit. Defined as one or more of following:

- **7.1** Female patients ≥50 years of age on the day of inclusion, who have been postmenopausal for at least 1 year;
- **7.2** Female patients <50 years of age at the day of inclusion, who have been postmenopausal for at least 1 year and serum FSH levels >40 mIU/mL as well as serum oestrogen levels <30 pg/mL or a negative oestrogen test;
- **7.3** minimum 6 weeks post-surgical sterilisation OR a negative urine pregnancy test at the screening visit AND one or more of following:
  - **7.3.1** Correct use of reliable contraception methods
  - **7.3.2** General sexual abstinence from the time of screening/during the study until a minimum of 30 days after the last administration of study medication if this is already established as the patient's preferred and usual lifestyle
  - **7.3.3** Having only female sexual partners
  - **7.3.4** Sexual relationship with sterile male partners only

Table 2-1. PRIORITY study inclusion criteria.

eGFR, estimated glomerular filtration rate; FSH, follicle stimulating hormone; HbA1c, glycated haemoglobin; T2DM, type 2 diabetes mellitus; UACR, urine albumin to creatinine ratio; WHO, world health organisation.

1. Systolic BP <110 or >160 mmHg at baseline
2. Diastolic BP >100 mmHg at baseline
3. T1DM according to WHO criteria
4. HbA1c <6.5% (48 mmol/mol) AND T2DM of > 5 years duration AND never treated with an antidiabetic drug of any kind
5. Treatment with more than one RAAS blocking agent (ACE-I, ARB or direct renin inhibitor)
6. Lithium treatment
7. Known or suspected hypersensitivity to spironolactone or to any of its excipients
8. Use of potassium sparing diuretics, such as: spironolactone, eplerenone or amiloride
9. Serum potassium level >5.4mmol/L at screening visit
10. Hyponatraemia determine by the investigator
11. Current cancer treatment or within 5 years from baseline (except basal cell skin cancer or
squamous cell skin cancer)
12. Any clinically significant disorder, except for conditions associated with T2DM which in the investigators opinion could interfere with the results of the trial
13. Cardiac disease defined as: heart failure (NYHA class III–IV) and/or diagnosis of unstable angina pectoris and/or MI, stroke, PTCA or CABG within the last 3 months
14. Non-diabetic CKD
15. Liver cirrhosis and abnormal liver function tests within the last 3 years
16. Addison's disease
17. Breastfeeding
18. Intend to become pregnant or not use adequate birth control within the study period
19. Known or suspected abuse of alcohol or narcotics
20. Inability to understand the informed consent form
21. Participation in any other intervention trial within 30 days of inclusion in PRIORITY

Table 2-2. PRIORITY study exclusion criteria.

ACE-I, ACE-inhibitors; ARB, angiotensin II receptor blockers; BP, blood pressure; CABG, coronary artery bypass grafting; CKD, chronic kidney disease;; eGFR, estimated glomerular filtration rate; HbA1c, glycated haemoglobin; NYHA, New York Heart Association; PRIORITY, proteomic prediction and renin angiotensin aldosterone system inhibition prevention of early DN in type 2 diabetic patients with normoalbuminuria; PTCA, percutaneous transluminal coronary angioplasty; RAAS, renin-angiotensin-aldosterone system; T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; UACR, urine albumin to creatinine ratio; WHO, world health organisation.

Patients attending for screening who did not meet the pre-specified inclusion and exclusion criteria did not progress further in the trial. Urinary proteomic testing was not performed for these patients. In cases for whom it was deemed appropriate (i.e. blood pressure marginally out with target for inclusion or potassium level considered to be spurious), re-screening of participants at least one week after the initial screening assessment was permitted.

2.2.3 Local recruitment strategies

A variety of strategies were developed in order to identify potential Glasgow participants. These are briefly summarised below.

2.2.3.1 Secondary care clinics

With the approval of the West of Scotland Research Ethics Committee and local consultants in diabetes and endocrinology, members of the study team screened T2DM clinic lists within the Greater Glasgow area using the TrakCare and Clinical Portal systems. This was done 4 weeks in advance of the clinic date. Any patient deemed potentially suitable to participate was posted an information letter including study team contact details and an individual reply slip to allow them to express an interest in participating (Appendix 1). Following receipt of reply slips or contact by other means, members of the study team made telephone contact with interested patients to discuss the study in more detail. Participant information sheets (PIS; Appendix 2) were then dispatched either by post or email and appointments were made for preliminary screening visits.

2.2.3.2 Scottish diabetes research network (SDRN)

The SDRN was commissioned by the Chief Scientist Office (CSO) in 2006 as a means to facilitate generation of high quality diabetes research in Scotland. The

network aims to enhance patient participation in research and has developed a national register of individuals who have given prior consent to being contacted for research studies.

With ethical approval in place an application was submitted to the SDRN to facilitate a registry search for individuals within the Glasgow area whose clinical characteristics met basic study inclusion and exclusion criteria. The resultant list included patient name; preferred contact method; address; date of birth; eGFR; diabetes medication; and cardiovascular disease status. The registry does not hold UACR information and the list was therefore re-screened by the author using the local Clinical Portal system to determine whether or not patients were normoalbuminuric. After this process, individuals who were deemed suitable for inclusion were sent an invitation letter and if interested in participating were able to contact the study team directly by telephone or email, or by return of an included reply slip (Appendix 1). Subsequent to this initial contact a PIS was dispatched and appointment made for screening visit (Appendix 2).

2.2.3.3 Retinal screening service

The Greater Glasgow and Clyde retinal screening service provides annual retinal photography for all individuals with diabetes aged 12 and over in the area. Patients can attend a number of fixed screening sites within Glasgow and a mobile service is also available to cover outlying areas. With the approval of the service manager invitation letters (Appendix 1) were displayed at retinal screening centres within Glasgow. These documents contained study team contact details as well as a reply slip, allowing interested individuals to contact the study team for further information. Following this initial contact and if deemed suitable for inclusion a PIS was dispatched and appointment made for screening (Appendix 2).

2.2.3.4 Advertising in local media

In order to reach T2DM patients not routinely attending review clinics or retinal screening services, ethical approval was granted to place information notices in news publications. On two separate occasions during the course of the study a 1/8 page advertisement was placed in the West of Scotland edition of the Daily Record newspaper. This conveyed some basic information about the study, as well as contact details and a study specific email address allowing interested individuals to contact the study team directly for further information. Following initial contact, a PIS was sent by post or email and screening appointment arranged.

2.2.3.5 Recruitment from primary care

This strategy began with recruitment of patients from GP practices affiliated with the NHS Greater Glasgow and Clyde Clinical Research Facility. This scheme is a formal arrangement between a selection of GP practices within the area and NHS R&D through which a number of mechanisms are approved for identification of potential recruits into clinical studies. With the agreement of practice management or the principal GP study nurses were able to search practice databases for individuals who met the study inclusion and exclusion criteria. A list of individuals who could be approached was then created and reviewed by practice medical staff to ensure that no inappropriate invitations were made. Invitation letters were then produced, signed by the principal GP and sent to individuals eligible to participate. As with other strategies, these letters included contact details for the study team and a tear-off reply slip allowing interested individuals to make direct contact (Appendix 1). Those wishing to participate were then sent a study PIS and appointments made for screening (Appendix 2).

Around 100 local practices are formally affiliated with the University of Glasgow. However, a number of the highest recruiting centres were already engaged in identifying participants for another large diabetes study with very similar

inclusion and exclusion criteria. This left a more limited pool of affiliated practices to approach. For this reason ethical approval was sought to extend invitations to GP practices on a city-wide basis, irrespective of existing affiliation with the University. The mechanisms for screening of practice lists and invitation of potential participants was as detailed above.

2.2.3.6 Pilot study participants

As detailed in section 2.1, a local pilot study was developed and conducted in advance of the main trial and this will be described in more detail in chapter 4. Any individuals who had taken part in the pilot project and were eligible for the PRIORITY trial were sent an invitation letter by the study team, containing contact details and a reply slip to indicate willingness to participate (Appendix 1). Those who expressed an interest were sent a PIS and an appointment was confirmed for screening (Appendix 2).

2.2.4 Study design

The key aspects of the design of the PRIORITY trial are illustrated in Figure 2-1. In summary, this multicentre project is being conducted at 17 institutions throughout Europe. Part-observational study and part-randomised controlled trial, the objective is to risk stratify 2000 normoalbuminuric T2DM patients for later development of DN using the CKD273 urinary proteomic classifier. Those identified as “high-risk” are randomised in a double-blind manner to receive either spironolactone 25mg or placebo, while “low-risk” individuals enter an observational study. The visit schedule is summarised in Figure 2-2 and described below. Recruitment opened in February 2014 and closed in September 2016, and the follow up period will end in September 2018.

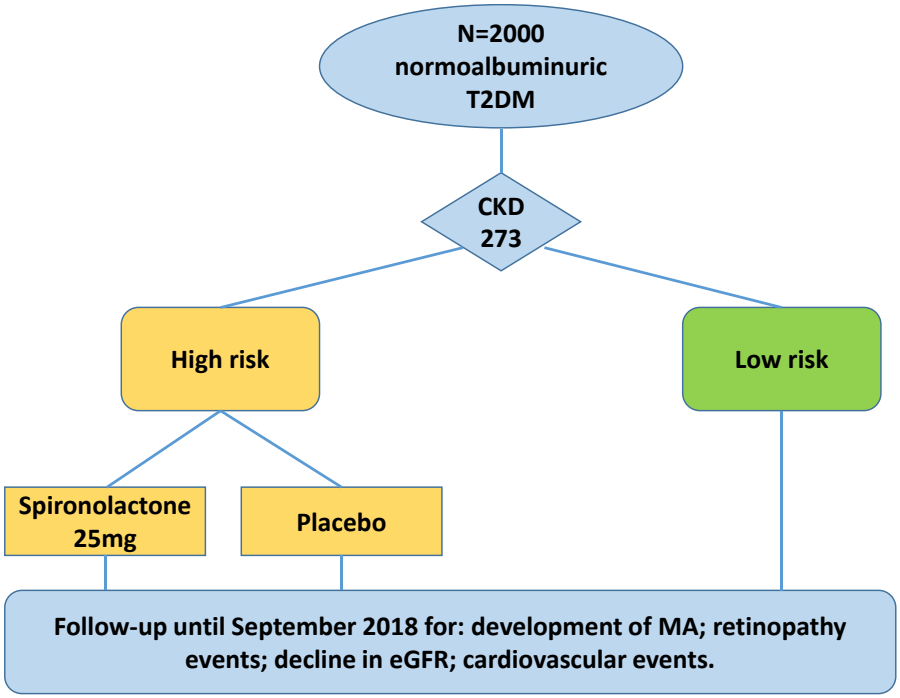

Figure 2-1. PRIORITY study design.

T2DM, type 2 diabetes; MA, microalbuminuria; eGFR, estimated glomerular filtration rate.

2.2.4.1 Screening visit

The first study visit for all participants was the screening visit. At this stage the PIS was discussed, any questions about the trial were answered and an informed consent form was signed (Appendix 3). Concurrent medications were reviewed briefly, primarily to determine whether the patient was already being treated with an ACE-I or ARB. Height, weight, blood pressure (average of 3 measurements taken after 10 minutes rest in sitting position) and heart rate were documented. A spot sample of urine was obtained, dipstick analysis performed (and urine pregnancy testing in female participants with childbearing potential) and stored at -80°C for later proteomic analysis. Stability testing has confirmed that classifier score is not significantly affected by timing of sample collection, handling or storage methods, nor by subject activity level [381], therefore no specific advice regarding activity levels, dietary or medication changes was given prior to study visits. In addition blood samples were taken for

measurement of electrolytes and HbA1c. Patients were not given any specific advice about avoiding heavy exercise before the study visit.

Participants were subsequently provided with the materials necessary to obtain 3 consecutive first morning urine samples at home and post these back to the study team for measurement of UACR, as well as with PIS specific to the local sub-studies which will be discussed in more detail later.

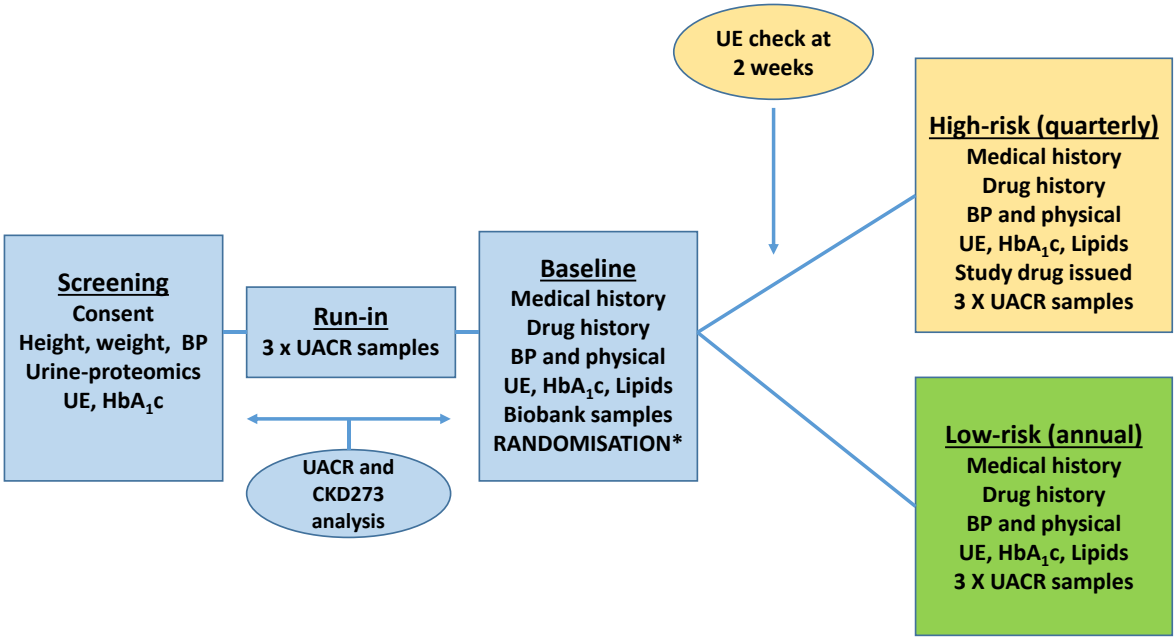

Figure 2-2. PRIORITY study visit schedule.

BP, blood pressure; UE, urea and electrolytes; HbA1c, glycated haemoglobin; UACR, urine albumin: creatinine ratio. *Participants in high-risk group only.

2.2.4.2 Run-in period

In the 8-12 week interval between screening and baseline visits, study participants collected 3 consecutive first morning void samples for measurement of UACR and returned these to the study team using pre-paid postage materials provided at screening. Received samples were stored at -80°C and shipped to the Steno Diabetes Research Centre in Gentofte, Denmark on a 3-weekly basis for measurement of UACR. From there, samples from participants who were confirmed to be normoalbuminuric were shipped to Mosaiques Diagnostics in Hanover, Germany for urinary proteomic analysis. Results were received by the study team in advance of participants' baseline visits. Individuals entering the high-risk group based on urinary proteomics were contacted by the study team at least 1 week prior to the baseline visit in order to inform them of the result.

2.2.4.3 Baseline visit

The baseline visit was scheduled for 8-12 weeks from screening date. On arrival, results of screening investigations were discussed with participants and study personnel confirmed that they are happy to continue in the trial. The PIS for the local sub-studies was reviewed and informed consent obtained if participants were interested in this aspect of the study (appendices 10 and 11). Medical history, tobacco and alcohol use and a full list of concomitant medications including drug; dose; frequency; start and stop dates; and indication for treatment was then documented. Blood pressure was measured and a full physical examination performed by the study doctor. Investigation of any abnormalities not previously documented was arranged by the study team at the discretion of the principal investigator. Blood and urine samples were taken for local measurement of electrolytes, eGFR, HbA1c and lipid profile, as well as for local biomarker sub-studies which will be described later, and for storage in the PRIORITY study biobank at the Steno Diabetes Research Centre. Participants in the high-risk group were randomised and provided with study medication as well as a participant alert card (Appendix 4) and arrangements made for a 2-week safety visit. Following these key study procedures, patients who consented to participate in the local sub-studies then underwent an assessment of vascular function which will be described in more detail later. After the baseline visit an information letter was dispatched to the GP of each participant detailing which arm of the study they had been allocated to (Appendix 5).

2.2.4.4 High risk group – safety visit

Two weeks from randomisation, high-risk participants were asked to return for a brief safety visit. Study personnel enquired about any adverse events since the baseline visit, blood pressure was re-checked and a blood sample was taken for measurement of electrolytes and eGFR.

2.2.4.5 High risk group – quarterly study visits

Participants in the high-risk group returned for 3-monthly review by the study team from inclusion until the end of the study. During these visits medical history was reviewed with particular reference to any changes in medication or adverse events in the preceding 3 months. Blood pressure was recorded and a symptom-directed physical examination performed. Blood samples were taken for local measurement of electrolytes, eGFR and HbA1c, (lipid profile is checked on an annual basis) and participants were asked to return 3 consecutive first morning void urine samples for measurement of UACR. Study medication was returned, compliance determined (defined as taking 80-110% of allocated treatment within a 3 month period) and a new supply issued.

2.2.4.6 Low-risk group – annual visits

Participants in the low-risk group were invited to return for review on an annual basis until the end of the trial. Medical history was recorded with particular reference to cardiovascular events and retinopathy status, and concomitant medications reviewed. Blood pressure was recorded and a symptom-directed physical examination was performed. Blood samples were taken for local measurement of electrolytes, eGFR, HbA1c and lipid profile and participants were asked to return 3 consecutive first morning void urine samples for measurement of UACR.

2.2.4.7 End of study

At the final visit, study procedures will be performed as described in the annual visits section above. In addition blood samples will be taken for storage in the PRIORITY study biobank at the Steno Diabetes Research Centre. After the end of the trial, participants will continue to receive standard diabetes care in either a primary or secondary care clinic according to local guidelines.

2.2.4.8 Endpoints

Development of confirmed MA (UACR>30mg/g) in at least 2 out of 3 consecutive first morning void samples with at least a 30% increase from study inclusion, or UACR more than 40mg/g is the primary endpoint in the PRIORITY trial. Additional secondary endpoints that will be evaluated both in the whole study population and in the intervention group are listed below.

- Comparison of a composite of fatal and non-fatal cardiovascular events (myocardial infarction, coronary artery bypass graft, percutaneous coronary intervention, stroke, hospitalisation for heart failure) and all-cause mortality.
- Comparison of incidence of retinopathy and laser-treatment.
- Changes in geometric mean UACR throughout the study, assessed both as slope of UACR and absolute change from study inclusion.
- Development of macroalbuminuria (UACR >300mg/g) in at least 2 out of 3 consecutive first morning void urine samples.
- Development of eGFR < 60ml/min/1.73m^2 in participants whose eGFR was >60ml/min/1.73m^2 at inclusion.
- Changes in eGFR throughout the study period, assessed both as slope of eGFR and absolute change from study inclusion.

2.2.5 Statistical considerations

Statistical considerations relevant to the main PRIORITY trial are discussed below. Statistical methods used in the complementary projects are detailed in chapters 3, 4, 5, 6 and 7.

2.2.5.1 Sample size

The expected proportions of T2DM patients developing MA within the study period were: 24% of patients in the high-risk group receiving active study medication; 40% in the high-risk group receiving placebo; and 8.5% in the low-risk group. Using the sample size formula for two proportions test (α= 0.05 ß=0.80) randomized 1:1, 129 participants were required in each arm of the intervention group. To account for a 10% expected drop-out rate, it was planned to randomise 300 high-risk patients. High-risk patients were expected to comprise 15% of included patients. In order to identify 300 high-risk patients, 2000 patients were be included in total. To account for an estimated screening failure rate of 25% a total of 2700 individuals were screened.

2.2.5.2 Proteomic classification

The study population are risk-stratified based on CKD273 classifier score at inclusion. The threshold for classification of high-risk individuals was determined to be 0.154. This figure was determined from previously published data [268, 295].

2.2.6 Randomisation and unblinding

2.2.6.1 Randomisation

The interactive web response system (IWRS) for randomisation and allocation of study medication was developed by the Robertson Centre for Biostatistics, University of Glasgow. All participants with a high-risk urinary proteomic score at screening who were eligible to continue in the trial were computer-randomised in a 1:1 manner to either the intervention or placebo arms of the trial using a block randomisation process. Randomisation was stratified according to whether the participant was already prescribed ACE-I or ARB in

order to ensure that the number of patients on these agents was balanced between the active treatment and placebo groups.

2.2.6.2 Blinding and unblinding procedure

Investigators and study participants are blinded to the type of intervention allocated. Placebo and active drug are indistinguishable from each other in terms of appearance, pack labelling and instructions for use. All study medication packs are labelled with a unique pack identification number. At the time of randomisation each high risk participant was allocated a pack number by the IWRS. Allocation of subsequent pack numbers is triggered by an electronic request from study personnel in advance of quarterly study visits.

In the event of a serious medical occurrence that could be linked to study medication when it is essential to confirm whether the affected patient is treated with spironolactone, emergency unblinding can be performed. This is carried out via a telephone response system and the contact number is printed on participant alert cards. After emergency unblinding an email alert is generated to the study sponsor, study management and the Robertson Centre for Biostatistics. No more than 3 unblinding requests are permitted within 24 hours to avoid malicious use of the system. Planned unblinding will be performed by staff at the Robertson Centre for Biostatistics at the end of the trial after the final analysis plan is determined and a date agreed by the scientific steering committee.

2.2.7 Adverse events

The study protocol defines adverse events (AEs) as “any untoward medical occurrence in a trial participant after baseline”. It is not necessary to demonstrate a causal relationship to study medication. These are considered serious adverse events (SAEs) in the event that they result in death; are life-

threatening; require hospitalisation; result in persistent incapacity; or are congenital defects.

AE information is collected at study visits, with particular reference to the following:

- type of event
- start and end dates of event
- grading according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) [382]
- causality of study drug
- treatment measures taken
- outcome

These incidences are documented in the individual patient record as well as on the study electronic case report form (eCRF). The same evaluation process is required for SAEs with the caveat that these must also be documented on paper SAE reporting forms and faxed or emailed to the study sponsor within 24 hours of study staff becoming aware of the event. For the PRIORITY trial, SAE monitoring is performed by the Institute of Clinical Pharmacology at Hanover medical school with authorisation of the sponsor.

2.2.7.1 Hyperkalaemia

Hyperkalaemia is often a concern in trials evaluating intensified RAAS blockade. Participants have potassium levels checked at every study visit, and inclusion in the trial is not permitted if screening serum potassium is greater than 5.4mmol/L. A study-specific management protocol is followed in the event that a participant is found to be hyperkalaemic.

2.2.8 Sample processing

Processing of blood and urine samples required in the context of the PRIORITY trial is described below. Additional biomarker analysis performed as part of the local sub-studies will be described within the relevant results chapters.

2.2.8.1 Albuminuria measurement

As detailed above, study participants are asked to collect 3 consecutive first morning void samples for measurement of UACR during the run-in period then at each routine study visit thereafter throughout their inclusion in the trial. These are collected at home and then returned to the study team using pre-paid postage kits provided at screening. Upon receipt a dipstick test is performed on the most recent sample. If there is evidence of urinary tract infection the samples are discarded and a repeat collection is arranged following appropriate treatment. Samples are then stored at -80°C and shipped on dry ice to the Steno Diabetes Centre for analysis on a 3-weekly basis.

Following receipt at the central laboratory samples are thawed and centrifuged at 1680g for 10 minutes before measurement of urine albumin and creatinine (Vitros 5600 immunoanalyser, Ortho Clinical Diagnostics, France). The geometric mean UACR from all 3 samples as well as each individual UACR result is then entered onto the eCRF to be accessed by the local study team with any screening failures highlighted.

2.2.8.2 Urinary proteomic sample preparation

As detailed above, participants are asked to provide a spot urine sample at the screening visit. A dipstick test is performed and if there is evidence of infection the sample is discarded and a repeat obtained following appropriate treatment. Samples are then stored at -80°C and shipped on dry ice to the central laboratory on a 3-weekly basis. Once normoalbuminuria is confirmed on the

corresponding run-in specimens samples for proteomic processing are shipped to Mosaiques Diagnostics in Hanover, Germany for analysis.

0.7µL of urine is required for CE-MS processing. Shortly before analysis the samples are thawed and diluted with 0.7ml of 2mol/L urea and 10mmol/L NH_4OH containing 0.02% sodium dodecyl sulphate. In order to remove proteins with molecular weight greater than 20kDa samples are spun at 3000g using a Centrisart ultracentrifugation device (Sartorius, Göttingen, Germany) until 1.1ml of filtrate has been collected. The filtrate is then desalinised using a PD-10 column (GE Healthcare Biosciences, Stockholm, Sweden) which is pre-equilibrated using 0.01% NH_4OH in high-performance liquid chromatography-grade water (Figure 2-3). Samples are then lipophilised using a freeze-drier (Speed-Vac RVC 2-18/Alpha 1-2, Christ, Osterode a.H., Germany), suspended in high-performance liquid chromatography-grade water and stored at 4°C until CE-MS analysis.

Figure 2-3. Desalinisation using PD-10 column.

2.2.8.3 CE-MS sample processing

CE-MS analysis is performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, USA) coupled to a micro-time of flight (micro-TOF) -mass spectrometer (Bruker Daltonic, Germany) as shown in Figure 2-4. The initial step involves passage of the sample through a buffer filled capillary, with resultant separation of peptides based on migration time. To do this silica capillaries are first rinsed with running buffer containing 30% methanol and 0.5% formic acid for 3 minutes. Approximately 700nl of sample is then injected into the capillary and a charge of +30kV applied at the inlet for separation of peptides. The length of the capillary is maintained at a temperature of 35°C during processing. Prior to introduction of the next sample the capillary is rinsed with 0.1M NaOH, followed by water and finally running buffer.

Figure 2-4. Capillary electrophoresis – mass spectrometry.

Capillary electrophoresis (left) coupled to micro-TOF mass spectrometer (centre) illustrating electrospray interface (right).

The capillary electrophoresis system is coupled to a micro-TOF-mass spectrometer (Bruker Daltronic, Germany.) The sample is introduced into the MS system as a nano-ion spray; the electro-ionisation interface shown in Figure 2-4 (Agilent technologies, Palo-Alto, Ca, USA) is grounded with potential fixed to -4kV. Mass spectra are accumulated every 3 seconds over a range of mass:charge ratios (350-3000) followed by calculation of the mean. Data acquisition is automatic via the capillary electrophoresis program. A vast amount of information is generated in a single CE-MS run as often more than 1000 spectra are produced for each sample. As a result a number of analytical steps are required in order to process this information correctly. The repeatability and stability of this technique has been confirmed between individuals in the same laboratory, as well as between laboratories [381].

2.2.8.4 CE-MS data processing

Data analysis is performed using a bespoke Mosaiques-Visu software tool. The software detects each CE-MS peak and calculates the charge based on isotopic distributions and conjugated masses. The Mosaiques-Visu software then deconvolutes the data, allowing multiple mass spectral peaks generated for each peptide at differentially charged states to be recorded as a single mass using a probabilistic clustering algorithm. Only signals observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio of at least 4 were considered for inclusion. As multiple analytical and patient-specific confounding factors can affect the reliability of these analyses, data are normalised based on the abundance of 29 “housekeeping” peptides which are not affected by patient age, gender or disease states as an internal standard [383]. The result of this processing is a list of mass-spectral peaks characterising each peptide by its molecular mass, signal intensity and CE migration time. Peptides in different samples are considered to be identical if mass deviation is minimal.

2.2.8.5 Urinary peptidome database

All detected features in the urine samples that pass quality control criteria are then deposited in a Microsoft Structured Query Language database to allow further exploration of and comparison with other samples. Features in different samples are considered identical if mass deviation is lower than ±50ppm at 800Da, increasing to ±75ppm for 15kDa features. Features detected in specific clusters are assigned to the respective protein IDs. Those that cannot be related to a specific cluster are attributed a value of 0. A number of features appear sporadically in only a handful of samples, these entities are of questionable significance only those detected in more than 20% of urine samples are further investigated as a "noise-filtering" process.

2.2.8.6 Generation of CKD273 classification factor

In order to allow direct comparison between individuals or patient groups the data generated in a CE-MS run is transformed into a single numerical score, termed a "classification factor". This is calculated in high dimensional parameter using MosaCluster software which employs support vector machine (SVM) based mathematical modelling. The software operates by generating polypeptide models combining peptides which are differentially expressed between affected patients and healthy controls. Each of these peptides represents one dimension in the n-dimensional parameter. Statistical variability of the SVM classification process has been consistently shown to be less than 10%, both under the same operating conditions over a short period of time and when determined by different operators with different devices over a longer time period [268].

2.2.8.7 Routine clinical biochemistry

Clinical biochemical parameters including urea and electrolytes; HbA1c and lipid profile are measured locally within NHS Greater Glasgow and Clyde clinical

laboratories. Following entry of these data, the study eCRF generates each participant's eGFR using the CKD-EPI formula [149].

2.2.9 Data storage

Details of each study visit were stored locally in hard copy case report forms, identifiable only by patient's unique study numbers. These documents were held within a locked filing cabinet and accessed only by members of the immediate study team. In addition, consent forms were scanned onto the local Clinical Portal database, thereby making local clinicians involved in treating these patients aware that they are participants in a clinical trial. Details of study visit were also entered onto the Clinical Portal system by study personnel to ensure that this information is available to participants' GPs online.

As well as being stored locally, information pertaining to each study visit is also manually input into the study-specific eCRF by study personnel who have been trained in operation of the system and have authorised access by use of a unique password and ID. Verification and management of data within the eCRF is overseen by staff at Hannover Clinical Trials Centre, and in addition any entries which appear to be erroneous are highlighted and "queried" electronically by the system. This data is stored in pseudonymised form. The link document through which study numbers can be linked with personal information including participant name and date of birth is stored in paper copy in the local study-specific site file. This file is held in a separate room from the paper CRFs.

3. The safety and effectiveness of mineralocorticoid antagonists in combination with ACE-inhibitors and/or angiotensin receptor blockers in patients with chronic kidney disease: A systematic review and meta-analysis

3.1 Introduction

The global prevalence of CKD is estimated at between 8 and 16% and continues to rise [384]. Irrespective of aetiology CKD is associated with significantly increased risk of cardiovascular death, cardiovascular events and hospitalisation [285, 385]. Although the magnitude of risk is higher in dialysis dependent patients [385] many individuals with earlier stage CKD die from cardiovascular disease before progressing to renal replacement therapy. In fact, the risk of cardiovascular events is increased by 43% even in patients with CKD stages 3a and b [285]. As a result, CKD places a huge burden on healthcare services as well as patients and their families.

To date the most effective treatment strategies to slow disease progression and thereby reduce the incidence of end-stage renal disease are control of blood pressure and proteinuria [144] both of which are predictive of mortality in adults with CKD [315] [386]. The beneficial effects of RAAS blockade with ACE-inhibitors or angiotensin receptor blockers in CKD patients are well established [315, 387-389] and these agents are standard of care for control of blood pressure and proteinuria in both diabetic and non-diabetic CKD [144]. Although these routinely-prescribed first-line medications effectively reduce cardiovascular and renal events as well as blood pressure and urine protein or albumin excretion, many patients still progress to ESRD or die from cardiovascular events. A number of studies have therefore investigated the effectiveness of dual RAAS blockade [320, 322, 390] on progression of renal and cardiovascular disease, and meta-analyses have shown that combined RAAS blockade is more effective than monotherapy in terms of reduction of blood pressure and proteinuria [391-393]. However, in general any positive effects on

blood pressure or albuminuria have been offset by risks of hyperkalaemia, acute kidney injury and symptomatic hypotension and as such this strategy is not recommended in routine clinical practice [144].

Recent years have seen a renewed appreciation of the role of aldosterone as a mediator of cardiovascular and renal disease beyond its effects on fluid and electrolyte homeostasis and thereby blood pressure control. These "non-classical" effects include induction of vascular and glomerular sclerosis; inflammation; and vasoconstriction [113, 394, 395]. RAAS blockade with long-term ACE-I or ARB monotherapy can potentially result in incomplete suppression of circulating aldosterone levels, a phenomenon termed "aldosterone breakthrough". Small studies have shown that "aldosterone breakthrough" is associated with more rapid decline in GFR, progression of albuminuria and worsening symptomatology in chronic cardiac and renal disease [133, 396, 397] perhaps because any residual aldosterone attenuates the cardio- or reno-protective effects of these agents. These insights have stimulated interest in the use of MRAs in combination with ACE-I or ARB in patients with CKD to control proteinuria and delay disease progression.

A number of trials examining the effects of addition of MRA to ACE-I or ARB therapy in patients with CKD of any aetiology have been published within the past decade [323, 325-341, 398-402]. These studies are small and generally of insufficient duration to report impact of treatment on hard clinical endpoints, however the results were first meaningfully combined in a 2009 meta-analysis by the Cochrane Collaboration [392] which was later updated in 2014 [393]. The overall conclusion of these projects was that although addition of MRA significantly reduced proteinuria and blood pressure these beneficial effects were outweighed by increased risk of hyperkalaemia, a factor known to limit prescribing of these agents in patients with CKD [403, 404]. Although the methodology behind these analyses is undoubtedly sound, a number of additional trials have been published since the latest update [338, 340, 341] and several confounding issues are evident. Firstly, a proportion of included studies permitted addition of other antihypertensive agents to MRA in the treatment arm during the course of the trial, thus rendering it impossible to dissociate the effect of MRA from those of alternative agents added. Secondly, these analyses

focussed mainly on published proteinuria data only, consolidating a number of disparate measures. As a result, meaningful meta-analysis of these effects was not feasible. Given that a variety of measures of spot or 24 hour urinary protein or albumin excretion are reported in CKD studies it is possible that not all available data on protein/albumin leak was analysed and a more robust determination of these effects is required. Thirdly, the risk of hyperkalaemia reported is a reflection of the number of participants developing a serum or plasma potassium level above the pre-defined limits in individual trial protocols rather than the true incidence of clinically significant hyperkalaemia requiring treatment, which is potentially of greater interest to the clinicians responsible for the care of patients with CKD.

In view of these factors an updated meta-analysis of trials combining MRA with ACE-I and/or ARB therapy in patients with CKD was performed using unpublished summary data from original authors where possible including from three studies not considered in the most recent Cochrane review [393]. Outcomes of particular interest were impact of MRA on: blood pressure; urinary protein and albumin excretion; renal excretory function; potassium (including incidence of clinically significant hyperkalaemia necessitating medical treatment); as well as the hard clinical endpoints all-cause death; cardiovascular morbidity and mortality; and requirement of renal replacement therapy where these data were available. By excluding studies where MRA was combined with additional antihypertensive agents in the intervention arm, this meta-analysis aim to report as purely as possible the effect of MRA without the confounding impact of additional therapies. This work was carried out by the author together with Dr Alison Taylor, clinical research fellow in nephrology.

3.2 Methods

A literature search was performed by two authors (the author and Dr Alison Taylor) independently using PubMed (1966-1st Dec 2014), EMBASE (1947-1st Dec 2014) and the Cochrane Clinical Trials Database. The full search strategy is shown in (Appendix 6).

3.2.1 Trial Type

Randomised controlled trials in human subjects of both selective and non-selective MRAs compared to placebo, or open label trials using MRA as additional therapy compared to standard care in the control arm, in participants with CKD stage 1-5 for delay or prevention of disease progression were analysed. The initial period of randomised crossover studies was also considered for inclusion. Trials were considered eligible if MRA was used alone, or in combination with ACE-I, ARB, or both, i.e. additional MRA as dual or triple RAAS blockade. Studies where additional antihypertensive therapy was added to MRA in the intervention arm were excluded, as were studies directly comparing MRA to non-RAAS blocking antihypertensive agents.

3.2.2 Participants

Studies including participants with CKD stage 1-5 with albuminuria or proteinuria were considered eligible for the analysis. Studies in patients requiring renal replacement therapy were not included in the formal analysis but the terms haemodialysis; peritoneal dialysis; and renal transplantation were used in the search strategy to ensure all appropriate trials were identified. These studies were later used in a separate sub-analysis of mortality outcomes.

3.2.3 Interventions

Trials of both selective and non-selective MRAs compared to placebo; ACE-I; ARB; or both were included in the analysis. Four weeks was considered to be the minimum acceptable duration of intervention and studies of less than 4 weeks duration were excluded.

3.2.4 Outcome Measures

The primary outcome measures included the effect of addition of MRA on the following:

a) End of treatment systolic and diastolic blood pressure (mmHg)
b) End of treatment urinary albumin or protein excretion, measured as either 24 hour collection or spot albumin: or protein: creatinine ratio
c) End of treatment renal excretory function including: serum creatinine (μmol/L); isotopic glomerular filtration rate (GFR, mL/min); estimated GFR (eGFR, ml/min/1.73m^2); creatinine clearance (ml/min). In cases where several measures of renal function were reported, these data were meta-analysed using the following hierarchy:
    i. isotopic GFR;
    ii. creatinine clearance from 24 hour collection;
    iii. eGFR using the MDRD or CKD-EPI formulae;
    iv. estimated creatinine clearance using Cockcroft-Gault formula.
d) End of treatment serum or plasma potassium, incidence of potassium level above pre-defined study upper limit, and incidence of clinically significant hyperkalaemia necessitating intervention with potassium-lowering therapies.
e) Death, requirement of renal replacement therapy and cardiovascular events.

3.2.5 Data Collection

A list of titles and abstracts was produced using the search strategy shown in (Appendix 6). These were assessed independently by two reviewers (the author and Dr Alison Taylor) who discarded those not meeting the pre-defined inclusion criteria. Full texts of the remaining trials were then independently assessed and considered for inclusion. Any discrepancies between reviewers were settled by a third author (Dr Paddy Mark, reader in nephrology). Data were then retrieved from the full texts of published papers using specific extraction forms as shown

in (Appendix 7). If required data were not given in the published manuscript study authors were contacted to request further information.

3.2.6 Assessment of Bias

Trial quality was assessed independently by two reviewers (the author and Dr Alison Taylor) using the Cochrane Collaboration risk of bias assessment tool [405]. Factors considered were:

a) adequate sequence generation;
b) allocation concealment;
c) blinding of participants; trial personnel and outcome assessors;
d) reporting of incomplete outcome data;
e) suggestion of selective outcome reporting;
f) intention-to-treat analysis.

3.2.7 Statistical Analysis

Statistical analysis was performed by the author and Dr Alison Taylor with the support of Dr David Preiss (senior clinical research fellow, Nuffield Department of Public Health, University of Oxford). Random effects meta-analysis was performed for continuous and categorical outcomes in order to manage between-trial heterogeneity introduced by analysing varied trial populations. For continuous outcomes weighted mean differences were calculated using one of two different approaches depending on the data available for analysis:

a) final visit results for the outcome of interest were compared between intervention and control arms after using meta-analysis to verify that baseline data were not different between the trial arms;

b) where sufficient data were available, change in weighted mean difference in the outcome of interest from baseline to end-of-trial was calculated by meta-analysis.

For categorical outcomes risk ratios (RRs) were calculated from available data as the ratio of cumulative incidence and 95% confidence intervals for trial participants at baseline and for those who developed the outcome of interest.

Authors variously reported urinary protein or albumin excretion using either 24 hour collection values, spot albumin or protein: creatinine ratios. Due to the distribution of data, standard deviations were not routinely available for these measures in many of the included trials, making meaningful analysis challenging. Where data were available, percentage change from baseline in any measure of urinary protein or albumin excretion was analysed using weighted means and weighted standard deviations in exploratory analyses.

Heterogeneity between trials was quantified using the I^2 statistic to provide a measure of the proportion of overall variation attributable to inter-trial heterogeneity, with $p<0.10$ considered significant. Publication bias was determined for the most commonly reported outcomes using funnel plots and Egger tests. All statistical analyses were conducted using Stata version 13 (StataCorp, College Station, Texas).

3.3 Results

3.3.1 Literature Search and Trial Characteristics

3.3.1.1 Search Results

The combined search of PubMed, EMBASE and the Cochrane database resulted in identification of 299 citations. After removal of duplicate citations 143 titles and abstracts remained for screening. A further 87 citations were excluded at this stage due to: combined intervention with MRA and additional

antihypertensive agents (8 studies); description of outcome measures not pre-specified for this analysis (5 studies); cohort of participants requiring renal replacement therapy (11 studies); description of additional outcomes in a duplicate cohort (4 studies); non-randomisation (4 studies); intervention of less than 4 weeks duration (2 studies); trial results later retracted (1 study). Two review articles were also removed at this stage. After full text assessment of the remaining 56 articles, 19 trials including a total of 1646 participants [323, 325-341, 398] were selected for inclusion. The summary study flow chart is shown in Figure 3-1.

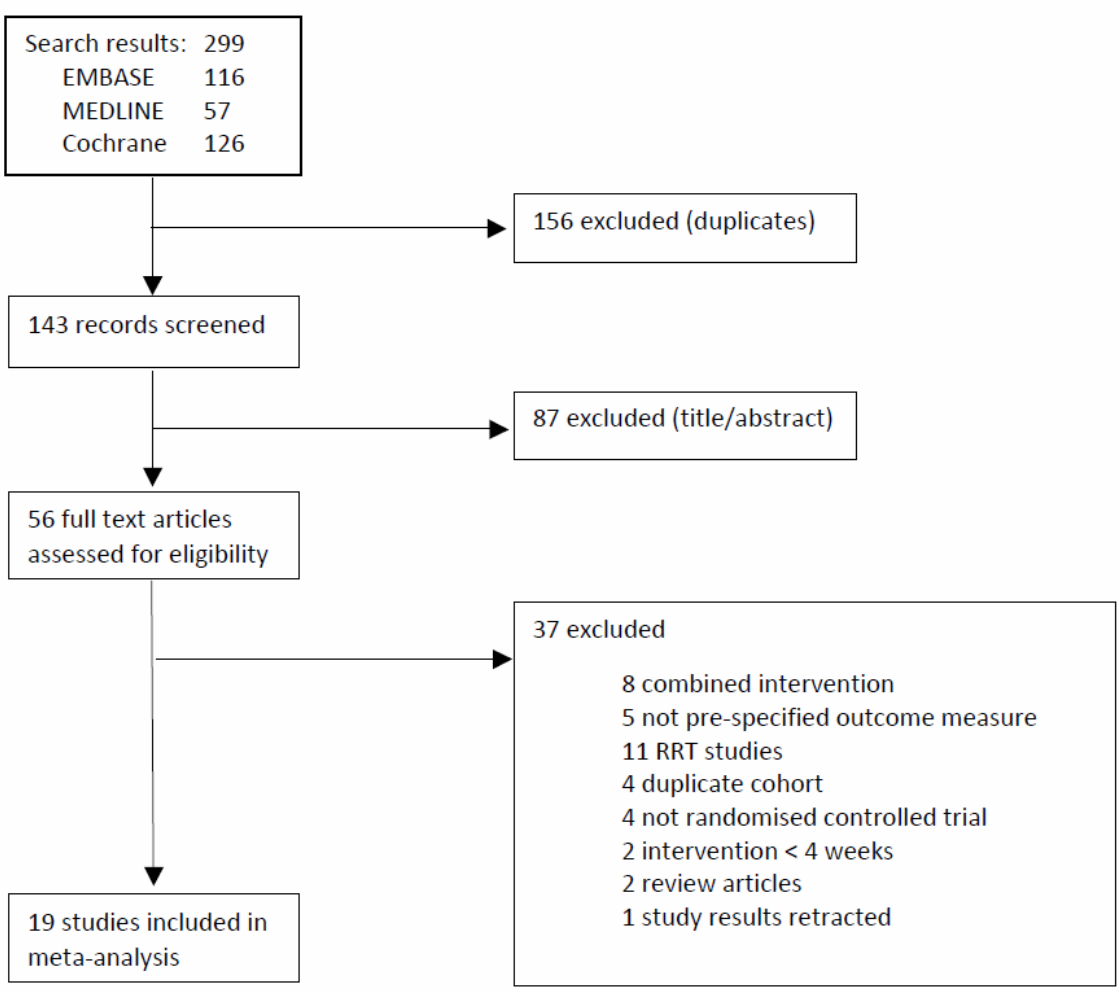

Figure 3-1. Study flow chart.

Reproduced from Currie et al, BMC Nephrology 2016;17: 237

3.3.1.2 Supplemental Information

Where required data were not reported in the published manuscript original authors were contacted by email with data extraction form attached (Appendix 7) and relevant fields highlighted. As a result, additional unpublished summary data was supplied by 8 authors of 10 (53%) trials [325-327, 329, 330, 335, 338-340, 398] for inclusion in the final analysis.

3.3.1.3 Trial Characteristics

a) Study Design

Of the 19 studies selected for inclusion 5 were randomised placebo-controlled trials; 7 trials had randomised controlled design comparing intervention with addition of MRA to standard care as control group; and 7 were randomised crossover studies. Participant groups were varied: 6 trials included participants with non-diabetic CKD of mixed aetiology; 8 trials recruited participants with diabetic nephropathy; 2 studies focussed on participants with CKD and hypertension; and 3 included participants with both diabetic and non-diabetic CKD. Trial duration ranged from 8 to 52 weeks, the smallest trial recruited 18 participants and the largest 359.

b) Intervention

Fourteen trials used Spironolactone 25-50mg as intervention and 5 trials used Eplerenone at doses ranging from 25-100mg per day. Eighteen trials added Spironolactone or Eplerenone to ACE-I or ARB, compared to ACE-I or ARB alone (i.e. dual RAAS blockade compared to monotherapy) while one study added Spironolactone to ACE-I and ARB (i.e. triple compared to dual RAAS blockade).

c) Endpoints

The majority of trials reported at least one of 4 different urinary protein or albumin excretion measures as the primary endpoint. Authors reported either 24 hour urinary protein or albumin excretion; urine albumin: creatinine ratio; or urine protein: creatinine ratio. In 3 trials protein or albumin excretion was a secondary outcome measure where blood pressure, pulse wave velocity (PWV) and left ventricular mass index (LVMI) respectively were the primary outcomes of interest [335, 336, 340].

Varied methods of reporting renal excretory function were also used. Where eGFR was calculated methods included the MDRD and CKD-EPI tools, while the Cockcroft-Gault method was used to measure creatinine clearance. Three trials measured isotopic GFR using ^{51}Cr-EDTA [325, 329, 398]. Characteristics of participants and interventions in included trials are shown in Table 3-1.

Study	Kidney disease	No. of patients included	Intervention Group	Control group	Co-intervention	Study duration	Baseline eGFR (ml/min/1.73m^2)	Endpoints
Abolghasmi 2011	CKD with resistant hypertension	41	Spironolactone 25-50mg	Placebo	multi-drug regime including ACE-I+/-ARB	12 weeks	Not available	BP, potassium, creatinine, urinary sodium
Ando 2014	CKD with hypertension	314	Eplerenone 50 mg	Placebo	ACE-I+/-ARB of at least 8 weeks duration	1 year	Treatment 67.7±14.3 Control 68.6±13.6	UACR, creatinine, eGFR, urinary L-FABP, 24hr urinary sodium, incidence of cerebrovascular and cardiovascular events
Bianchi 2006	Non-diabetic CKD (idiopathic GN)	165	Spironolactone 25mg	ACE-I+/-ARB	ACE-I+/-ARB	1 year	Treatment 62.4±21.9 Control 62.2±19.0	24 hr urinary protein, BP, creatinine, eGFR potassium
Boesby 2011 (XO)	Non-diabetic CKD	40	Eplerenone 25-50mg	multi-drug regime including ACE-I+/-ARB	multi-drug regime including ACE-I+/-ARB	8 weeks	59±26	24 hr urinary albumin, BP, potassium, creatinine clearance
Boesby 2013	Diabetic and non-diabetic CKD	26	Eplerenone 25-50mg	ACE-I+/-ARB	ACE-I+/-ARB	24 weeks	36±10	cfPWV, AIx, AASI, 24hr urinary albumin
Chrysostomou 2006*	Diabetic and non-diabetic CKD	41	Spironolactone 25mg	Placebo as ARB; Placebo as Spironolactone	ACE-I alone; ACE-I+ARB	3 months	Not available	24hr urinary protein, BP, creatinine, creatinine clearance, potassium
Edwards 2009	Non-diabetic CKD with no renovascular diagnosis	112	Spironolactone 25mg	Placebo	ACE-I/ARB	36 weeks	Treatment 49±12 Control 53±11	LVMI, cfPWV, aortic distensibility, AIx, BP

Table 3-1. Summary of included studies.

Continues on following pages. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

Study	Kidney disease	No. of patients included	Intervention Group	Control group	Co-intervention	Study duration	Baseline eGFR (ml/min/1.73m^2)	Endpoints
Epstein 2006+	Diabetic nephropathy	359	Eplerenone 50mg or 100mg	Placebo	ACE-I	12 weeks	ACE±EPL 50 73(62.1-83.6) ACE±EPL 100 75 (62.8-85.9) Control 74 (60.5-82.2)	UACR, potassium, BP, eGFR
Guney 2009	Non-diabetic CKD	24	Spironolactone 25mg	ACE-I+/-ARB	ACE-I+/-ARB	6 months	Treatment 63.0±22.71 Control 56.3±35.6	UPCR, urinary TGF-β1, eGFR, creatinine, potassium, BP, aldosterone
Mehdi 2009	Diabetic nephropathy	81	Spironolactone 25mg	Placebo or ARB	ACE-I	48 weeks	Not available	UACR, BP, creatinine clearance, potassium
Nielsen 2012 (XO)	Diabetes with microalbuminuria	21	Spironolactone 25mg	Placebo	ACE-I/ARB	60 days	Not available	24hr urinary albumin, BP, GFR, urinary L-FABP, urinary NGAL, urinary KIM-1
Rossing 2005 (XO)	Diabetic nephropathy	20	Spironolactone 25mg	Placebo	ACE-I+/-ARB	8 weeks	Not available	24 hr urinary albumin, BP, GFR
Saklayen 2008 (XO)	Diabetic nephropathy	24	Spironolactone 25-50mg	Placebo	ACE-I/ARB	3 months	Treatment 61.9±23.4 Control 54.4±20.1	BP, creatinine, potassium, UPCR
Schjoedt 2005 (XO)	Diabetic nephropathy	20	Spironolactone 25mg	Placebo	ACE-I+/-ARB	2 months	Not available	24hr urinary albumin, BP, GFR

Study	Kidney disease	No. of patients included	Intervention Group	Control group	Co-intervention	Study duration	Baseline eGFR (ml/min/1.73m^2)	Endpoints
Tylicki 2008 (XO)	Non-diabetic CKD	18	Spironolactone 25mg	ACE-I+ARB	ACE-I+ARB	8 weeks	107.8 (93-140.9)	24hr urinary protein, BP, creatinine, potassium, PRA, urinary NAG, urinary PIIINP
Tylicki 2012 (XO)	Non-diabetic CKD	18	Eplerenone 50mg	ARB+Aliskiren	ARB	8 weeks	Not available	UACR, BP, creatinine clearance, potassium
van den Meiracker 2006	Diabetic nephropathy	53	Spironolactone 25-50mg	Placebo	ACE-I/ARB	1 year	Treatment 93.1±45 Control 66.3±35.1	24hr urinary protein, BP, creatinine, eGFR, potassium
Wang 2013	Diabetic and non-diabetic CKD	208	Spironolactone 20mg	multi-drug regime including ACE-I+/-ARB	multi-drug regime including ACE-I+/-ARB	16 weeks	Treatment 65.8±22.2 Control 66.5±24.3	24hr urinary protein, creatinine, potassium, eGFR, BP, aldosterone
Ziaee 2013	Diabetes with microalbuminuria	60	Spironolactone 25mg	ACE-I	ACE-I	12 weeks	Treatment 79.8±18 Control 82.5±19.1	UACR, BP, potassium, eGFR

Data are mean±SD or median (IQR). UACR, urine albumin:creatinine ratio; UPCR, urine protein:creatinine ratio; ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CKD, chronic kidney disease; NG, glomerulonephritis; L-FABP, liver-type fatty acid binding protein; XO, crossover study design; cfPWV, carotid-femoral pulse wave velocity; AIx, augmentation index; AASI, ambulatory arterial stiffness index; LVMI, left ventricular mass index; TGF-β1, transforming growth factor-β1; NGAL, neutrophil gelatinase associated lipocalin; KIM-1, kidney injury molecule-1; PRA, plasma renin activity; NAG, n-acetyl-β-D-glucosaminidase; PIIINP, amino-terminal propeptide of type III procollagen; *this study had 4 arms; +this study had 3 arms

3.3.1.4 Trial Quality

Trial quality was variable as assessed using the Cochrane Collaboration tool [405] as shown in Table 3-2. Sequence generation was adequately described in 9 (47%) trials and allocation concealment was adequate in 8 (42%). Both participants and investigators were blinded in 12 (63%) trials and intention-to-treat analysis was performed in 4 (21%) studies. Dropouts were adequately accounted for in 16 (84%) trials and there were no differences in dropout rates between intervention and control arms.

Trial	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants (performance bias)	Blinding of personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Intention to treat analysis
Abolghasmi 2011	**Unclear risk** (don't state how participants were randomised)	**Unclear risk**	**Low risk** (double-blind)	**Low risk** (double blind)	**Low risk** (not stated in paper but unlikely that biochemical outcomes would be influenced)	**Unclear risk** (don't report whether all patients completed study)	**Low risk** (protocol not available but all prespecified outcome measures reported)	**Not performed**
Ando 2014	**Low risk** (computer generated list)	**Low risk** (list created by central statistician, block size concealed to all investigators)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** (data collection and management personnel and statisticians all blinded for duration)	**Low risk** (losses to follow up disclosed – 18, should not affect results as predicted drop-out rate in sample size calculation was 10%)	**Low risk** (All prespecified outcomes were reported)	**Yes**

Table 3-2. Risk of bias assessment.

Continues on following pages. XO, crossover study design. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

Trial	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants (performance bias)	Blinding of personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Intention to treat analysis
Bianchi 2006	**Low risk** (computer generated list)	**Unclear risk** (further description of allocation not included)	**High risk** (open label)	**High risk** (open label)	**High risk** (open label)	**Low risk** (drop-outs disclosed)	**Low risk** (all prespecified outcomes reported, most drop outs from high risk group were as a results of K+ but this was disclosed in paper)	**Not performed**
Boesby 2011	**Unclear risk** ("investigator drew sealed opaque envelopes" but no detail on how randomisation list created)	**Low risk** (sealed, opaque envelopes)	**High risk** (open label XO)	**High risk** (open label XO)	**High risk** (open label XO)	**Low risk** (drop-outs disclosed)	**Low risk** (all prespecified outcomes reported)	**Modified** (drop outs after randomisation still included in final analysis)
Boesby 2013	**Unclear risk** ("randomisation done by GCP-unit" but no detail on how)	**Unclear risk** (not documented)	**High risk** (open label)	**High risk** (open label)	**High risk** (open label)	**Low risk** (drop-outs disclosed, groups still equal)	**Low risk** (all prespecified outcomes reported)	**Not performed**

Trial	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants (performance bias)	Blinding of personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Intention to treat analysis
Chrystosostomou 2006	**Unclear risk** ("randomisation done by clinical trial pharmacists not involved in study")	**Low risk** (simple randomisation)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** (unblinded after 3 months but unlikely to have influenced outcome measures)	**Low risk** drop outs disclosed but only 1 so unlikely to affect outcome)	**Low risk** (all prespecified outcomes reported)	**Yes**
Edwards 2009	**Unclear risk** (no detail on how sequence generated)	**Unclear risk** (no detail on how allocation performed)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** (not stated in paper but unlikely that biochemical outcomes would be influenced)	**Low risk** (drop outs disclosed)	**Low risk** (all prespecified outcomes reported)	**Not performed**
Epstein 2006	**Unclear risk** (no detail on how randomisation performed)	**Unclear risk** (no detail given)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** (not stated in paper but unlikely that biochemical outcomes would be influenced)	**Low risk** (drop outs fully disclosed)	**Low risk** (all prespecified outcomes reported)	**Not performed**
Guney 2009	**Unclear risk** (no detail on how randomisation performed)	**Unclear risk** (no detail given)	**High risk** (not blinded)	**High risk** (not blinded)	**High risk** (not blinded)	**Low risk** (drop outs fully disclosed)	**Low risk** (all prespecified outcomes reported)	**Not performed**

Trial	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants (performance bias)	Blinding of personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Intention to treat analysis
Mehdi 2009	**Low risk** (computer randomisation, by diabetes type)	**Low risk** (performed by staff at investigational study drug unit)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** (not stated in paper but unlikely that biochemical outcomes would be influenced)	**Low risk** (drop outs disclosed but only 1 so unlikely to affect outcome)	**Low risk** (all prespecified outcomes reported)	**Yes**
Nielsen 2012	**Low risk** (computer generated randomisation)	**Low risk** (unknown block size and frequency)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** (not stated in paper but unlikely that biochemical outcomes would be influenced)	**Unclear risk** (don't report whether all patients completed study)	**Low risk** (all prespecified outcomes reported)	**Unable to comment - ?assume all participants completed study**
Rossing 2005	**Low risk** (computer generated randomisation)	**Low risk** (sealed envelopes)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** ("code not broken until all data entered into a database which was locked for editing")	**Low risk** (drop outs disclosed but only 1 so unlikely to affect outcome)	**Low risk** (all prespecified outcomes reported)	**Not performed**
Saklayen 2008	**Unclear risk** ("sequence generated randomly by a clinical trials pharmacist")	**Unclear risk** (no detail given)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** ("investigators blinded until code was broken at end of study")	**Low risk** (drop outs fully disclosed)	**Unclear risk** (intended outcome measures not stated in methods section)	**Not performed**

Trial	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants (performance bias)	Blinding of personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Intention to treat analysis
Schojedt 2005	**Low risk** (computer generated)	**Low risk** (concealed with computer generated envelopes)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** ("code not broken until all data entered into a database which was locked for editing")	**Low risk** (drop outs fully disclosed)	**Low risk** (all prespecified outcomes reported)	**Not performed**
Tylicki 2008	**Low risk** (computer generated)	**Low risk** ("independent of study personnel")	**High risk** (not blinded)	**High risk** (not blinded)	**High risk** (not blinded)	**Low risk** (no drop outs)	**Low risk** (all prespecified outcomes reported)	**Not performed/required**
Tylicki 2012	**Low risk** (computer generated)	**Unclear risk** (no detail given)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** (not stated in paper but unlikely that biochemical outcomes would be influenced)	**Low risk** (no drop outs)	**Low risk** (all prespecified outcomes reported)	**Not performed/required**
Van der Meiracker 2006	**Low risk** (computer generated)	**Unclear risk** (no detail given)	**Low risk** (double blind)	**Low risk** (double blind)	**Low risk** (not stated in paper but unlikely that biochemical outcomes would be influenced)	**Low risk** (drop outs fully disclosed)	**Unclear risk** (intended outcome measures not stated in methods section)	**Not performed**

Trial	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants (performance bias)	Blinding of personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Intention to treat analysis
Wang 2013	**Unclear risk** (minimal detail on method of randomisation)	**High risk** (open randomisation)	**High risk** (not blinded)	**High risk** (not blinded)	**High risk** (not blinded)	**Low risk** (drop outs fully disclosed)	**Low risk** (all prespecified outcomes reported)	**Not performed**
Ziaee 2013	**Unclear risk** (no detail on how randomisation performed)	**Unclear risk** (no detail on how randomisation performed)	**High risk** (not blinded)	**High risk** (not blinded)	**High risk** (not blinded)	**Unclear risk** (don't report whether all patients completed study)	**Unclear risk** (intended outcome measures not stated in methods section)	**Not performed**

3.3.1.5 Heterogeneity

Heterogeneity was considerable for meta-analysis of the majority of outcomes (final visit systolic and diastolic blood pressure; final visit serum creatinine, albumin: creatinine ratio, protein: creatinine ratio, 24 hour urinary protein and albumin excretion; final visit potassium). Limited heterogeneity was seen for other outcomes (change from baseline systolic and diastolic blood pressure; final visit creatinine clearance, eGFR, GFR hierarchy; change from baseline potassium and risk of hyperkalaemia). The I^2 values for these variables are given in Tables 3-4 and 3-5 and Figures 3-5 to 3-10.

3.3.1.6 Publication Bias

There was a suggestion of publication bias for systolic blood pressure as determined by funnel plot (Figure 3-2) and Egger test (p=0.08), but not for end of study GFR (Figure 3-3, Egger test p=0.89) or hyperkalaemia risk (Figure 3-4, Egger test p=0.81).

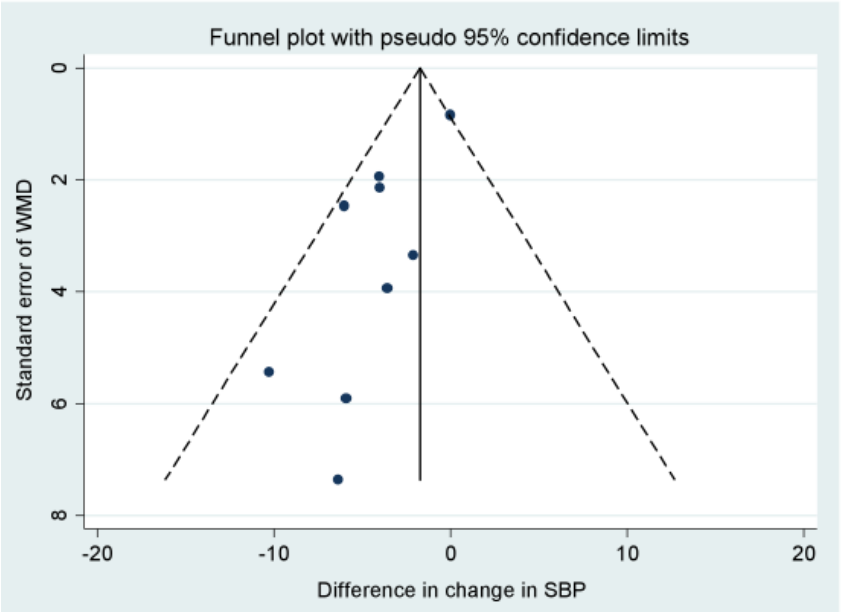

Figure 3-2. Funnel plot for systolic blood pressure.

SBP, systolic blood pressure; WMD, weighted mean difference. Reproduced from Currie et al, BMC Nephrology 2016;17: 237.

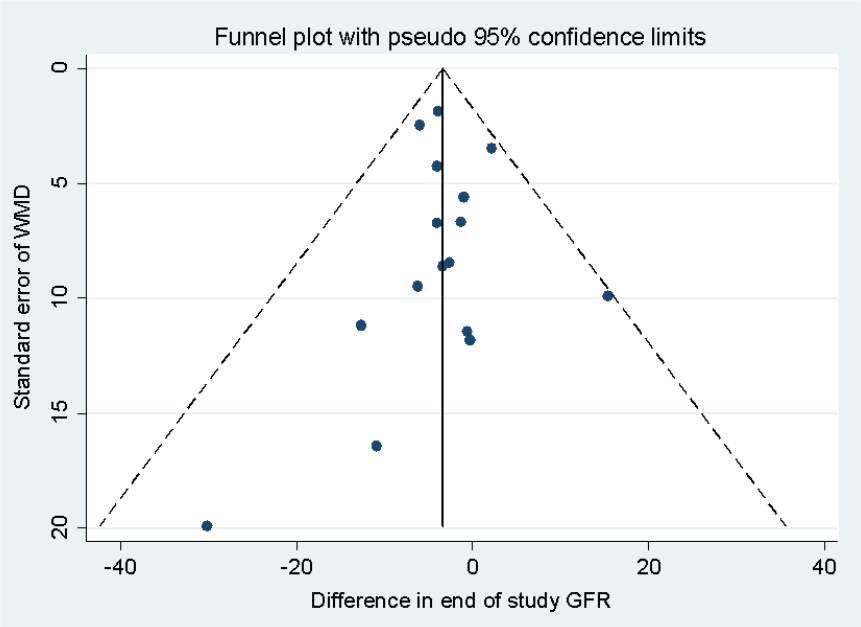

Figure 3-3. Funnel plot for end of study glomerular filtration rate (GFR).

eGFR, estimated glomerular filtration rate; WMD, weighted mean difference. Reproduced from Currie et al, BMC Nephrology 2016;17: 237.

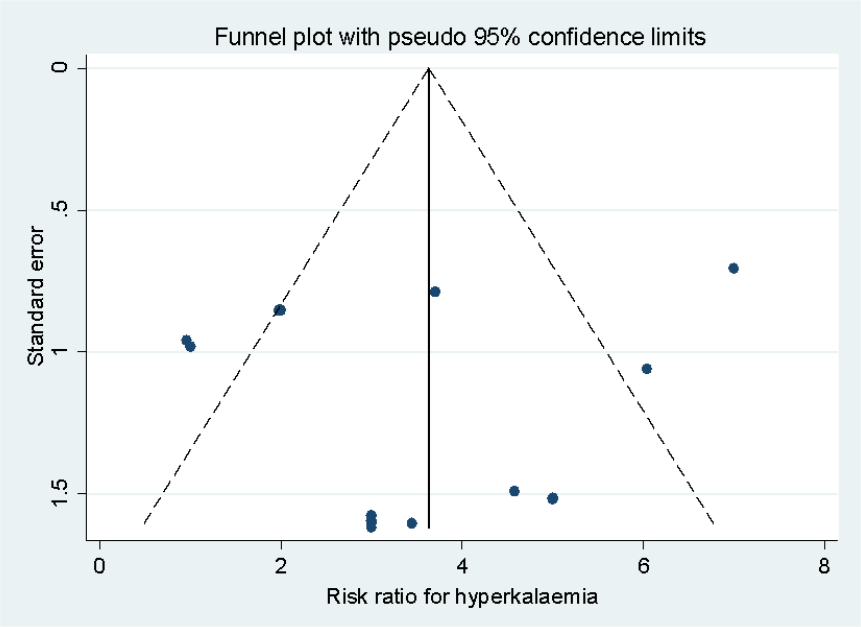

Figure 3-4. Funnel plot for risk ratio for hyperkalaemia.

WMD, weighted mean difference. Reproduced from Currie et al, BMC Nephrology 2016;17: 237.

3.3.2 Clinical Outcomes

Meta-analysis of baseline data for all outcomes of interest showed that these were balanced across studies (Table 3-3), confirming that use of end-of-trial meta-analysis was an appropriate analysis strategy to employ.

3.3.2.1 Effect of MRA treatment on blood pressure

Addition of MRA led to significant reductions in both systolic and diastolic blood pressure in comparison to ACE-I and/or ARB alone. Based on final visit blood pressure results, MRA led to a 5.7mmHg reduction (95% CI -9.04, -2.34) in systolic blood pressure. In the 9 trials where change from baseline was available, addition of MRA again resulted in a 3.3mmHg (95% CI -5.56, -1.04) reduction in systolic blood pressure compared to ACE-I and/or ARB alone (Figure 3-5). Enhanced RAAS blockade with MRA also produced a 1.7mmHg reduction (95% CI -3.37, -0.10) in final visit diastolic blood pressure, and in trials where change from baseline was available for analysis a 2.8mmHg reduction (95% CI -3.35, -2.33) was seen with the addition of MRA to standard RAAS blockade (Table 3-4).

Variable	Units	No. of trials	No. patients in treatment	No. patients in placebo/control	Effect size (95% CI)	I^2 (p value)
Systolic BP	mmHg	17	693	686	0.46 (-0.75, 1.66)	0.0% (0.981)
Diastolic BP	mmHg	17	693	686	0.63 (-0.18, 1.43)	0.0% (0.739)
Serum Potassium	mmol/L	17	708	702	-0.01 (-0.05, 0.04)	0.0% (0.992)
Creatinine	µmol/L	18	646	640	-0.20 (-4.50, 4.10)	25.1% (0.160)
Estimated GFR	ml/min/1.73m^2	9	459	454	-0.10 (-3.13, 2.94)	35.1% (0.137)
Creatinine Clearance	ml/min	6	132	130	-3.30 (-9.64, 3.04)	16.5% (0.307)
Urinary protein creatinine ratio	mg/mmol	4	146	150	0.06 (-0.68, 0.20)	19.6% (0.292)
Urinary albumin creatinine ratio	g/g creatinine	7	364	360	3.44 (-8.82, 15.71)	64.8% (0.009)
24 hr urinary protein excretion	g/24 hours	4	145	140	0.04 (-0.14, 0.22)	0.0% (0.815)
24 hr urinary albumin excretion	mg/24 hours	6	151	155	-3.26 (-42.14, 35.62)	0.0% (0.716)

Table 3-3. Comparison of baseline data in meta-analyses

BP, blood pressure; GFR, glomerular filtration rate. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

Variable	Measurement	No. of study groups	No. patients in intervention	No. patients in placebo/control	Effect size (95% CI)	I^2 (p value)
Systolic BP (mmHg)	Change from baseline	9	260	266	-3.30 (-5.56, -1.04)	40.0% (0.101)
	Final visit	16	666	659	-5.69 (-9.04, -2.34)	81.8% (0.000)
Diastolic BP (mmHg)	Change from baseline	9	260	266	-2.84 (-3.35, -2.33)	0.0% (0.799)
	Final visit	16	666	659	-1.73 (-3.37, -0.10)	68.3% (0.000)

Table 3-4. Effect of MRA with ACE-I and/or ARB compared with ACE-I/ARB monotherapy on end of treatment blood pressure.

BP, blood pressure. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

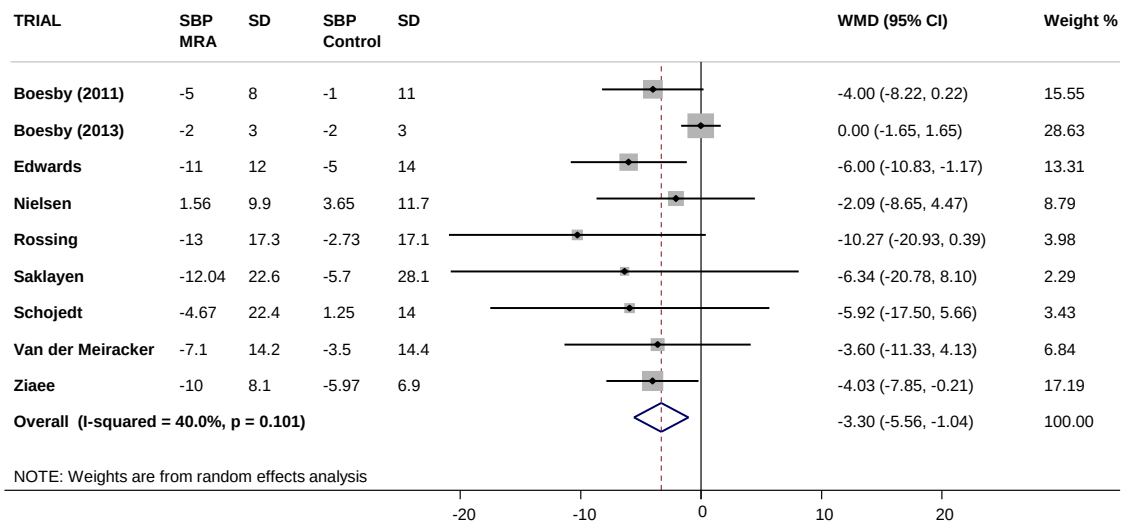

Figure 3-5. Effect of addition of MRA to ACE-I and/or ARB on blood pressure.

Based on change from baseline blood pressure. SBP, systolic blood pressure; MRA, mineralocorticoid receptor antagonist; WMD, weighted mean difference. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

3.3.2.2 Effect of MRA treatment on renal excretory function

Based on end-of-trial results, addition of MRA to ACE-I and/or ARB led to a non-significant increase in serum creatinine of 3.8µmol/L (95% CI -2.14, 9.79). In studies where creatinine clearance was reported meta-analysis revealed a non-significant reduction of 2.5 ml/min (95% CI -7.05, 2.04) using final visit values. However, when the hierarchy of GFR results were analysed as previously detailed above, a small but statistically significant reduction was seen with addition of MRA using final visit values (-3.15 ml/min/1.73m^2; 95% CI -5.36, -0.95) as shown in Figure 3-6.

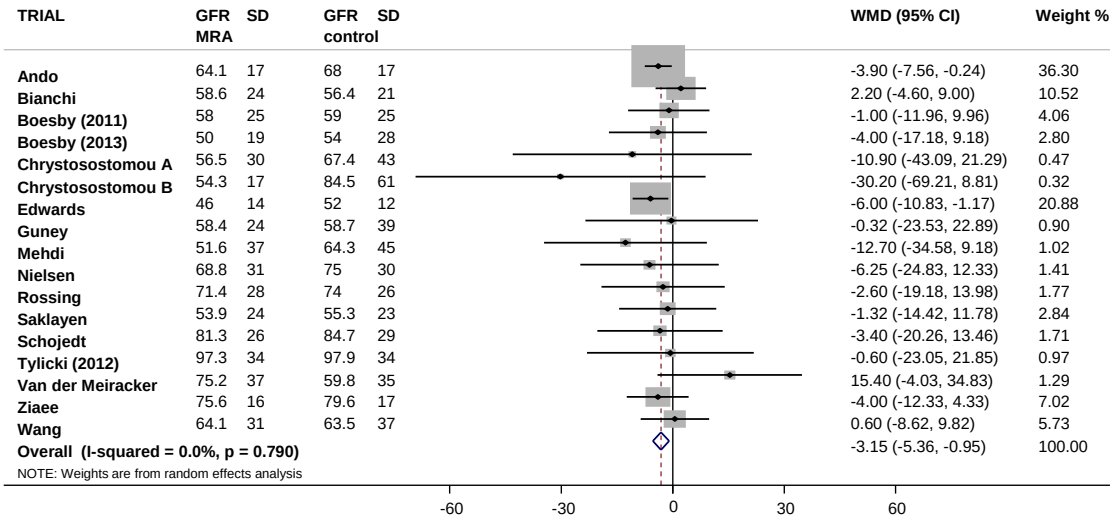

Figure 3-6. Effect of addition of MRA to ACE-I and/or ARB on end-of treatment renal excretory function.

Analysis based on end-of-treatment renal excretory function. In cases where multiple measures were reported analysis was based on the following hierarchy: isotopic GFR; creatinine clearance from 24 hour collection; eGFR using MDRD or CKD-EPI formulae; estimated creatinine clearance using Cockroft-Gault formula. GFR, glomerular filtration rate; MRA, mineralocorticoid receptor antagonist; WMD, weighted mean difference. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

3.3.2.3 Effect of MRA treatment on urinary albumin/protein excretion

A variety of measures of urinary protein or albumin excretion were reported in included studies which are summarised in Table 3-5. In trials where UACR was reported addition of MRA led to a non-significant reduction of 10.91mg/mmol (95% CI -26.15, 4.32) using end-of-trial results. No authors reported change from baseline ACR. In studies reporting 24 hour UAE, MRA led to a significant reduction of -332.91 mg/24hrs (95% CI -624.08, -41.02). Three study groups reported change in 24 hour albumin excretion from baseline and meta-analysis again confirmed a significant reduction of 292.23 mg/24hrs (95% CI -422.19, -162.27).

Variable	Measurement	No. of study groups	No. patients in intervention	No. patients in placebo/control	Effect size (95% CI)	I^2 (p value)
Creatinine (µmol/L)	Final visit	16	601	595	3.83 (-2.14, 9.79)	50.4% (0.011)
Creatinine Clearance (ml/min)	Final visit	6	132	130	-2.51 (-7.05, 2.04)	0.0% (0.599)
eGFR ($ml/min/1.73m^2$)	Final visit	13	626	617	-2.71 (-4.85, -0.57)	0.0% (0.727)
GFR hierarchy*	Final visit	17	692	682	-3.15 (-5.36, -0.95)	0.0% (0.790)
Urinary ACR (mg/mmol)	Final visit	7	355	351	-10.91 (-26.15, 4.32)	83.4% (0.000)
Urinary PCR (g/g creatinine)	Final visit	4	146	150	-0.91 (-1.35, -0.46)	58.4% (0.065)
24 hour urinary albumin excretion (mg/24 hours)	Final visit	6	151	155	-332.91 (-624.80, -41.02)	66.5% (0.011)
	Change from baseline	3	90	94	-292.23 (-422.19, -162.27)	0.0% (0.606)
24 hour urinary protein excretion (g/24 hours)	Final visit	2	124	121	-0.41 (-0.90, 0.09)	77.1% (0.037)

Table 3-5. Effect of MRA with ACE-I and/or ARB compared with ACE/ARB monotherapy on end of treatment renal excretory function and urinary protein/albumin excretion.

eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate; ACR, albumin: creatinine ratio; PCR, protein: creatinine ratio. Reproduced from Currie et al, BMC Nephrology 2016;17: 237.

In trials where urine protein: creatinine ratio was used, MRA added to ACE-I and/or ARB led to a significant change in end-of-trial values (-0.91g/g creatinine; 95% CI -1.35, -0.46). No trials reported change from baseline protein: creatinine ratio. In studies where 24 hour urinary protein excretion was recorded, addition of MRA resulted in a non-significant reduction (-0.41 g/24hrs; 95% CI -0.90, 0.09) in end-of-trial values.

Analysis of absolute values in the case of urinary protein or albumin measures may be misleading as these data are non-parametric; in order to combine the data in a more appropriate manner relative change from baseline in any measure of protein/albumin excretion was assessed where these results were available. Using a difference in means analysis in order to allow inclusion of data from all 19 trials, addition of MRA resulted in a 38.7% (weighted SD 21.5%) reduction in any measure of urinary protein or albumin excretion. Focussing only on data from the 5 trials where percentage change in urinary protein/albumin excretion was available enhanced RAAS blockade with the addition of MRA led to a weighted mean difference of -31.03% (95% CI -35.34, -26.72) as shown in Figure 3-7.

3.3.2.4 Effect of MRA treatment on potassium

Weighted means analysis confirmed that additional MRA treatment did lead to increased potassium levels based on end-of-trial values (0.21mmol/L; 95% CI 0.08, 0.33) although there was significant heterogeneity between included studies (I^2=82.7%, p=<0.001). When analysed as change from baseline where these data were available using random effects meta-analysis a similar increase was seen with addition of MRA (0.19mmol/L; 95% CI 0.12, 0.270).

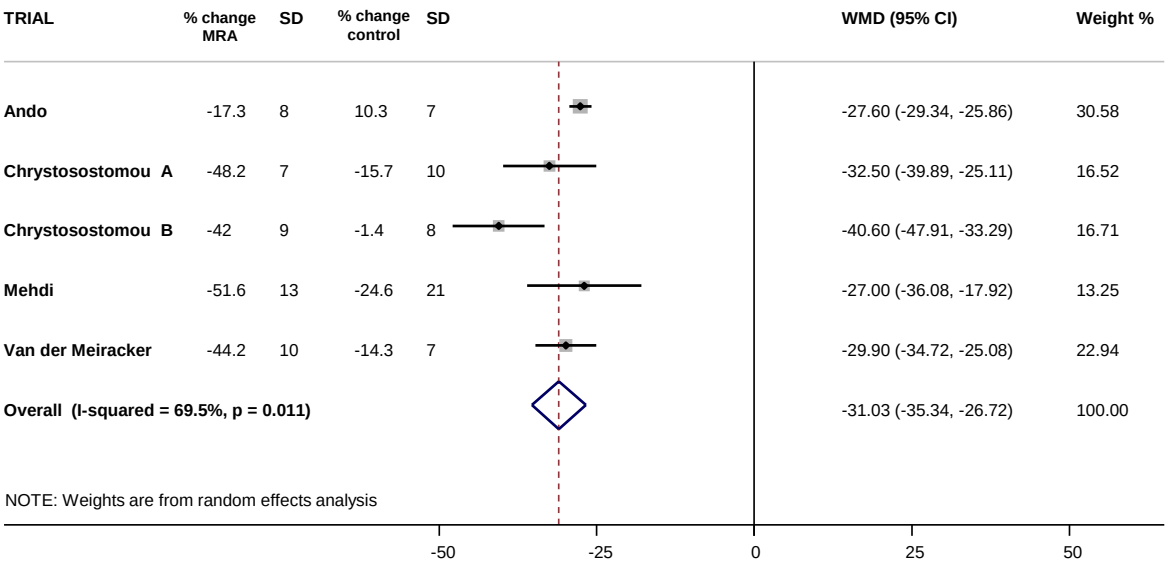

Figure 3-7. Effect of addition of MRA to ACE-I and/or ARB on urinary protein or albumin excretion.

Analysis based on change from baseline UPCR; 24 hour urine protein; UACR or 24 hour urine albumin in the 5 studied where this was reported. MRA, mineralocorticoid receptor antagonist; WMD, weighted mean difference. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

Risk of developing hyperkalaemia above the pre-defined trial upper limit was increased threefold with the addition of MRA to ACE-I and/or ARB as shown in Figure 3-8 (RR 3.02; 95% CI 1.75, 5.18). Number needed to harm for one year of MRA treatment calculated from trials where at least one case of hyperkalaemia was reported was 10 (95% CI 5, 27). Neither baseline serum creatinine (p=0.21) nor diabetes status (p=0.38) conferred an increased risk of hyperkalaemia based on included studies (Figure 3-9). Relative risk of being withdrawn from the intervention arm of the trial due to hyperkalaemia was increased to a similar extent (RR 3.21; 95% CI 1.19, 8.71). Number needed to harm over one year of treatment in trials where therapy was discontinued in at least one participant was 23 (95% CI 7, 267).

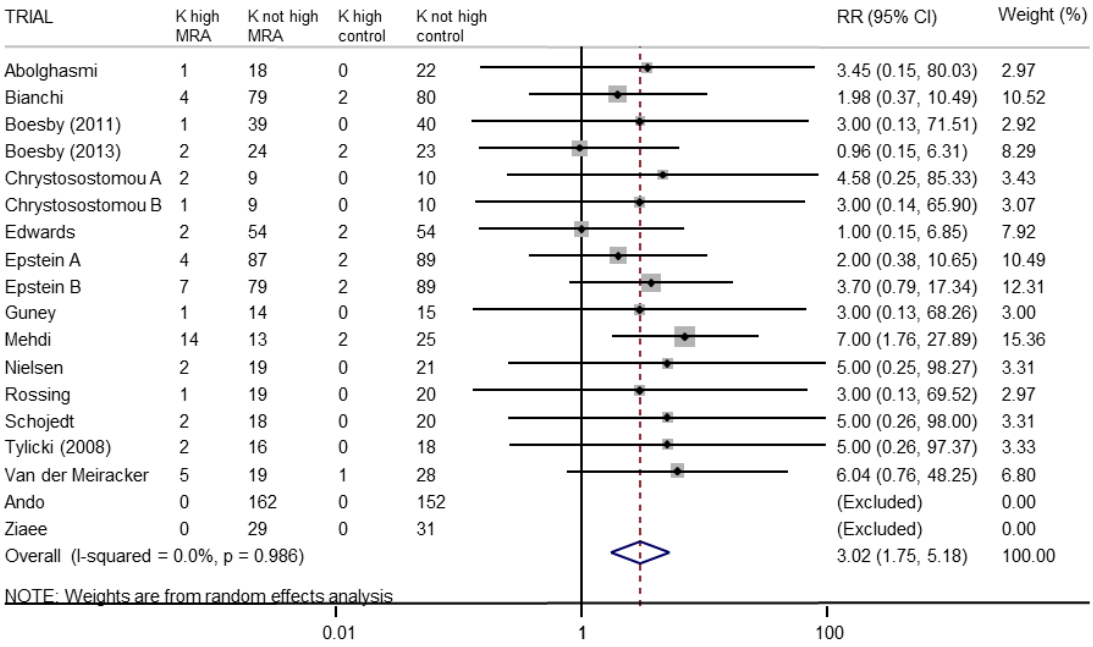

Figure 3-8. Relative risk of developing hyperkalaemia above the threshold pre-defined by investigators in each study with addition of MRA to ACE-I and/or ARB.

K, potassium; MRA, mineralocorticoid receptor antagonist; RR, relative risk. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

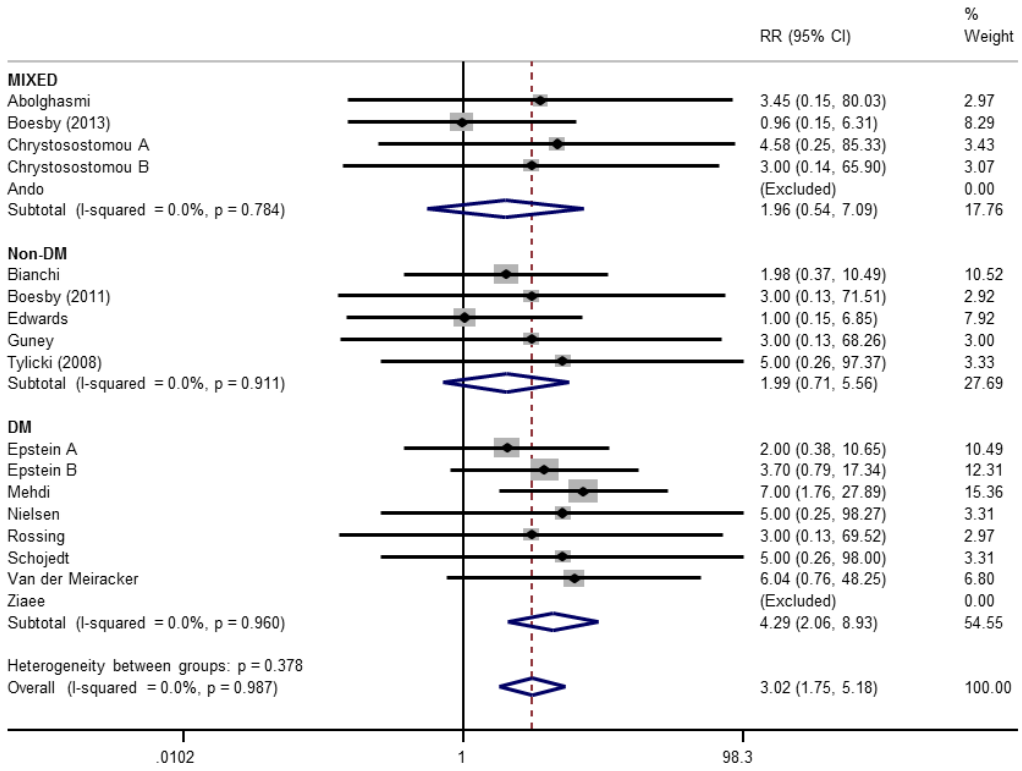

Figure 3-9. Relative risk of developing hyperkalaemia above study thresholds according to diabetes status.

Mixed refers to studies including both diabetic and non-diabetic patients; non-DM refers to studies where patients with diabetic nephropathy were not included; DM refers to studies including only patients with diabetic nephropathy. DM, diabetes mellitus; RR, relative risk. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

Focusing on clinically significant hyperkalaemia which necessitated medical intervention, only 1 trial reports hospitalisation of a study participant due to hyperkalaemia (plasma potassium 7.1mmol/L after 2 weeks of therapy necessitating infusion of IV insulin and dextrose and cardiac monitoring) [398].

3.3.2.5 Vascular parameters

Two trials reported the effects of addition of MRA to ACE-I and/or ARB on surrogate cardiac and vascular markers including: LVMI [335], ejection fraction (EF) [335]; augmentation index corrected for heart rate of 75 beats/minute (AIx@75) [335, 340] and carotid-femoral pulse wave velocity (cfPWV) [335, 340].

In one study in 112 participants with non-diabetic CKD stage 3A, addition of MRA resulted in significant improvement in LVMI compared to placebo (-14±13g vs +3±11g respectively, p=<0.01). There was no effect on EF with MRA over the 36 week treatment period [335]. Two groups reported the effect of MRA on cfPWV. In the trial described above, addition of MRA significantly improved cfPWV over a 36 week treatment period in comparison to placebo (-0.8 ± 1.0 vs -0.1 ± 0.9 m/s respectively, p=<0.01). Significant improvement in AIx@75 was also seen (-5.2 ± 6.1% vs -1.4 ± 5.9%, p=<0.05) [335]. In another study of effects of 24 weeks MRA treatment in patients with both diabetic and non-diabetic CKD stage 3-4, addition of MRA resulted in a statistically significant 4.4% reduction in AIx@75 (p=0.04) and of 0.1m/s in cfPWV, although this was not statistically significant (p=0.8) [340].

3.3.2.6 Morbidity and mortality

Included studies were small and study duration was no longer than 1 year, therefore trials were not powered to determine the effects of addition of MRA therapy on hard clinical outcomes. There were no reported of participants commencing dialysis or requiring renal transplantation during the course of the included studies. Morbidity and mortality data from included studies are summarised in Table 3-6. One study reported a participant death in the intervention arm, the cause of which was unknown [338], but no deaths were reported among the 18 remaining included trials. Two trials reported cardiovascular morbidity [328, 338]. In one of these trials, the duration of which was one year, a single study participant allocated to the intervention arm developed atrial fibrillation while another participant in the control group

suffered a stroke [338]. Another study, lasting 48 weeks, reported 6 cardiovascular events in the intervention group (2 strokes, 2 hospitalisations for heart failure, 1 myocardial infarction and 1 coronary artery bypass graft) whilst one participant in the control arm suffered a stroke [328].

Trial	Number of participants (control)	Number of participants (MRA)	Event	Events in control arm	Events in MRA arm
Ando 2014	152	162	Death	0	1 (0.6%)
			Atrial fibrillation	0	1 (0.6%)
			CVA	1 (0.7%)	0
Medhi 2009	27	27	Heart failure	2 (7.4%)	0
			CVA	2 (7.4%)	1 (3.7%)
			MI	1 (3.7%)	0
			CABG	1 (3.7%)	0

Table 3-6. Morbidity and mortality reported in included studies.

MRA, mineralocorticoid receptor antagonist; CVA, cerebrovascular accident; MI, myocardial infarction; CABG, coronary artery bypass graft. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

3.3.2.7 Mortality in Renal Replacement Therapy Trials

Studies in patients requiring renal replacement therapy were excluded from the main analysis, however all-cause mortality data was available from 6 trials [399-402, 406, 407]. The populations and interventions evaluated in these trials are summarised in Table 3-7. There was no heterogeneity between trials included in this analysis (I2=0.0%, p=0.543). Random effects meta-analysis demonstrated a significant reduction in all-cause mortality in patients randomised to MRA treatment in comparison to controls (RR 0.40; 95% CI 0.23, 0.69) as shown in Figure 3-10.

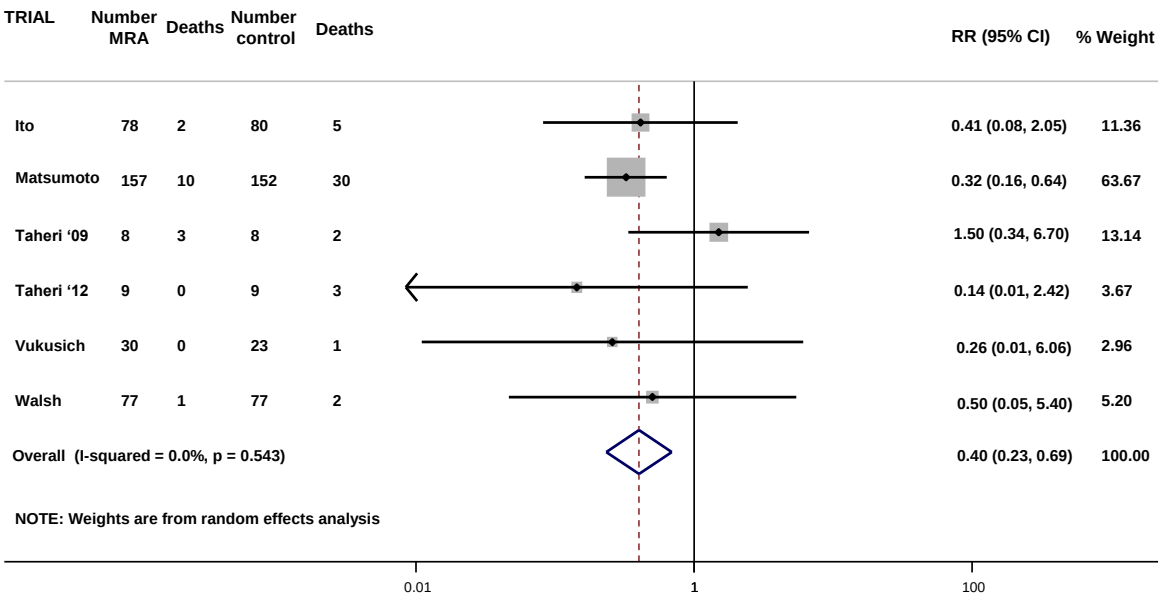

Figure 3-10. Effect of addition of MRA to ACE-I and/or ARB on all-cause mortality in patients requiring dialysis.

MRA, mineralocorticoid receptor antagonist; RR, relative risk. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

Trial	Intervention	Duration	Population	Number of Participants (control)	Number of participants (MRA)	Deaths (control arm)	Deaths (MRA arm)
Ito, 2014	Spironolactone 25mg	2 years	Peritoneal dialysis	80	78	5 (6.3%)	2 (2.5%)
Matsumoto, 2014	Spironolactone 25mg	3 years	Haemodialysis	152	157	30 (19.7%)	10 (6.4%)
Taheri, 2009	Spironolactone 25mg	6 months	Haemodialysis with EF < 45%	8	8	2 (25%)	3 (37.5%)
Taheri, 2012	Spironolactone 25mg	6 months	Peritoneal dialysis	9	9	3 (33.3%)	0
Vukusich, 2010	Spironolactone 50mg	2 years	Haemodialysis	23	30	1 (4.3%)	0
Walsh, 2015	Eplerenone 50mg	13 weeks	Haemodialysis	77	77	2 (2.5%)	1 (1.3%)

Table 3-7. Mortality reported in excluded RRT studies

RRT, renal replacement therapy; MRA, mineralocorticoid receptor antagonist; EF, ejection fraction. Reproduced from Currie et al, BMC Nephrology 2016;17: 237

3.4 Discussion

The world prevalence of CKD is increasing, attributable at least in part to the alarming global epidemics of hypertension, obesity and diabetes [408]. Current guidelines suggest RAAS blockade with ACE-I or ARB to slow disease progression in patients with CKD irrespective of aetiology [144]. Despite the routine prescribing of these agents in clinical practice many patients continue to progress to end stage renal disease, or die from cardiovascular complications of their disease [285]. Studies focusing on enhanced RAAS blockade with combined ACE-I and ARB therapy have raised concerns due to adverse effects and this strategy is not currently recommended [144]. Accumulating evidence points towards the pivotal role of aldosterone in the development and progression of CKD, and clinical studies included in this review have shown the beneficial effects of addition of MRAs to ACE-I and/or ARB including reduction of urinary protein or albumin excretion and blood pressure. Concerns remain that this combination has the potential to cause hyperkalaemia and further deterioration in renal function in patients with pre-existing CKD. Furthermore, the effects of addition of MRA on hard clinical endpoints such as all-cause mortality or cardiovascular outcomes remains unclear.

This meta-analysis confirmed that enhanced RAAS blockade through addition of MRA to ACE-I and/or ARB therapy is associated with significant reductions in blood pressure and urinary protein or albumin excretion at the cost of a small reduction in GFR and a quantifiable risk of hyperkalaemia. Data on hard clinical endpoints including morbidity and mortality are limited meaning that meta-analysis of impact of MRA on these outcomes was not possible. These findings are in keeping with the previously published meta-analyses [392, 393]. These publications, however, have analysed mainly published protein or albumin excretion data; and due to the disparate measures of proteinuria and albuminuria used in these studies and inability to obtain additional data from authors in many cases, meta-analysis of the effects of MRA on proteinuria or albuminuria was not possible. In this meta-analysis additional unpublished summarised data were obtained from a number of authors, resulting in more comprehensive assessment of the impact of MRA on urinary protein or albumin excretion. By excluding studies where MRA was combined with additional

antihypertensive agents in the intervention arm, this meta-analysis reports purely the effect of MRA without the confounding impact of additional therapies.

The association between albuminuria and progression to ESRD is well-documented, resulting in its evolution into a surrogate outcome measure in many clinical trials. Although clinical studies confirming the beneficial impact of reducing albumin or protein excretion on morbidity and mortality are lacking, a recent meta-analysis including data from over 78,000 patients reported that a 30% reduction in albuminuria confers 23.7% reduction in risk of progression to ESRD, irrespective of the drug class used [156]. This study demonstrates that addition of MRA to RAAS blockade with ACE-I and/or ARB in patients with CKD has the potential to reduce urinary protein or albumin excretion by more than 30%, which could theoretically translate into even greater benefits in terms of risk of progression of renal, and potentially cardiovascular disease [409, 410].

It is possible that reductions in proteinuria and albuminuria seen with MRA are not entirely blood pressure independent, however disentangling this relationship is impossible in the presence of substantial blood pressure lowering associated with addition of MRA across all included trials. Plotting the percentage reduction in proteinuria/albuminuria (any measure) against systolic blood pressure at final visit across all included studies is suggestive of an association between the two. However, it should be borne in mind that intensive blood pressure lowering beyond 120mHg systolic does not seem to confer additional reno-protective benefits, and in some cases has been shown to be associated with accelerated progression of renal disease [411, 412].

Hyperkalaemia is an inherent risk associated with prescription of RAAS blocking agents, particularly when considering dual or in some trials triple therapy. A population-based time series analysis indeed suggested that an increase in prescribing of spironolactone in patients with heart failure was associated with higher rates of hyperkalaemia [413], and studies of dual RAAS blockade in patients with diabetic and non-diabetic CKD have been associated with increased rates of hyperkalaemia in the intervention arm [320, 322]. Certainly this meta-analysis confirms a mean increase from baseline of 0.19mmol/L with addition of

MRA to ACE-I and/or ARB, and a 3-fold increased relative risk of developing hyperkalaemia. But how do we define hyperkalaemia? In the context of this analysis the relative risk of hyperkalaemia must be carefully interpreted as the risk of serum or plasma potassium rising above the upper limit pre-defined by the trial investigators, rather than clinically significant hyperkalaemia necessitating medical treatment. In fact, from all 19 trials included in this analysis, only 1 reported a single incidence of a study participant being admitted to hospital for administration of IV insulin and dextrose to treat a plasma potassium of 7.1 mmol/L [398]. Hyperkalaemia as defined in included studies ranged from potassium of 5.5-6 mmol/L, values which in clinical practice many nephrologists would not routinely treat. It should also be considered that the toxic effects of a given potassium level are to an extent depended on baseline potassium and the rate of increase, rather than simply its numerical occurrence, factors which in general are not considered within the context of clinical trials. Furthermore, it has been demonstrated that although potassium values do rise with regular dosing of RAAS blocking agents, the pattern appears to be that of an early initial increase thereafter followed by a steady-state rather than continued rise [414]. Taking into consideration the potential to achieve substantial reductions in urinary protein excretion, a quantifiable risk of developing hyperkalaemia should not necessarily dissuade clinicians from prescribing these agents, particularly if close biochemical monitoring is employed and participants with high-normal baseline potassium values are excluded from treatment. Recent data suggest that the nonsteroidal MRA Finerenone effectively reduced albuminuria in patients with DN with less incidence of hyperkalaemia (1.8% of treatment group) and no cases of serum potassium >6mmol/L [415] making it a promising agent for future study. In this study patients with serum potassium of >4.8mmol/L were excluded, again highlighting the potential importance of selectively targeting therapy towards those at lower risk of complications. Moreover, a recent phase 2 study in patients with DN and hyperkalaemia on RAAS inhibitors confirmed that the potassium-binding polymer Patiromer resulted in significant reduction in potassium over a 4 week treatment period [416] although the majority of patients had CKD stage 2-3 and effects of this agent in more advanced disease are yet to be determined.

Worsening of renal excretory function is another concern frequently associated with use of MRAs or enhanced RAAS blockade. In this meta-analysis the addition of MRA in patients with CKD who are already established on ACE-I and/or ARB was associated with an approximate reduction of 3.15ml/min/1.72m^2 in any measure of GFR, a value which although statistically significant would be unlikely to cause concern in a clinical setting in the majority of cases. In fact it is has been shown that an initial decline in GFR is associated with a more favourable course of renal function, and it has been shown that those individuals who experience greater decline in GFR in the short term stand to benefit most [417]. Applicability of these results to the general CKD population remains limited by the short duration of trials included and the effects of this strategy on longer term renal outcomes is unclear. In addition, no data is available on change in GFR upon withdrawal of treatment to determine whether these trends are reversible.

The trials included in this analysis were short (maximum duration 1 year) and underpowered to determine the impact of addition of MRA to ACE-I and/or ARB on longer term renal outcomes, morbidity or mortality. The present analysis cannot therefore draw conclusions regarding long-term safety and efficacy of combination treatment with MRA and ACE-I and/or ARB, despite postulated benefits in terms of reduction in protein or albumin excretion [156, 409, 418]. Moreover, mean baseline GFR was >35 ml/min/1.73m^2 in all included studies, meaning effects of treatment in more advanced disease remain to be determined. The ongoing Benefits of Aldosterone Receptor Antagonism in Chronic Kidney Disease (BARACK-D) study aims to follow more than 2000 participants with stage 3b CKD over the course of a 36 month randomised controlled trial of spironolactone versus placebo, and will address some of these unanswered questions [419].

Strengths of this analysis include independent systematic literature-searching, assessment of study quality and data extraction by two independent reviewers following a pre-specified strategy. In addition, 8 authors (10 trials) provided supplemental unpublished information resulting in a more complete dataset and the ability to perform more comprehensive analysis of the effects of addition of MRA on urinary protein or albumin excretion. Furthermore, supplemental data

was obtained from 3 trials not included in the previous meta-analysis [338, 340, 341] resulting in accumulation of data from a higher number of participants (1646 compared to 1549), making this the largest analysis to date.

A significant limitation of this analysis is that included studies are relatively small and powered to detect differences in surrogate endpoints. Duration of these trials was also short and long-term follow up data on clinical endpoints such as mortality and progression of CKD are lacking. Seven (37%) of the included trials had a crossover rather than randomised controlled trial design and reporting of methodology was variable such that adequate assessment of trial quality was not feasible in all cases. The marked heterogeneity in measures of urinary protein or albumin excretion reported made this analysis particularly challenging and likely means this outcome is still not as comprehensively assessed as it could be. Whilst these results demonstrate a significant reduction in any measure of urinary protein or albumin excretion and studies have shown that there is high agreement between albumin: creatinine ratio and 24 hour excretion values [20] standardisation of reporting of proteinuria or albuminuria across clinical trials would enable far more consistent and reliable analysis in the future, particularly if investigators continue to use these measures as surrogates for progression of renal disease [420].

In conclusion, addition of MRA represents a promising therapeutic strategy for reduction of blood pressure and proteinuria or albuminuria in patients with CKD already established on standard RAAS blockade. The risk of hyperkalaemia associated with this treatment combination is quantifiable and could be minimised by selecting appropriate patients for treatment and close monitoring of biochemistry, while use of nonsteroidal MRAs may further minimise this risk in the future. Well-designed, larger and longer-term studies are needed to determine the safety and efficacy of enhanced RAAS blockade on hard clinical endpoints such as progression to ESRD, all-cause mortality and cardiovascular outcomes.

4. Pilot Study

4.1 Introduction

This chapter will focus on local validation of the CKD273 biomarker for diagnosis of DN, as well as preliminary exploration of its relationship to subclinical organ damage.

4.1.1 Biomarkers

Biomarkers are defined as characteristics which are "objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [421]. The current guideline-endorsed biomarkers for diagnosis and monitoring of DN are albuminuria and eGFR [144] but for reasons already discussed in section 1.4 neither provide sufficient diagnostic sensitivity or specificity for early disease detection. This is particularly relevant as we enter the era of personalised medicine, driving to direct early targeted intervention towards the individuals at highest risk of progressive disease [373, 376, 380]. Although innumerable studies have described biomarkers with potential for preclinical prediction of DN no single candidate has yet made the leap from bench to bedside, as translation into clinical practice requires a number of key biomarker evaluation steps as shown in Figure 4-1 [379].

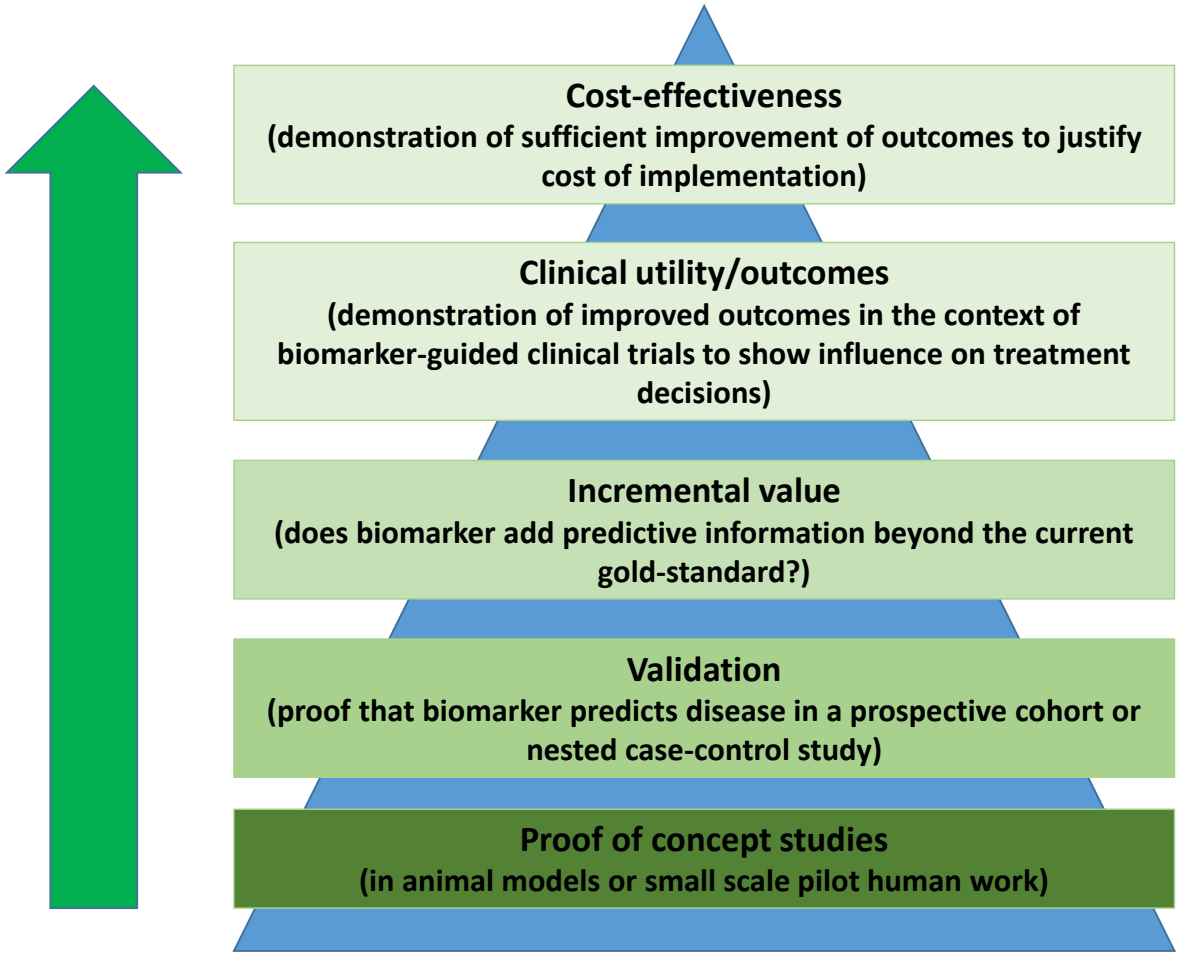

Figure 4-1. Stages of biomarker development.

Adapted from Currie G and Delles C. Current Diabetes Reports, 2016; 16: 104.

4.1.2 Studies evaluating the CKD273 biomarker

Use of proteomics to identify novel biomarkers for DN is an attractive concept, as multi-parametric classifiers have the potential to simultaneously capture numerous underlying mechanistic aspects of a heterogeneous disease process. As well as offering diagnostic advantages, use of proteomics also has potential for exploration of the pathogenesis of complex conditions. Studies evaluating the diagnostic and predictive performance of the CKD273 classifier in CKD patients and more specifically DN have already been described in section 1.6. To date, CKD273 is the urinary proteomic classifier that has been most rigorously

evaluated in a number of independent studies of both case-control and longitudinal design [422] and this progress is summarised graphically in Figure 4-2. It should be emphasised, however, that proof of clinical utility and health economic analysis have not yet been achieved.

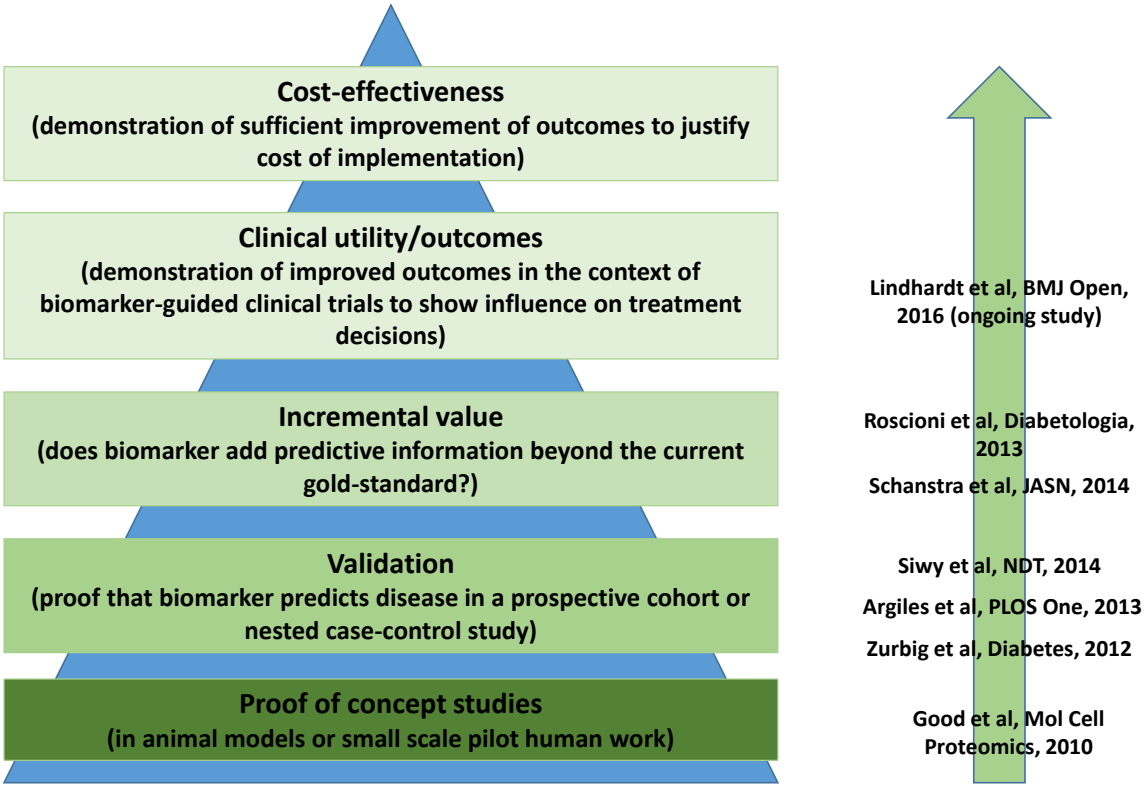

Figure 4-2. Progress in evaluation of CKD273 biomarker.

Illustration of published studies evaluating the CKD273 biomarker and progress through stages of biomarker evaluation.

4.1.3 Microalbuminuria as a cardiovascular biomarker

The evidence for use of MA as an indicator of renal disease is robust and it remains the gold standard clinical index of DN in routine practice. In addition, there is little doubt that MA also identifies individuals who are at high cardiovascular risk as shown by Mogensen (seminal paper published in 1984) [21]. In T2DM the presence of MA is associated with a 2.4-fold increased risk of cardiovascular death [423], and similar relationships are seen in the healthy and hypertensive populations [160, 424-426]. This risk is not determined by the categorical presence of MA *per-se*, but should rather be considered as a continuum which extends both beyond MA into the macroalbuminuric range [427]; as well as to even lower levels which are well within the normoalbuminuric range [145, 166].

The mechanisms which link MA with cardiovascular disease remain incompletely understood, and can be only partially explained by other cardiovascular risk factors such as smoking, hypertension and unfavourable lipid profile. As described in chapter 1, "The Steno Hypothesis" proposes that MA reflects generalised vascular dysfunction, simultaneously affecting the microvasculature supplying the retina and kidneys, as well as the intima of larger vessels. They reasoned that higher degrees of UAE were associated with transcapillary escape of albumin as an indicator of vascular permeability, and albuminuric patients were shown to have higher circulating levels of endothelial dysfunction markers compared to normoalbuminuric individuals matched for disease duration. These findings were independent of traditional risk factors such as blood pressure; diabetes duration and HbA1c and led the authors to postulate that difference in ECM composition and therefore increased permeability may offer a potential explanation [97]. Numerous publications have since described links between MA and both circulating and clinical endothelial function measures in patients with and without diabetes [105, 109, 428, 429]. Similarly, circulating markers of inflammation are associated with the presence and extent of MA [96, 103, 430].

Endothelial dysfunction and inflammation are important early events in the initiation and progression of atherosclerotic disease, and therefore offer a plausible link between MA and cardiovascular risk. As a potential early

biomarker of DN in normoalbuminuric patients, could CKD273 therefore simply be an indicator of generalised vascular dysfunction and early atherosclerotic disease rather than a specific renal biomarker? In a low-risk cohort of normoalbuminuric T2DM patients such as those participating in the PRIORITY trial, hard clinical outcomes will take many years to develop. As a first step, exploration of intermediate vascular phenotypes and circulating biomarkers may provide some useful information.

4.1.4 Assessment of subclinical organ damage

A number of clinical tools for measurement of early functional or morphological alterations in the vasculature in advance of diagnosis of overt cardiovascular disease have been developed, and some are now endorsed for stratification of intermediate-risk patients in clinical guidelines [431]. These are summarised in Figure 4-3. For the purposes of this study two of the less operator-dependent measurements were selected: central haemodynamics and carotid intima-media thickness (cIMT).

4.1.4.1 Central haemodynamic indices

During systole pressure waves are generated and propagate towards the peripheral vasculature. Eventually these waves are reflected backwards as a result of reduced vascular diameter; bifurcations or vascular stiffness gradients. Merging of these reflected waves with the antegrade wave lead to its amplification, with the result that peripheral pressures are often higher than central aortic pressure [432]. It is this central, rather than peripheral pressure that is exerted on the heart, brain and kidneys. As vessels stiffen the reflected wave arrives earlier, whereas with vasoconstriction its amplitude is altered. These processes result in increased central pressure and traumatic degeneration of the elastic component of the arterial wall [433].

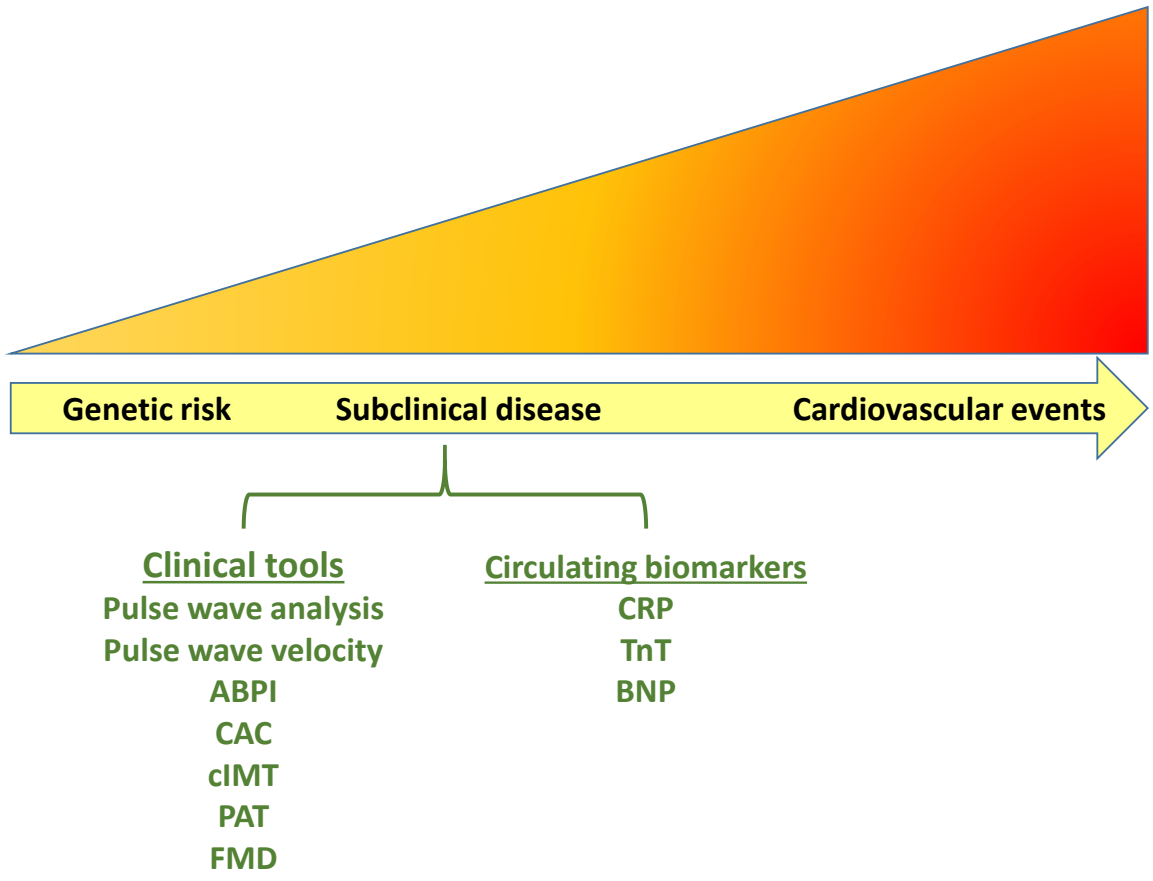

Figure 4-3. Markers of subclinical cardiovascular organ damage.

ABPI, ankle brachial pressure index; CAC, coronary calcium score; cIMT, carotid intima media thickness; PAT, peripheral arterial tonometry; FMD, flow mediated dilatation; CRP, C-reactive protein; TnT, high sensitivity cardiac troponin T; BNP, brain natriuretic peptide.

Central haemodynamics can be assessed by indices of central blood pressure as well as measures of wave reflection. These can be measured invasively, but in the context of clinical studies non-invasive methods are often preferable. **Pulse wave analysis** records pressure waves from carotid, radial or brachial arteries and uses a transfer function to extrapolate central pressure indices, augmentation pressure and augmentation index (AIx): the ratio of augmentation pressure to pulse pressure [434]. Many of these measurements can be made with validated automated devices; some have been shown to independently predict all-cause mortality and cardiovascular events; and antihypertensive therapies have shown that improvement in some of these parameters can translate into risk reduction [435, 436].

4.1.4.2 Carotid intima media thickness

Early in the development of atherosclerotic disease the vessel subintimal layer is infiltrated by lipids and inflammatory cells, a precursor to atheroma and vascular fibrosis. Imaging can be used to detect these early structural changes and a thickened intima-media complex serves as an indicator of atherosclerosis [437]. Measurement of cIMT and presence of plaque can be determined using B-mode transcutaneous ultrasound by measuring the distance from intima to adventitia, typically visible as a double-line on imaging of the arterial wall [438]. The European Society of Cardiology guidance specifies a measurement of 0.9mm as the cut-off for increased cIMT [439] however this marker is heavily influenced by age and therefore a single cut-off may lead to misclassification of some age-groups [437]. Many studies have demonstrated higher cIMT in individuals at cardiovascular risk [438], and its predictive power for future cardiovascular events is well established, with every 0.1mm increase in cIMT corresponding to 16% increased cardiovascular risk in one meta-analysis [440]. Changes in cIMT, however, do not appear to have a prognostic implication [440] and cIMT measurement has modest utility for reclassification of individuals at intermediate cardiovascular risk [441].

4.2 Aims

This study aimed to:

- validate the CKD273 urinary proteomic classifier for diagnosis of DN;
- explore the relationship between CKD273 and subclinical organ damage.

4.3 Methods

This section provides a general description of recruitment, protocols and techniques used in the conduct of this pilot study (Figure 4-4). A full description of the design of the PRIORITY trial can be found in chapter 2.

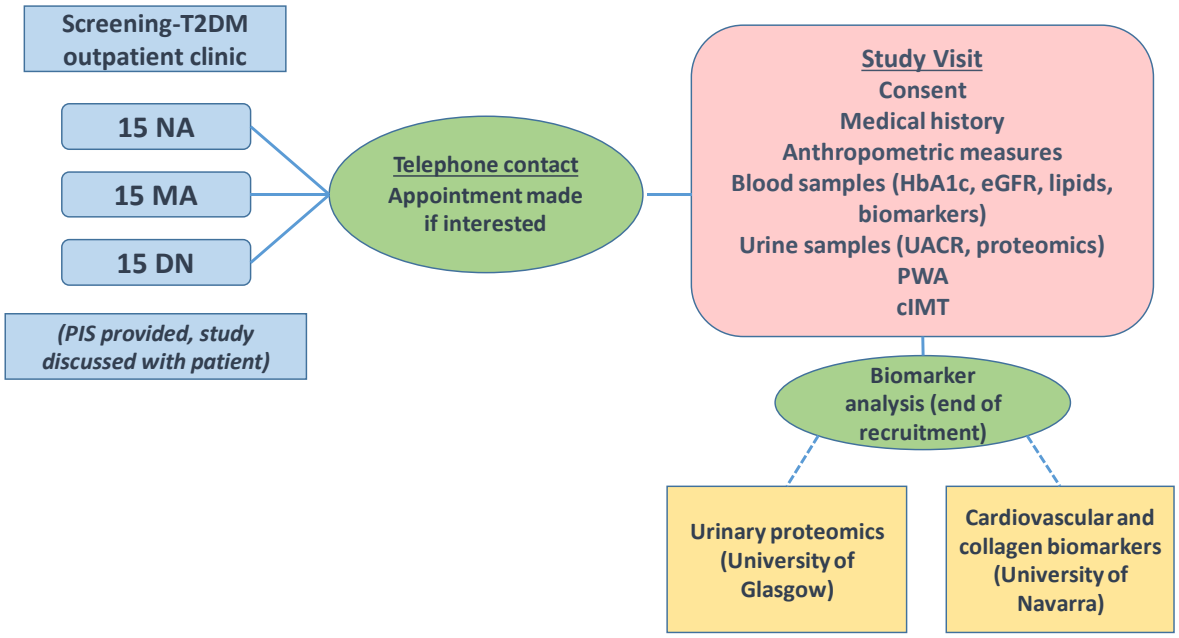

Figure 4-4. Pilot study outline.

T2DM, type 2 diabetes; NA, normoalbuminuria; MA, microalbuminuria; DN, diabetic nephropathy; PIS, participant information sheet; HbA1c, glycated haemoglobin; eGFR, estimated glomerular filtration rate; UACR, urine albumin: creatinine ratio; PWA, pulse wave analysis; cIMT, carotid intima-media thickness.

4.3.1 Ethical approval

The PRIORITY pilot study was approved by the West of Scotland Research Ethics Committee 4 (reference 13/WS/0154) and NHS Greater Glasgow and Clyde Research and Development (reference GN13CA187) in June 2013. The PIS can be found in (Appendix 8).

4.3.2 Participant selection and recruitment

Individuals with T2DM were identified from secondary care clinics at Stobhill Hospital in Glasgow in discussion with consultants and specialist nurses responsible for these clinics. For this pilot project a convenient sample size of 45 patients across the spectrum of DN was decided upon: 15 nonalbuminuric patients; 15 with MA and 15 with overt DN confirmed by consultant diabetologist and/or nephrologist. Of note, diagnosis of DN was primarily made on the basis of detection of albuminuria and/or reduced eGFR rather than by renal biopsy. Patients attending the clinic were approached by a member of the study team and provided with both verbal and written information about the project. Those who expressed an interest in participating were given an invitation letter as well as PIS, and an appointment was made for the study visit after a follow up telephone call within 1 week of the clinic appointment. Individuals with T2DM, aged between 18 and 75 years who were able to provide written informed consent and willing and able to attend a single study visit were deemed eligible to participate. Individuals with type 1 diabetes; any condition that would affect interpretation of clinical vascular studies (e.g. atrial fibrillation; ventricular bigeminy; cardiac pacemaker); uncontrolled blood pressure (>180mmHg systolic and/or >100mmHg diastolic); patients with anuric ESRD and those with non-diabetic CKD were excluded from the study.

4.3.3 Study visits

All study visits were held at the British Heart Foundation Glasgow Cardiovascular Research Centre in a quiet room, temperature controlled to 22-24°C. Participants were advised to abstain from caffeinated beverages for at least 6 hours prior to their scheduled appointment. Upon arrival, the PIS was discussed and any questions about the study were answered prior to obtaining informed consent (Appendix 9). Study procedures were then performed as described below.

4.3.3.1 Basic clinical parameters

A brief medical history and list of concurrent medications was obtained. Height and weight were recorded and body mass index (BMI) calculated. After being seated for 10 minutes resting heart rate and blood pressure were measured. Blood pressure was recorded using the Omron MX2 automated device, which was regularly calibrated by the medical physics department. The average of 3 consecutive readings was documented. If blood pressure was found to be above 140/90mmHg the patient was advised to see their GP for a repeat measurement within the next 2 weeks. Peripheral blood samples were then taken and participants were asked to provide a spot urine sample.

4.3.3.2 Pulse wave analysis

Pulse wave analysis was performed using the SphygmoCor system (Atcor Medical, Australia) with participant lying supine and the dominant wrist resting on a pillow in a slightly dorsiflexed position. Blood pressure was re-checked and this value input into the SphygmoCor software for reference. The radial pulse was palpated digitally, then the applanation tonometer (Millar Instruments, Houston, USA) was applied over the radial artery at the site of maximal pulsation. The tonometer was connected to a dedicated laptop computer for data collection and analysis. Once a satisfactory waveform was obtained sequential readings were recorded over a 9 second period before the proprietary software determined an average arterial waveform as shown in Figure 4-5. The software then calculated central blood pressure and AIx. As AIx is highly dependent on heart rate this value was also automatically corrected to heart rate of 75 beats per minute (AIx@75). The mean of 3 readings meeting the quality checks described below was recorded for analysis.

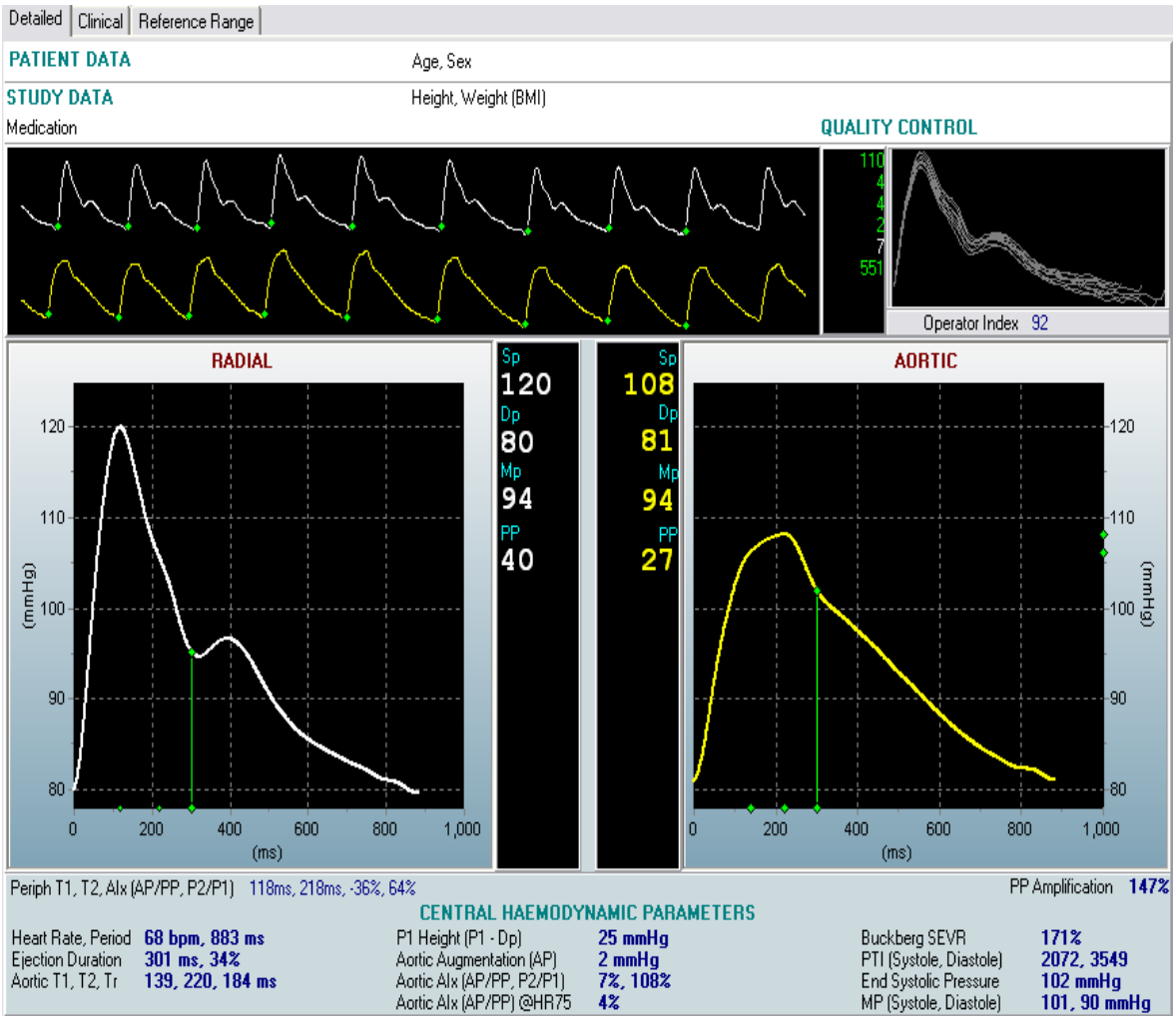

Figure 4-5. Pulse wave analysis.

Representative example of pulse wave analysis output generated using the SphygmoCor device (Atcor Medical, Australia).

The SphygmoCor software utilises an internal quality control measure termed the “operator index”, where a score of 100% is the highest achievable. Measurements where operator index was ≥ 80% were deemed acceptable for the purposes of this study. In addition any low amplitude waveforms were discarded. All measurements were performed by the author and prior to commencing the study 20 test measurements were reviewed by an independent observer to ensure they met acceptable standards.

4.3.3.3 Carotid intima media thickness

Carotid ultrasonography was performed in a dedicated, temperature controlled room using the Acuson Sequoia 512 scanner and L7 5-12 MHz linear array probe (Siemens, Erlangen, Germany). Participants were asked to remove clothing from their upper body and given a gown to wear in order to optimise exposure, they were then asked to lie in the supine position. Longitudinal brightness-mode (B-mode) still images and video clips were then recorded from the distal 1cm of the common carotid artery; carotid bulb; and internal carotid artery bilaterally; taking care to ensure the image was horizontal and to maximise the length over which the double-line pattern representing thickness of the tunica intima and media was visible. After the vascular images were obtained, Doppler flow velocity in the internal carotid artery was measured as a safety check to exclude significant stenosis. Following discussion with the local stroke unit lead sonographer it was decided that any participant where flow velocity was >1.25m/s (which could correspond to a > 50% stenosis) would be referred on for a confirmatory scan and then to the stroke service for further investigation and optimisation of cardiovascular risk where necessary. Each scan was saved in Digital Imaging and Communications in Medicine (DICOM) format for later offline measurement using the Syngo Arterial Health Package (Siemens Healthcare Limited, Surrey UK). All scans were performed by the author, trained in vascular ultrasound techniques at the Department of Vascular Medicine, Academic Medical Centre, Amsterdam. All images were read in batches by the author, blinded to participant identity. The mean cIMT was calculated for common carotid; bulb and internal carotid; and the overall mean of these results was also calculated for later data analysis. Figure 4-6 shows a sample cIMT measurement at the common carotid artery.

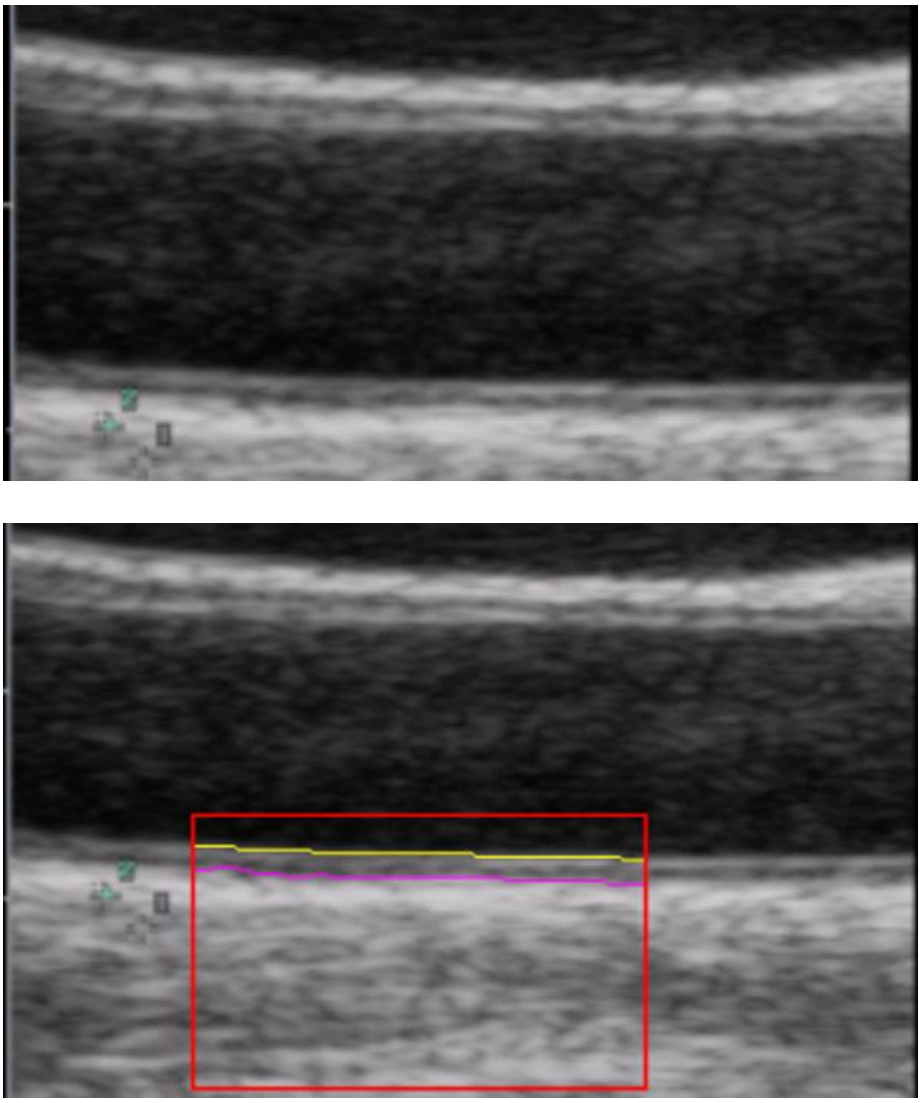

Figure 4-6. Carotid IMT scanning.

Upper frame shows longitudinal B-mode ultrasound view of the common carotid artery with classical “double line” representing the intima and adventitia. Lower frame shows automated measurement of same image using Syngo AHP software.

4.3.4 Urine samples

Each participant was asked to provide a spot urine sample in a sterile foil bowl. This was then split between a universal container and 3 x 1ml aliquots. The universal container was labelled with participant name, date of birth and community health index (CHI) number and sent to the local NHS Greater Glasgow and Clyde biochemistry lab at the Western Infirmary for measurement of UACR. The 3 x 1ml aliquots were then frozen at -80°C and stored for later analysis. Once the study was complete, 1ml of stored urine from each participant was used for CE-MS analysis as described in chapter 2. This was performed locally at the University of Glasgow by Dr Bill Mullen and colleagues.

4.3.5 Blood samples

Peripheral blood samples were taken from the antecubital fossa using a standard tourniquet and vacutainer system. Samples were collected as follows:

- 2 x 7ml serum tube
- 2 x 7ml EDTA tube
- 7ml Lithium heparin tube

One serum and one EDTA tube were each labelled with participant name, date of birth and CHI number before being sent to the NHS Greater Glasgow and Clyde Biochemistry laboratory at the Western Infirmary for measurement of electrolytes, eGFR and HbA1c. As the local laboratory only numerically reports those eGFR values that are <60ml/min/1.73m^2, eGFR was formally calculated using the MDRD formula. The remaining samples were prepared for storage as detailed below.

Upon arrival in the laboratory samples were centrifuged at 2700rpm at 4°C for 10 minutes. They were then aliquoted as follows:

- Serum tube - 3 x 500µL serum
- EDTA tube - 3 x 500µL plasma
- Lithium heparin tube - 2 x 500µL plasma

Samples were then labelled with participants unique study identifier only, and frozen at -80°C for later analysis.

4.3.5.1 Biomarker studies

STNFR 1 was selected as a novel predictor of DN, as described in chapter 1. Plasma levels were measured using enzyme-linked immunoassay (ELISA) (Life Technologies, Paisley, UK) by Jim McCulloch, chief laboratory technician.

A vast array of potential candidates relating to varied aspects of vascular pathophysiology have been proposed as biomarkers for refining cardiovascular risk prediction. High-sensitivity cardiac troponin T (TnT) and N-terminal pro brain natriuretic peptide (NT-proBNP) (both measured by ELISA, Roche Diagnostics, Basel, Switzerland) were selected as markers of cardiovascular risk for the purposes of this study. In addition, **CITP (**enzyme immunoassay; Orion Diagnostica, Espoo, Finland), **PICP** (ELISA, Quidel Corporation, San Diego, USA) and **TIMP-1** (ELISA, GE Healthcare, Little Chalfont, UK) were measured as indicators of collagen turnover.

- **NT-proBNP** is the precursor of biologically active BNP, which is secreted by cardiac tissue in response to stretch or damage. It is recognised not only as a marker of heart failure, but more recently also as a predictor of cardiovascular events in the general population as well as in individuals with pre-existing cardiovascular disease [442, 443].
- **TnT** is a sensitive and specific indicator of ischaemic myocardial damage and is widely accepted as a predictor of future cardiovascular outcomes

[444]. More recently the high sensitivity micronecrosis marker has also been demonstrated as a powerful predictor of mortality in a variety of patient groups [445, 446].

Cystatin C was also selected as a robust biomarker of renal disease, as described in chapter 1. Analysis was performed using ELISA (Biovendor, Brno, Czech Republic). Measurement of Cystatin C; hs-cTNT; PICP; CITP; and TIMP-1 was carried out at the University of Navarra, Pamplona, Spain by Dr Susana Ravassa.

4.3.6 Statistical analysis

Statistical analyses were performed using SPSS (SPSS, IMB Analytics, New York, USA) and Minitab16 (Pennsylvania, USA) software packages. Data distribution was assessed by manual inspection of histograms and the Kolmogorov-Smirnov test. Data that followed a normal distribution were expressed as mean ± standard deviation (SD), otherwise they were summarised using median and interquartile range. For continuous data differences between the 3 study categories were determined by one-way analysis of variance (ANOVA) if the data were normally distributed, or by Kruskall-Wallis test if not normally distributed. Tukey's family error rate was used to account for multiple comparisons. Two-sample t-tests and Mann Whitney U-tests were used for sub-group analysis on appropriately distributed data. Pearson's correlation coefficient was used to examine the univariate relationship between CKD273 classifier score and individual vascular parameters (using raw or transformed data as appropriate depending on distribution). P-values <0.05 were considered statistically significant.

4.4 Results

Forty five patients with T2DM consented to participate in this study between September 2013 and March 2014.

4.4.1 Clinical Characteristics

Table 4-1 shows the clinical characteristics of study participants. Patients with overt DN had a longer duration of diabetes; higher UACR and creatinine. Age; BMI; blood pressure; HbA1c; and eGFR were not significantly different across the 3 study categories. Number of current smokers was higher in the MA subgroup.

Variable	NA (n=15)	MA (n=15)	DN (n=15)	p-value
Age (years)	61±8	64±6	59±7	0.130
Gender (number males)	7	13	10	0.677
Current smokers	1	5	1	**0.024*
Diabetes duration (years)	8 (1-25)	11 (3-39)	20 (5-44)	**0.005*
BMI (kg/m²)	34.4±6.20	35.1±8.13	34.4±6.69	0.955
SBP (mmHg)	144±15	149±20	148±16	0.765
DBP (mmHg)	83±7	83±10	82±12	0.910
HbA1c (mmol/mol)	69 (41-108)	64 (47-120)	76 (43-120)	0.144
UACR (mg/mmol)	1.1 (0-3.3)	7.7 (2.6-22.5)	124.5 (0.8-412.6)	**<0.001*
Creatinine (µmol/L)	74 (55-106)	73 (4-129)	155 (81-777)	**<0.001*
eGFR (ml/min/1.73m²)	75.7±24.3	67.5±20.3	56.8±37.3	0.196

Table 4-1. Clinical characteristics of study participants according to DN status.

NA, normoalbuminuria; MA, microalbuminuric; DN, diabetic nephropathy; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; UACR, urine albumin:creatinine ratio; eGFR, estimated glomerular filtration rate. Data are mean±SD or median (range) depending on distribution. p-values are by one-way ANOVA or Kruskall-Wallis test depending on distribution of data for continuous variables, or by Fisher's exact test for categorical variables. * denotes statistical significance.

4.4.2 Validation of CKD273 for diagnosis of DN

There was an increase in CKD273 classifier score across study subgroups (normoalbuminuric, -0.169±0.373; microalbuminuric, 0.421±0.467; and overt DN 0.765±0.434; *p=<0.01* by one-way ANOVA). As shown in Figure 4-7, following correction for multiple comparisons CKD273 score was significantly different between normo- and microalbuminuric (-0.169±0.373 vs 0.421±0.467, p=0.011) and overt DN patients (-0.169±0.373 vs 0.765±0.434, p=<0.001). However the difference in classifier score between microalbuminuric patients and those with overt DN was not statistically significant (0.421±0.467 vs 0.765±0.434, p=0.121).

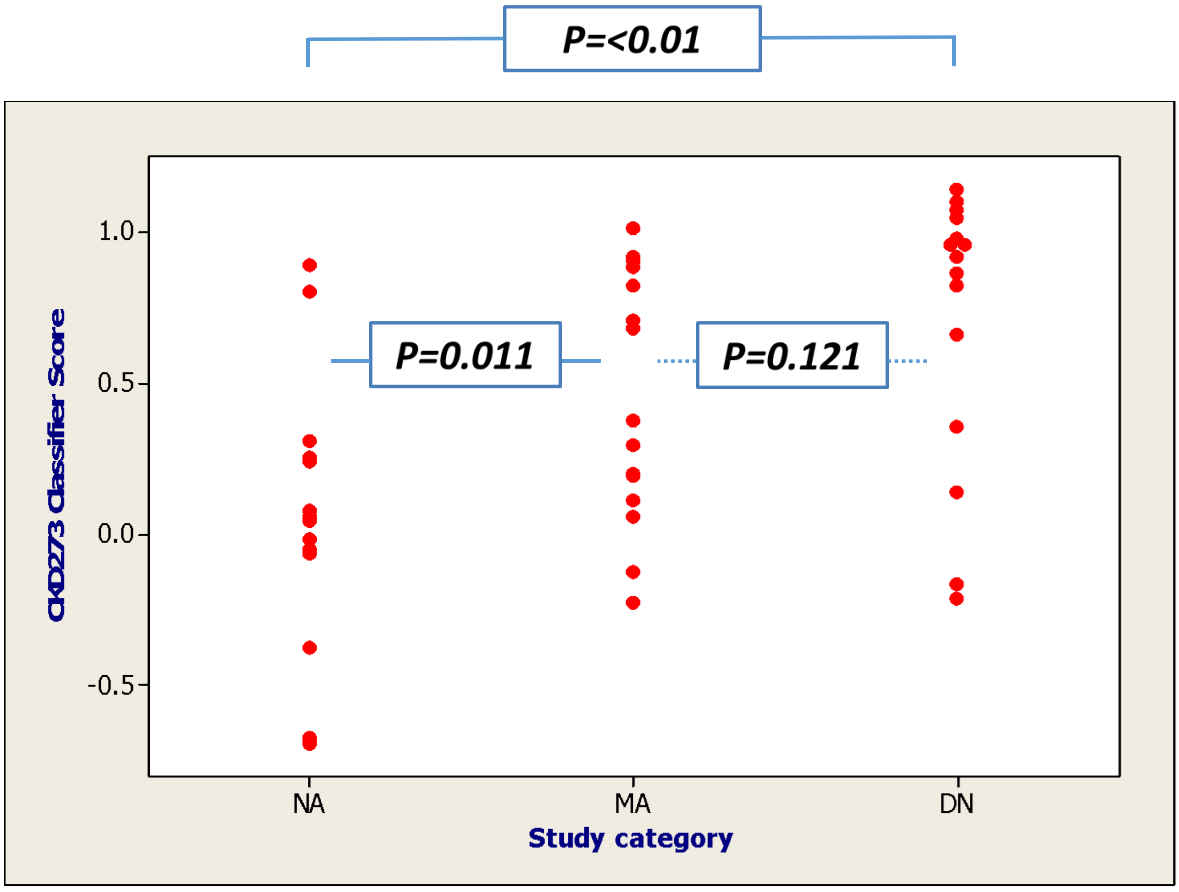

Figure 4-7. CKD273 classifier score across subgroups following correction for multiple comparisons.

NA, Normoalbuminuria; MA, microalbuminuria; DN, diabetic nephropathy. Comparison by one-way ANOVA with Tukey's correction for multiple comparisons. Solid lines indicate statistical significance, dotted line indicates non-significant difference.

There was a significant association between CKD273 and UACR as shown in Figure 4-8. The association with eGFR, however, was not statistically significant. Plasma STNFR1 and Cystatin C levels were measured as novel biomarkers of DN, as shown in Table 4-2. There was a significant association between CKD273 and both of these parameters as shown in Figure 4-9. Among traditional clinical parameters, CKD273 did not correlate with age (r=0.009, p=0.953); diabetes duration (r=0.264, p=0.08); BMI (r=0.088, p=0.564); systolic blood pressure (r=0.099, p=0.518); diastolic blood pressure (r=-0.064, p=0.674); or HbA1c (r=-0.122, p=0.426).

Variable	NA (n=15)	MA (n=15)	DN (n=15)	p-value
Cystatin C (ng/ml)	1019 (745-1386)	1146 (695-2014)	1746 (1003-5336)	**<0.001*
STNFR1 (ng/ml)	2.63 (1-49-3.57)	2.49 (1.52-5.29)	6.29 (4.2-29.5)	**<0.001*
NT-proBNP (ng/l)	68.6 (20.9-297)	151 (28-1432)	208 (50-2151)	**0.03*
TnT (µg/l)	0.01 (0.005-0.025)	0.015 (0.008-0.035)	0.025 (0.009-0.066)	**0.001*
Central pulse pressure (mmHg)	46±14	53±19	52±18	0.565
AIX@75 (%)	22 (7-38)	25 (9-32)	25 (4-35)	0.788
cIMT (mm)	0.77±0.20	0.80±0.12	0.74±0.13	0.594
CITP (ng/ml)	5.1 (2.9-12.9)	6.4 (2.3-14.5)	8.9 (0-44.8)	**0.018*
PICP (ng/ml)	66.2 (40.9-154.3)	79 (36.9-163.6)	127.5 (52.1-387.6)	**0.011*
TIMP-1 (ng/ml)	874.9 (92-1218)	1003.1 (507.4-1170)	1298 (889-2609)	**<0.001*

Table 4-2. Clinical characteristics of study participants according to DN status.

NA, normoalbuminuria; MA, microalbuminuric; DN, diabetic nephropathy; STNFR1, soluble tumour necrosis factor 1; NT-proBNP, n-terminal pro-brain natriuretic peptide; TnT, high sensitivity cardiac troponin T; AIX@75, augmentation index corrected for heart rate 75 beats per minute; cIMT, carotid intima media thickness; CITP, telopeptide of collagen type 1; PICP, procollagen type 1 carboxyterminal propeptide; TIMP-1, tissue inhibitor of metalloproteinase 1. Data are mean±SD or median (range) depending on distribution. P-values are by one-way ANOVA or Kruskall-Wallis test depending on distribution of data for continuous variables. * denotes statistical significance.

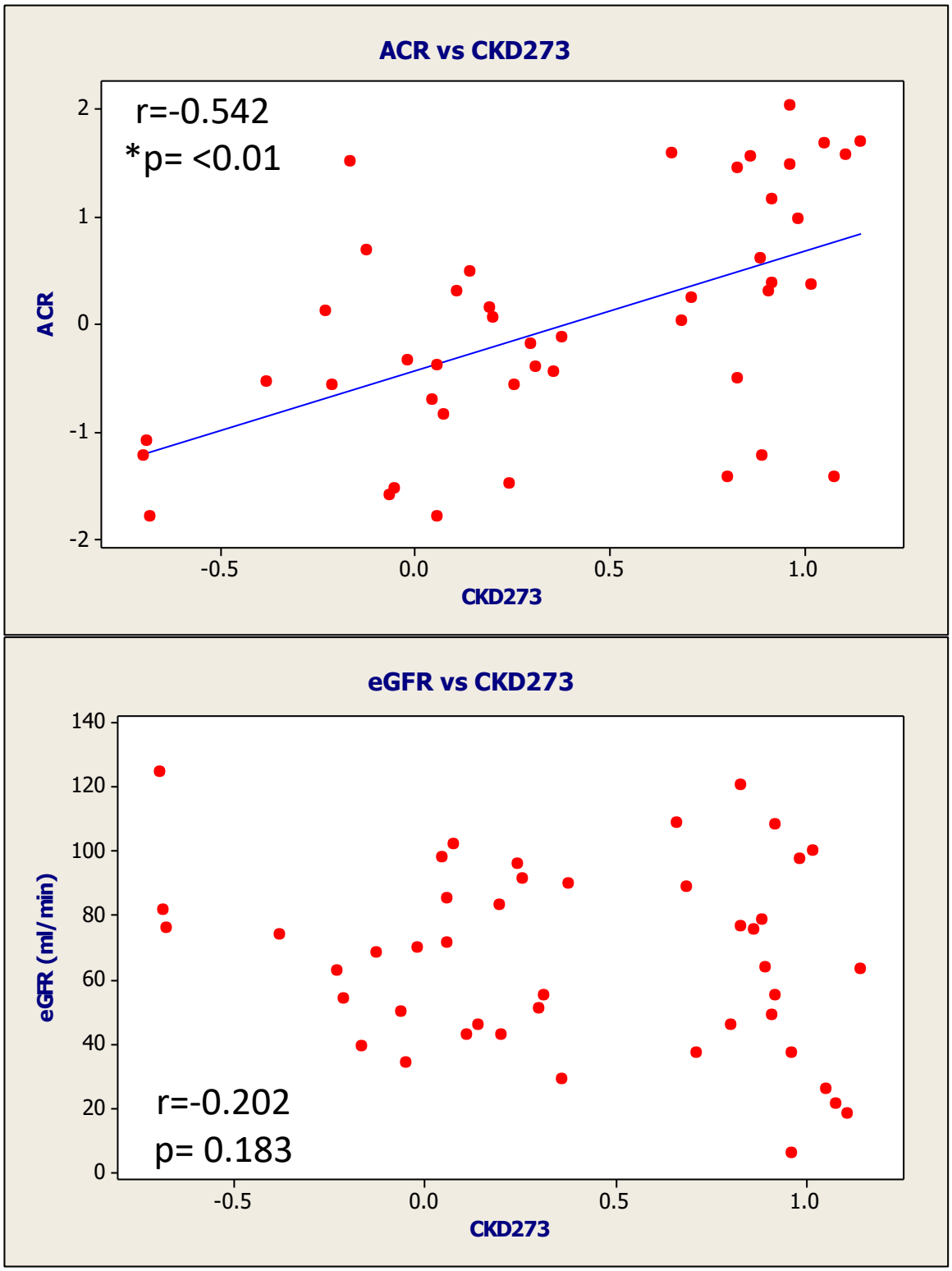

Figure 4-8. Association between CKD273 classifier and clinical indices of DN.

Correlations by Pearson's method using raw data for eGFR and transformed data for ACR. ACR, albumin: creatinine ratio; eGFR, estimated glomerular filtration rate. * denotes statistical significance.

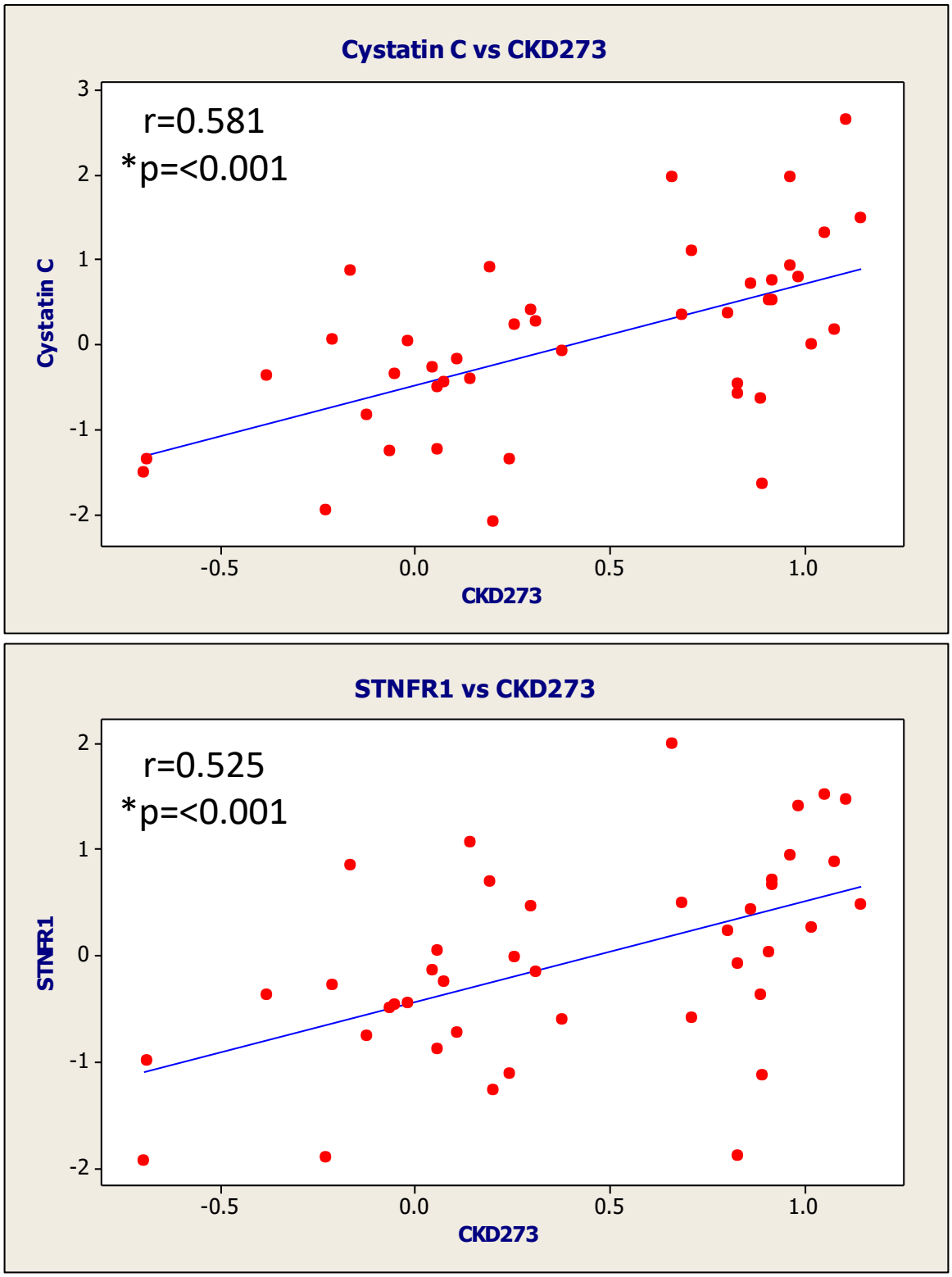

Figure 4-9. Association between CKD273 classifier and novel renal biomarkers.

Correlation by Pearson's method on transformed data. * denotes statistical significance. STNFR1, soluble tumour necrosis factor receptor 1.

Plotting a receiver operating curve, as shown in Figure 4-10, confirmed that 0.343 was the near-optimal threshold for diagnosis of DN in this test cohort, with sensitivity of 80% and specificity of 77%.

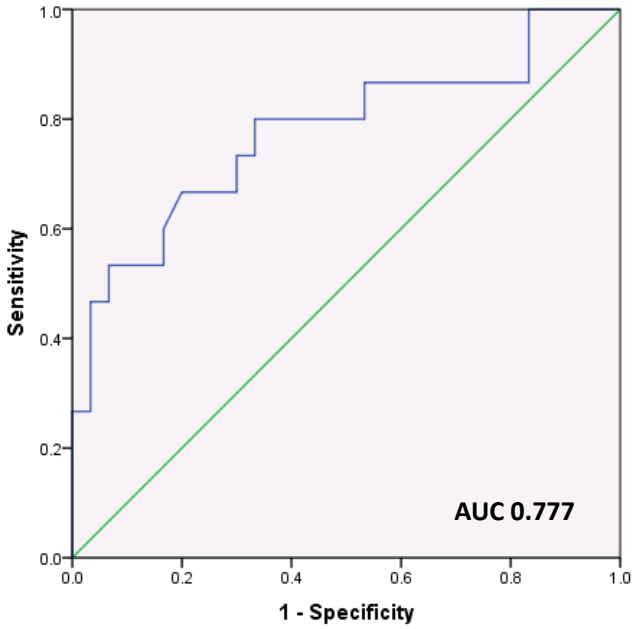

Figure 4-10. Sensitivity and specificity of CKD273 for diagnosis of DN determined by receiver operating curve.

AUC, area under the receiver operating curve.

4.4.3 CKD273 and vascular parameters

In order to explore the relationship between CKD273 classifier and subclinical vascular damage a number of clinical vascular phenotypes and circulating biomarkers were measured. Clinical markers of subclinical organ damage were not significantly different between study subgroups, as shown in Table 4-2. Nor were these correlated with CKD273 classifier score (r=0.109, p=0.478 for central pulse pressure; r=-0.199, p=0.196 for AIX@75; r=0.006, p=0.998 for cIMT). Circulating cardiovascular biomarkers, NT=proBNP and TnT, were significantly different across study categories as shown in Table 4-2. Significant associations were seen between these biomarkers and CKD273, as demonstrated in Figure 4-11.

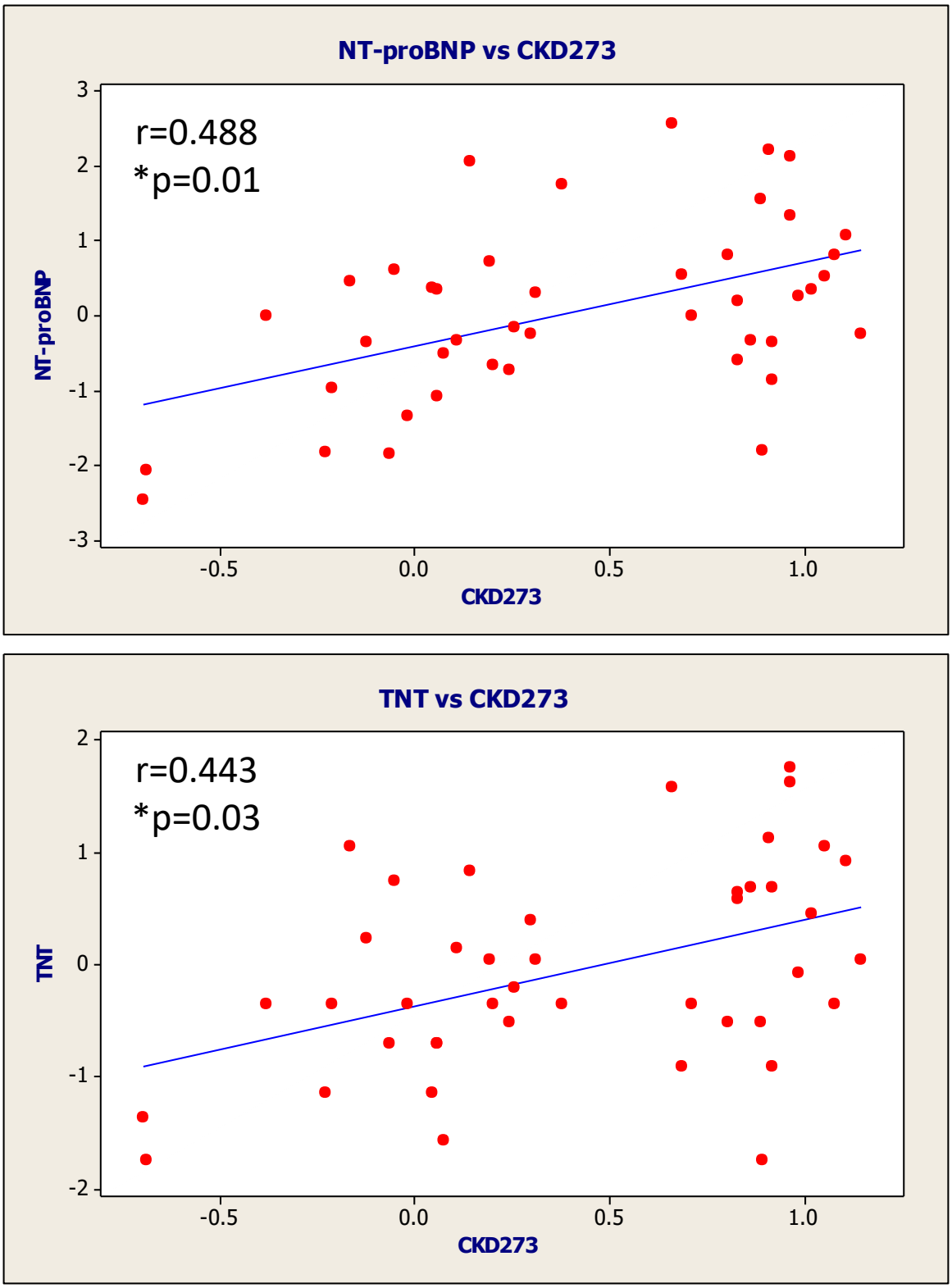

Figure 4-11. Association between CKD273 classifier and circulating cardiovascular biomarkers.

Correlation by Pearson's method on transformed data. * denotes statistical significance. NT-proBNP, N-terminal pro-brain natriuretic peptide; TnT, high sensitivity cardiac troponin T.

To offer potential mechanistic insights into these findings a selection of circulating markers of collagen turnover were measured. Results shown in Table 4-2 demonstrate that these markers were also significantly increased across study subgroups. Moreover, there were significant associations between these markers and CKD273 classifier score as shown in Figure 4-12.

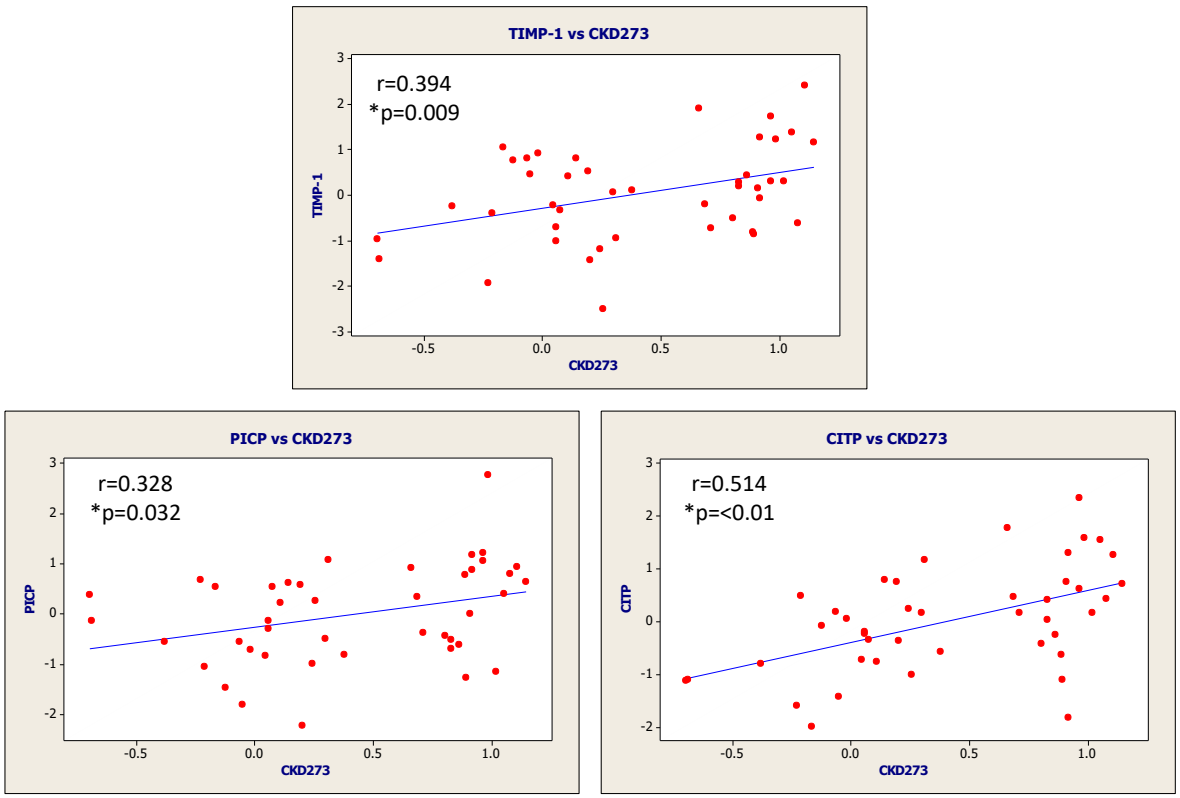

Figure 4-12. Association between CKD273 classifier and markers of collagen turnover.

Correlation by Pearson's method on transformed data. * denotes statistical significance. TIMP-1, tissue inhibitor of metalloproteinase 1; PICP, procollagen type 1 carboxyterminal propeptide; CITP, telopeptide of collagen type 1.

4.5 Discussion

The findings detailed above confirm that the CKD273 classifier is able to reliably distinguish T2DM patients with DN from those with normoalbuminuria. As expected, classifier score increased across the study subgroups which reflected the spectrum of disease. The difference between the MA and DN groups, however, did not reach statistical significance: this could be explained by small sample size; or alternatively may reflect the fact that CKD273 is being evaluated as an early marker of disease, thereby its utility in distinguishing two groups of patients who already have some evidence of established disease may be limited.

Previously published work established a CKD273 classifier cut-off of 0.343 for diagnosis of DN [268]. This has since been confirmed in several additional studies [295, 298] and based on receiver operating curve analysis the results described above are in keeping with this. Siwy et al recently demonstrated in 165 samples across multiple centres that this threshold accurately distinguished DN from control samples, with only 18 of 165 samples being misclassified. Receiver operating curve analysis in this study showed area under the receiver operating curve (AUC) of 0.95 for CKD273 [298]. From local analysis AUC for CKD273 was 0.77, again this may reflect limited sample size as other larger studies have confirmed higher AUC [268, 295]. Two out of the 15 (13.3%) normoalbuminuric patients had CKD273 scores above the threshold for detection of DN. Previous studies have shown that around 20% of normoalbuminuric patients can be expected to fall into the "high risk" category [268, 295, 298]; however it is impossible to speculate on the predictive power of the classifier in these patients based on a small cross-sectional study.

Collagen fragments are abundantly represented within the CKD273 classifier [268]. As described in chapter 1, altered collagen turnover is seen early in the pathogenesis of DN, and this pathway could offer one mechanistic explanation for the utility of CKD273 in early DN detection. As expected, markers of collagen turnover increased progressively across study subgroups and significant associations were demonstrated between CITP, PICP, TIMP-1 and the CKD273 classifier. It is tempting to speculate, therefore, that the classifier is detecting early collagen turnover within the GBM and mesangium however this may be an

oversimplification as it is well established that altered collagen turnover also plays a key role in the initiation and progression of other conditions, in particular cardiovascular disease [447, 448]. Moreover fragments of types 1 and 3 collagen are key components of the CKD273 classifier and these are typically associated with vascular rather than renal disease. Further exploration on the relationships between the CKD273 classifier and vascular and renal phenotypes is therefore warranted.

As expected, there was a significant association between CKD273 and UACR. Certainly albumin fragments are a component of the classifier [268], and by and large the clinical diagnosis of DN was based on albuminuria in this cohort. For this reason it is perhaps not surprising that no direct association with eGFR was found, as participants in the DN group had relatively preserved renal excretory function. Previous studies have confirmed a negative linear relationship between CKD273 score and eGFR [289, 290]. In view of the albuminuria-based discrimination between study subgroups it is not possible from this small sample to determine whether the CKD273 classifier captures multiple aspects of DN pathogenesis, or is mainly driven by albuminuria. For this reason exploration of its relationship to additional renal markers was a logical step. Cystatin C and STNFR1 were selected as two novel early biomarkers for CKD which have arguably the most robust evidence behind them to date [170, 171, 224, 225] and associations were demonstrated between these biomarkers and CKD273. It is generally expected that a proposed novel renal biomarker would correlate with other renal indices in a cohort of patients representing the spectrum of disease; the CKD273 classifier therefore is providing information that is already to some degree captured by traditional and novel clinical indices of renal disease. However, the correlation coefficients seen in this study are modest, suggesting that a significant proportion of the variability in CKD273 score cannot be explained by these existing biomarkers and the associated pathophysiological mechanisms alone.

No association was seen between CKD273 classifier and clinical measures of vascular structure and function nor did these measures differ between study categories. The lack of a significant difference in clinical markers of vascular damage between study subgroups is of uncertain significance. Again, this may

simply be an issue of sample size. In addition, measurement such as cIMT and AIx are generally recommended for restratification of "intermediate risk" individuals [431, 437]. The diagnosis of diabetes and presence of either MA or clinical albuminuria in the majority would certainly place them in a "high risk" category, rendering these vascular measures less useful. In addition, we cannot discount the effect of preventative therapies such as statins or RAAS blocking agents which were widely prescribed among the MA and DN subgroups.

It is possible that a degree of selection bias was also introduced by recruiting these participants exclusively from secondary care clinics. In general, T2DM patients are managed in primary care, with only those requiring more intensive risk factor control or developing complications being referred to hospital outpatient clinics, meaning that recruitment of higher risk normoalbuminuric patients into this subgroup is a possibility. Despite the lack of association with clinical measures of vascular structure and function, CKD273 classifier score was correlated with circulating cardiovascular biomarkers. Of course it should be borne in mind that these biomarkers are renally excreted, and so some degree of correlation with renal indices is to be expected. However, TnT and NT-proBNP predict cardiovascular outcomes independent of GFR [449] and it remains unclear therefore, whether CKD273 is identifying a degree of generalised microvascular disease affecting not only the kidney but other vascular beds such as the coronary microcirculation for example.

This study was limited by several factors. The small sample of 45 patients was decided upon for convenience as a pilot project, but is undoubtedly underpowered to draw clinically meaningful conclusions. In addition pooling results from three selected groups of patients to determine Pearson correlations may limit meaningful interpretation of these data, although this is perhaps less relevant given that no associations with vascular phenotypes were seen. The cross-sectional nature of the project means that it is impossible to determine the predictive power of CKD273 or its usefulness in informing therapeutic decision-making and this will be formally evaluated in the PRIORITY trial [380]. The albuminuria-based classification of patients is a confounding factor in interpretation of the clinical vascular data, and as the next progressive step evaluation of the relationship of these markers to CKD273 in a clean,

homogeneous cohort without the influence of albuminuria would be more useful. Furthermore, PWV rather than AIx is generally accepted as the “gold standard” non-invasive method for determination of arterial stiffness in the general population as well as different disease states [450] and this measurement may offer more clinically meaningful results.

5. Baseline characteristics of PRIORITY study cohort

5.1 Introduction

Recent years have seen a surge of interest in multimarker "omics" technologies for early detection of a variety of clinical conditions; fuelled in part by the concept that these panels have the potential to simultaneously capture changes in many disease-specific pathways simultaneously as well as the drive towards "precision medicine" - offering targeted treatments towards individuals at highest risk of disease. Although a multitude of proteomic panels have been described, the leap from bench to bedside is a long way off while reliable validation and health economic analyses are lacking. The CKD273 urinary proteomic biomarker is arguably the most rigorously evaluated proteomic biomarker to date and has been shown in small, retrospective analyses to have the potential to predict clinical DN in advance of any detectable changes in UAE or eGFR [268, 295, 296]. This could be of particular benefit as many patients continue to progress towards ESRD despite intervention with evidence-based therapies; and while prescribing of these agents for disease prevention in a healthy, unselected population is associated with delay in the onset of MA, this comes at the cost of an increased burden of side effects and adverse events [376].

The stages of biomarker evaluation have been described in chapter 4. In brief, association alone is insufficient to justify translation into the clinic. The ultimate proof of predictive potential in clinical practice is prospective validation in a large cohort; where therapy is guided by the biomarker in question. The PRIORITY trial is a unique proteomic biomarker-guided observational study and randomised controlled trial designed to determine the utility of CKD273 for early diagnosis and informing preventative interventions in DN [380]. The study is based on the rationale that it is difficult to identify the individuals who are at highest risk of progression to DN based on the current "gold standard" clinical markers, UAE and eGFR, which have been evaluated in detail in chapter 1. If the assumption that CKD273 does indeed have the potential to identify patients at the highest risk of progression to DN proves

correct, then detailed phenotyping of these individuals is of interest. They are normoalbuminuric with preserved eGFR and would be considered “low risk” from a clinical standpoint; yet their urinary proteome reflects alterations in clinically silent processes driving the development of CKD. Characterising these patients would serve several key purposes. First; this may improve our understanding of the pathogenesis of the condition, many aspects of which remain elusive. Second; a significant proportion of these proteomic panels are comprised of collagen fragments which can be altered in a multitude of disease processes and it is difficult therefore to determine the specificity of these classifiers for the disease process in question. Finally; proteomic technologies are expensive and not widely available, therefore if there is some other characteristic which sets these “high risk” individuals apart and can be measured more easily at a lower cost this too would be valuable information.

Much of the work described in this thesis was performed in parallel to the PRIORITY trial with these precise aims in mind. In the small cohort of patients with and without DN described in chapter 4, cross sectional exploration of determinants of the CKD273 classifier did not reveal any strong associations with traditional clinical parameters other than UACR. However, this work was on a small scale and confounded to an extent by the presence of varying degrees of CKD and UAE. A more detailed exploration of the relationship between CKD273 classifier score and markers of subclinical organ damage in a larger homogeneous cohort can be found in chapter 6. Participants in the studies described in chapter 6 were part of the PRIORITY cohort, however without comparing them to the trial cohort as a whole the local findings within Glasgow cannot be generalised. At the time of writing recruitment into the trial was ongoing, therefore a full description of the entire cohort at baseline is not feasible. This chapter will instead describe the composition of the entire PRIORITY study population in comparison to the local cohort at the end of the author’s active PhD studies.

5.2 Aims

This study aimed to:

- describe baseline characteristics of the PRIORITY trial population at end of January 2016 and compare these to the Glasgow trial cohort at the same time point in order to identify and potentially significant differences;
- explore traditional clinical parameters as determinants of CKD273 classifier score at baseline in the Glasgow trial cohort only, as doing so across the whole study population at this stage has the potential to introduce recruitment bias and compromise the integrity of the project.

5.3 Methods

The design and conduct of the PRIORITY trial has been extensively described in chapter 2. This section will therefore focus on methodology specific to collection, assimilation and analysis of the baseline data at end of January 2016.

5.3.1 PRIORITY study data access

All clinical data collected for participants of the PRIORITY trial were entered into a bespoke electronic case report form by local study team members. The system was designed by the team at Hannover Clinical Trials Centre (HCTC), a contract research organisation responsible for data management throughout the trial period. As a member of the study steering committee, the author approached the trial chief investigator and other committee members in January 2016 to request access to stored baseline data for all sites in order to conduct the analysis described in this chapter. The issue was discussed among steering committee members and it was agreed on the same date that a limited descriptive analysis could be performed. An arbitrary cut-off date of 31st

January 2016 was agreed upon to coincide with the end of the author's clinical PhD studies. Trial recruitment was ongoing at the time of writing.

5.3.2 Data extraction

After the required data were agreed upon by the consortium, data managers at HCTC extracted these from the central study database. Each required field was extracted as an individual password-protected Statistical Analysis System (SAS) file containing individual patient data for each participant in the trial, identified only by unique study ID which allowed identification of Glasgow participant data. These were then imported into SPSS (SPSS, IBM Analytics, New York, USA) individually for descriptive analysis.

5.3.3 Challenges encountered with data handling

Every effort was made to focus on data obtained at the study baseline visit to avoid including data from participants who subsequently failed screening or withdrew consent in advance of formal inclusion into the trial. This was not possible for all variables however due to both the structure of the database and the fact that some measures (such as height, weight and BMI) were only taken at screening. In view of the fact that the database was composed of a number of individual data files it was not possible to include only baseline data for each observation without merging each individual file into a "master" database. This was decided against for several reasons:

- This would have offered the author analysis options beyond the descriptive work agreed upon by the study steering committee and therefore it was decided not to manipulate the data further in that respect.
- As recruitment was ongoing at the time of writing, no full data clean-up had yet been performed. As a result a number of issues with the data

were identified during this exercise including: a variety of units being used for certain measurements (e.g. mmol/mol vs % for HbA1c) across different centres; varied reference values across centres; different sample types (e.g. serum versus plasma) and occasional errors with implausible values for some clinical observations. Given that the data were not finalised construction of a master database would have been inappropriate.

- This work was not intended to be a formal interim analysis on a clean, finalised dataset.

For the reasons described above it was therefore not possible to limit this descriptive analysis to a full cohort of patients with all data points available. In addition, the completeness of the data is dependent upon local data entry at study sites and the author or the team at HCTC had no influence over this process. As a result, the numbers of participants included in each sub-analysis for individual data points varies for different parameters. To make this clear the number of participants included in each sub-analysis is given in Table 5-1.

	Whole cohort	Whole cohort (excl. Glasgow)	Glasgow only	P-value
Age (yrs)	63 (24-74) n=1153	63 (27-74) n=945	62 (24-74) n=208	0.517
Gender (males/females)	725/428 n=1153	581/364 n=945	144/64 n=208	*0.036
Diabetes duration (yrs)	11.0 (0.3-52.4) n=1152	11.1 (0.3-52.4) n=944	9.8 (0.6-33.1) n=208	*0.001
RAAS blockade (Y/N)	903/557 (61.8%) n=1460	738/453 (62%) n=1191	165/104 (61.3%) n=269	0.848
Current smokers (%)	191/932 (17%) n=1123	165/802 (17.1%) n=967	26/130 (16.7%) n=156	0.903
Retinopathy status (none/simplex/ proliferative)	914(83%)/152(14%) /40 (4%) n=1106	776(85%)/102(11%) /37 (4%) n=915	138(72%)/50(26%) /3 (2%) n=191	*<0.001
Height (cm)	172 (139-205) n=1136	172 (139-205) n=939	170 (146-192) n=197	*<0.001
Weight (kg)	88 (47-164) n=1136	88 (47-164) n=939	88 (54-146) n=197	0.555
BMI (kg/m^2)	29.7 (17.9-57.1) n=1136	29.4 (17.9-57.1) n=939	30.9 (21-48.9) n=197	*<0.001
SBP (mmHg)	134 (94-183) n=1124	133 (97-177) n=931	136 (95-183) n=193	*<0.001
DBP (mmHg)	79 (49-109) n=1124	83 (49-101) n=931	79 (51-109) n=193	0.790
Heart rate (bpm)	75 (47-116) n=1124	74 (47-111) n=931	78 (51-116) n=193	*<0.001
HbA_1c (mmol/mol)	56 (31-115) n=1107	55 (31-115) n=917	61 (36-106) n=190	*<0.001
Cholesterol (mmol/l)	4.22 (1.3-8.6) n=1100	4.2 (1.3-8.6) n=910	4.3 (2.3-7.6) n=190	0.563
LDL (mmol/l)	2.2 (0.2-5.7) n=957	2.2 (0.2-5.7) n=784	2.1 (0.8-4.8) n=173	0.064
HDL (mmol/l)	1.2 (0.3-2.6) n=1085	1.2 (0.3-2.6) n=909	1.1 (0.6-2.4) n=176	0.492
Triglycerides (mmol/l)	1.6 (0.1-14.4) n=1107	1.6 (0.1-14.4) n=917	1.9 (0.7-4.6) n=190	*<0.001
eGFR ($ml/min/1.73m^2$)	90.6 (44.9-168.3) n=1111	90.7 (44.9-168.3) n=921	90.4 (46.2-123.4) n=190	0.175
ACR (mg/g)	5.3 (1.14-46.84) n=1189	5.3 (1.14-46.84) n=977	5.2 (1.33-34.51) n=212	0.868
CKD273 score	-0.334 (-1.50-0.94) n=1194	-0.358 (-1.50-0.94) n=982	-0.228 (-1.21-0.71) n=212	*<0.001
High/low risk (% high risk)	149/1045 (12.5%) n=1194	120/862 (12.2%) n=982	29/183 (13.7%) n=212	0.560

Table 5-1. Comparison of baseline characteristics between Glasgow participants and whole PRIORITY study cohort as of 31st January 2016.

Data are median (range). Comparisons between Glasgow cohort and remaining participants are by Mann Whitney U test. Categorical variables compared by chi-squared test. n=number of participants for which data available in each sub-analysis. P-value refers to comparison between Glasgow population and whole study cohort where * denotes statistical significance. eGFR calculated using CKD-EPI formula. CKD273 threshold for diagnosis of DN 0.343; for classification into PRIORITY study "high risk" group 0.154. RAAS, renin angiotensin aldosterone system; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein; eGFR, estimated glomerular filtration rate; ACR, albumin: creatinine ratio.

5.3.4 Determinants of CKD273 classifier

In order to avoid performing a formal interim analysis, and for the reasons described above pertaining to challenges encountered during data assimilation, it was decided that exploration of the determinants of CKD273 score would be limited to data from those participants in Glasgow who had been included in the trial between April 2014 and May 2016. Throughout this period the author had been responsible for overseeing participant recruitment into the study and these data were readily available in clean format locally as they had been collected in the implementation and analysis of the sub-studies conducted in parallel with the main trial, described in chapter 6.

5.3.5 Statistical Analysis

- **Comparison of Glasgow subset to whole PRIORITY study cohort as of January 2016**. Statistical analysis was performed using SPSS (SPSS, IBM Analytics, New York, USA). Distribution of data was determined by the Kolmogorov-Smirnov test as well as manual inspection of histograms and Q-Q plots. None of the data were normally distributed, therefore they were expressed as median (range). Comparisons between the whole PRIORITY study cohort and the Glasgow subset were made using the Mann-Whitney U test. Data from other centres were analysed only as part of the full study cohort as recruitment was ongoing and introduction of recruitment bias would therefore be a possibility; some centres had very small participant numbers and therefore individual centre comparisons would not be meaningful. P-values <0.05 were considered statistically significant.

- **Exploration of determinants of CKD273 classifier in the Glasgow sub-population described above**. Distribution was determined as already detailed. Data are given as median (range) or mean ± SD as appropriate. Correlations were performed using Pearson's method on raw data where distribution was normal, or on transformed data where this was not the case. General regression modelling was used to adjust analyses for confounding variables where necessary. P-values of <0.05 were deemed significant as above.

5.4 Results

The data described below are from the dataset extracted from the study database based on the predetermined cut-off of 31st January 2016. The completeness of the dataset is very dependent on timely and accurate data entry at each specific study site. As expected, there are a number of missing data points which may either result from delayed data entry, or the fact that study baseline visits have yet to be arranged for some participants. As agreed at the consortium meeting in January 2016, the author was granted access to these data for the purposes of a descriptive analysis only and therefore the dataset was not manipulated or merged in any way following extraction in Hannover.

5.4.1 Screened patients and screening failures

By 31st January 2016 there were data pertaining to 1475 study screening visits available on the study database. Table 5-2 shows the number of screened patients per study centre. At the time of writing Glasgow was the second highest recruiting centre in the consortium, as shown in Figure 5-1. Of these 1475 patients, 1327 had results of run-in sampling for UACR available on the central database on 31st January 2016. Ninety-eight (7%) were MA positive and 1189 were normoalbuminuric. UACR data had not yet been input for 40 participants. Of those who screen-failed as a result of microalbuminuria at run-

in, the median UACR was 48.5 (20.4-617) mg/g. Of note, MA was defined as UACR greater than 30mg/g in 2 of 3 consecutive run-in samples, therefore a participant could have geometric mean UACR from all 3 samples combined which fell below the 30mg/g threshold but still be excluded from the study. The total number of screening failures for any reason at 31st January 2016 was 305 (21%). This explains the discrepancy between the number of patients screened (1475) and the number of data points shown for each individual descriptive analysis, of which the majority are considerably lower. In addition, as detailed earlier a number of measures are only taken at screening, and therefore a higher number of data points were available for these parameters.

Centre	Patients screened
Steno Diabetes Centre, Gentofte	378
University Medical Centre, Groningen	167
University of Glasgow	**269**
Istituto de Ricerche Farmacologiche Mario Negri, Bergamo	157
Universita Karlova v Praze, Prague	74
Geniko Nosokomeio Athinas Ippokrateio, Hospital Diabetes Centre, Athens	75
Institut Klinické a Experimentální Mediciny, Prague	54
Instituto de Investigación Sanitaria de la Fundación Jiménez Díaz, Madrid	8
Klinikum st. Georg Gmbh, Leipzig	41
Cyril and Methodius University, Skopje	170
Diabetes Vascular Research Foundation, Hoogeveen	25
Universitair Ziekenhuis, Gent	20
University Medical Centre, Hoorn	37
Total	**1475**

Table 5-2. Number of screened patients per study site on January 31st 2016.

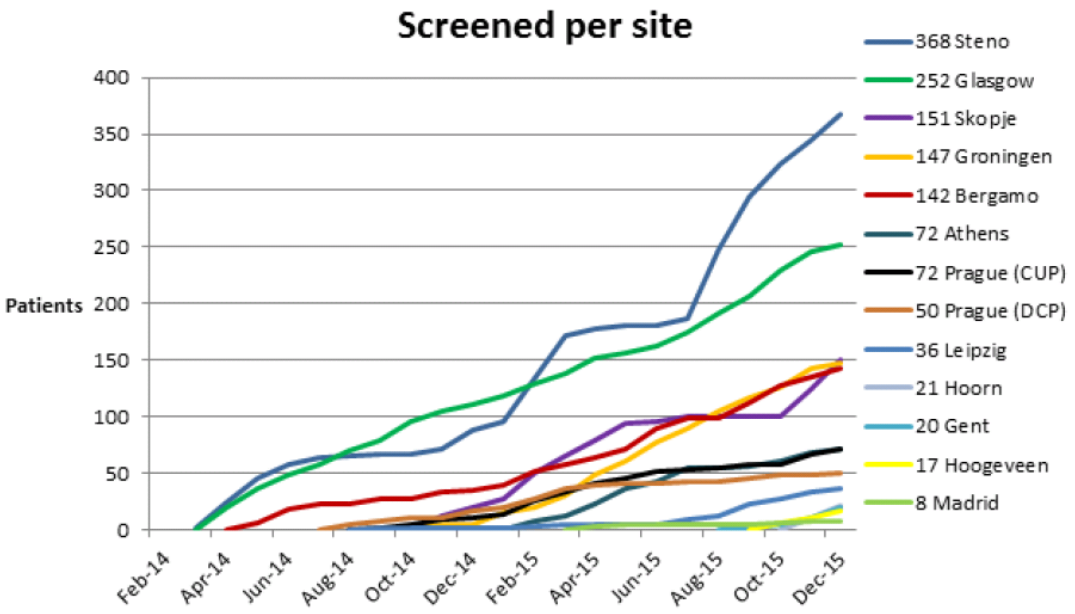

Figure 5-1. Illustration of recruitment progress per site taken from PRIORITY newsletter, January 2016.

This figure is taken from the consortium newsletter in February 2016. The number of participants included is generally updated several days before distribution, hence patients numbers may not precisely match table 5-2.

5.4.2 The PRIORITY study cohort in January 2016

Characteristics of the PRIORITY study cohort at the time of data extraction on 31st January 2016 are shown in the left hand column of Table 5-1. Median age of participants was 63 years and 63% of the cohort were male. Diagnosis of diabetes was made a median of 11 years prior to inclusion in the trial and the majority of participants (61.8%) were already prescribed a RAAS blocking agent (either ACE-I or ARB, current treatment with MRAs was not permitted at study entry). Current smokers comprised 17% of the cohort and 18% had either simplex or proliferative diabetic retinopathy. Median BMI fell at the upper end of the "overweight" category at 29.7kg/m^2 and blood pressure was within target range for T2DM patients with no nephropathy. Median HbA1c of 56 mmol/mol indicated good glycaemic control and lipid profile was acceptable (median cholesterol 4.2mmol/l; LDL 2.2 mmol/l; HDL 1.2 mmol/l; and triglycerides 1.6

mmol/l). Renal parameters were well within the currently accepted "normal range", with median eGFR of 90.6 ml/min/1.73m^2 and UACR of 5.3mg/g. From a clinical perspective one could conclude that this is a relatively low risk group of T2DM patients, yet according to proteomic risk scoring 12.5% were categorised as "high risk" for later development of DN.

5.4.3 How the Glasgow subgroup compares to the whole study population

The descriptive data specific to the Glasgow sub-population are determined from the same dataset extracted on 31st January 2016. In view of the difficulties described above the number of individual data points available for analysis ranged from 212 to 156 depending on the parameter in question. The Glasgow cohort did not differ from the whole study population in terms of age; use of RAAS blocking agents or smoking status. However some differences in risk factor profiles did emerge. The Glasgow cohort was more predominantly male in comparison to the whole study population (69.2% compared to 61.2%) and had a shorter median duration of diabetes (9.8 compared to 11.1 years). BMI and systolic blood pressure were also higher among Glasgow participants (30.9kg/m^2 compared to 29.4kg/m^2 for BMI; 136mmHg compared to 133mmHg for systolic blood pressure). Blood glucose was less well-controlled in the Glasgow participants (HbA1c 61mmol/mol compared to 55mmol/mol) and triglyceride levels were higher (1.9mmol/l compared to 1.6mmol/l), in keeping with the difference in BMI. Although these differences reached statistical significance, numerically they are modest and in a clinical setting would be unlikely to result in alterations in patient management. The prevalence of retinopathy was significantly higher in Glasgow compared to the rest of the study population (28% compared to 15.1%). The accuracy of these data are again dependent on entry at local sites and the means by which these data are collected. It is therefore not possible to draw further conclusions from a descriptive analysis such as this.

The focus of PRIORITY is of course on prediction of DN. From a renal standpoint it is clear that the Glasgow subgroup were comparable to the general study

population with no significant difference in eGFR (90.4ml/min/1.73m^2 compared to 90.7ml/min/1.73m^2) or UACR (5.2mg/g compared to 5.3 mg/g). Despite these results, median CKD273 classifier score was higher in Glasgow (-0.228 compared to -0.358). Overall 13.7% of Glasgow participants were "high risk", compared to 12.2% elsewhere, however this difference did not reach statistical significance. Whether this difference is the result of the variation in risk factor control described above cannot be inferred from a descriptive analysis based on an as yet incomplete dataset.

5.4.4 Determinants of CKD273 classifier in PRIORITY study participants

As described earlier, this analysis was based on data collected from baseline visits of PRIORITY participants in Glasgow between April 2014 and May 2016. In total 204 participants had attended a study baseline visit during the specified time period. Recruitment strategies were described in chapter 2 and Figure 5-2 illustrates the composition of the cohort based on these streams. The majority of the cohort were recruited from GP practices (55%); followed by secondary care clinics (20%), the SDRN (12%), news advertising (9%), retinal screening services (2%) and the initial pilot study described in chapter 4 (2%). The clinical characteristics of this group of patients, shown in Table 5-3, are numerically similar to the whole study cohort at baseline (Table 5-1) with the exception of higher CKD273 classifier score in Glasgow participants (although percentage of high risk participants similar to whole study cohort), higher prevalence of retinopathy and BMI in the obese rather than overweight range. Again, risk factors are within acceptable guideline-endorsed parameters and renal measures are comfortably within the clinically accepted “normal” range. Despite this 12.7% of this sub-population were deemed to be at high risk for later development of DN according to proteomic risk stratification.

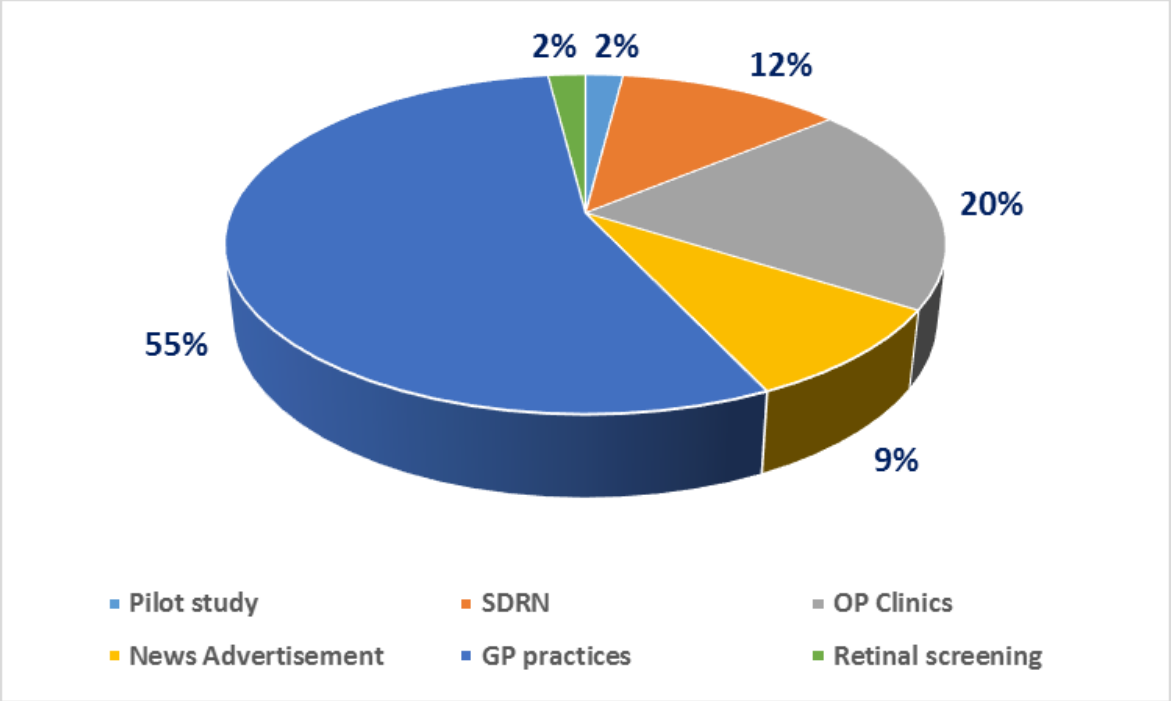

Figure 5-2. Composition of the Glasgow PRIORITY cohort in May 2016.

Figures are percentage of cohort from each recruitment strategy. SDRN, Scottish Diabetes Research Network; OP, outpatient.

Age (yrs)	**62 (24-74)**
Gender (males/females)	142/62 (70% male)
Diabetes duration (yrs)	9 (0.5-33)
RAAS blockade (Y/N)	119/85 (58% on treatment)
Current smokers (Y/N)	30/174 (15% smokers)
Retinopathy status (Y/N)	55/149 (27% retinopathy)
Height (cm)	170 (101-194)
Weight (kg)	88 (56-146)
BMI (kg/m^2)	31.2 (20.5-48.9)
SBP (mmHg)	136.5 (110-160)
DBP (mmHg)	79±9.1
HbA1c (mmol/mol)	61 (36-106)
Cholesterol (mmol/l)	4.2 (2.3-7.6)
LDL (mmol/l)	2.1 (0.8-4.8)
HDL (mmol/l)	1.1 (0.6-2.4)
Triglycerides (mmol/l)	1.9 (0.6-5.1)
eGFR (ml/min/1.73m^2)	88.5±15.2
ACR (mg/g)	4.9 (1.3-34.5)
CKD273 score	-0.249±0.381
Risk category (high/low)	26/178 (12.7% high risk)

Table 5-3. Clinical characteristics of Glasgow PRIORITY participants attending baseline visits between April 2014 and May 2016.

Data are mean±SD or median (range) as appropriate. eGFR calculated using CKD-EPI formula. CKD273 threshold for diagnosis of DN 0.343; for classification into high risk group for later development of DN 0.154. RAAS, renin angiotensin aldosterone system; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein; eGFR, estimated glomerular filtration rate; ACR, albumin: creatinine ratio.

One of the key limiting factors in identification of patients in the early stages of DN is the fact that traditional clinical parameters do not perform well as predictors of the condition. Whether any of these parameters contribute significantly to the CKD273 classifier score is not yet known and this issue will be addressed in the whole PRIORITY study cohort upon completion of recruitment. From this local sub-population, CKD273 score was only associated with age ($r=0.202$, $p=0.004$) and UACR ($r=0.209$, $p=0.003$) on univariate analysis as shown in Figure 5-3. There was no association with blood pressure ($r=0.054$, $p=0.446$ for systolic; $r=0.040$, $p=0.570$ for diastolic); disease duration ($r=-0.194$, $p=0.107$); BMI ($r=0.117$, $p=0.095$); HbA1c ($r=-0.035$, $p=0.616$); cholesterol ($r=-0.108$, $p=0.126$) or eGFR ($r=-0.069$, $p=0.326$). Results of multivariate regression modelling are shown in Tables 5-4 and 5-5. Model 1 included all parameters associated with classifier score on univariate analysis. While these factors all retained significance on multivariate analysis, they explained only 9.06% of the variability in CKD273 score. Model 2 included additional parameters which could contribute to DN risk, although not significantly associated with CKD273 score. Again, only age, BMI and ACR were statistically significant in this model which explained only 7.72% variability.

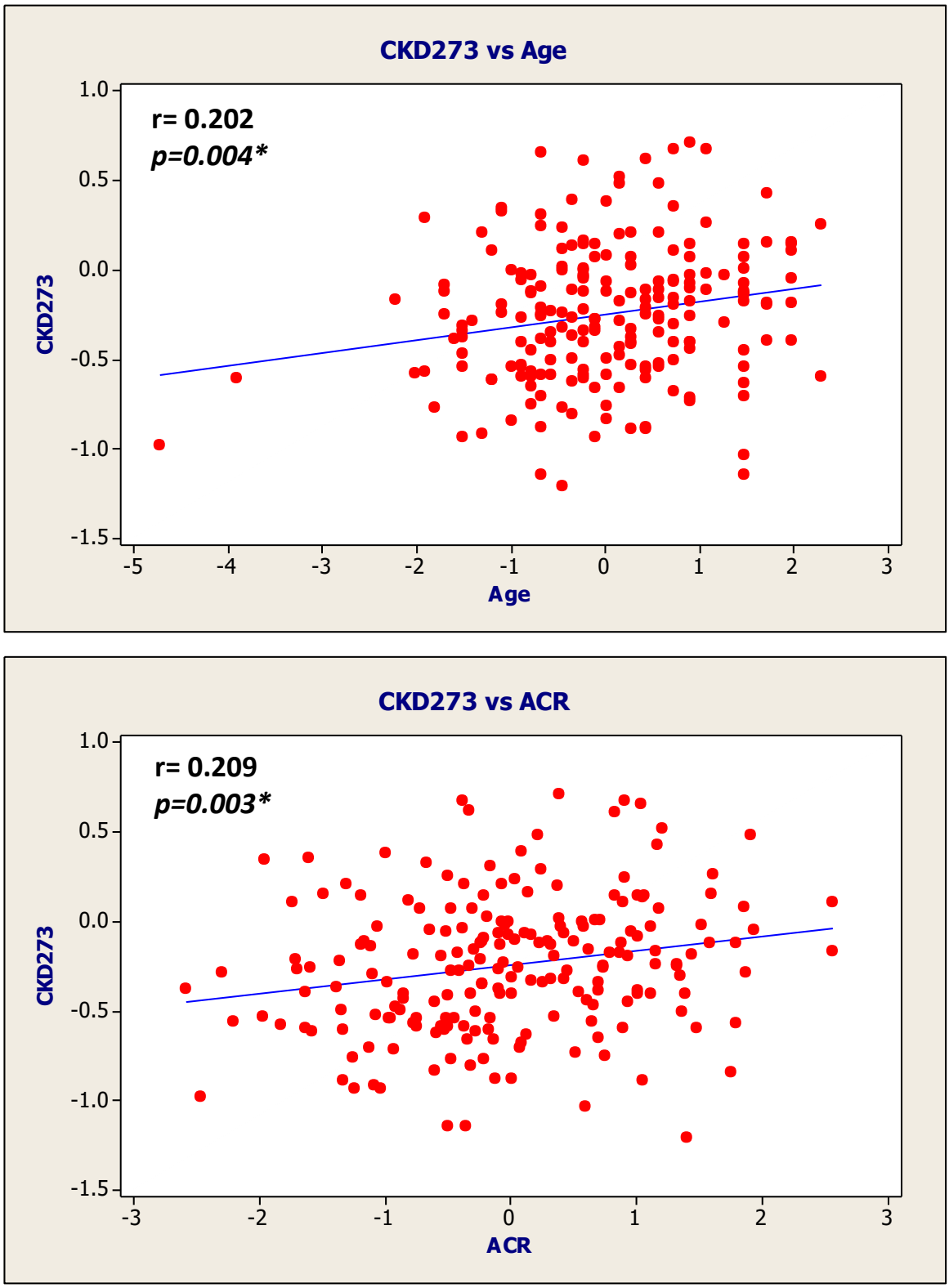

Figure 5-3. Relationship between CKD273, age and ACR on univariate analysis.

Correlations by Pearson's method on raw data for CKD273, ACR and age were transformed prior to analysis. * denotes statistical significance. ACR, albumin: creatinine ratio.

Variable	Coefficient	SE Coefficient	P-value
Constant	-0.249	0.025	**<0.001*
Age (years)	0.075	0.024	**0.002*
ACR (mg/g)	0.080	0.025	**0.002*
BMI (kg/m^2)	0.054	0.024	**0.025*

Table 5-4. Regression model 1 including all parameters associated with CKD273 on univariate analysis.

Adjusted R^2 9%. SE, standard error; ACR, albumin: creatinine ratio; BMI, body mass index.

Variable	Coefficient	SE Coefficient	P-value
Constant	-0.274	0.178	*0.125*
Age (years)	0.075	0.029	**0.009*
ACR (mg/g)	0.080	0.026	**0.027*
BMI (kg/m^2)	0.054	0.024	**0.025*
SBP (mmHg)	0.002	0.027	0.933
HbA1c (mmol/mol)	-0.006	0.025	0.806
eGFR (ml/min/1.73m^2)	0.002	0.001	0.887

Table 5-5. Regression model 2 including all parameters associated with CKD273 on univariate analysis as well as factors which could potentially contribute to DN risk.

Adjusted R^2 7.72%. SE, standard error; ACR, albumin: creatinine ratio; BMI, body mass index; SBP, systolic blood pressure; HbA1c, glycated haemoglobin; eGFR, estimated glomerular filtration rate.

5.5 Discussion

At the time of data extraction from the central database 1475 participants had been included in the study, although proteomic and UACR processing was ongoing for a number of these individuals. The prevalence of MA among these subjects was 7%, which is similar to that seen in the general population [451]. This low prevalence probably reflects the fact that this was not a random sample of the T2DM population but primarily focused screening in order to identify normoalbuminuric patients. The PRIORITY trial cohort at end-of-January 2016 reflect a relatively low-risk group of T2DM patients. With the exception of being overweight this group have well-controlled blood pressure; HbA1c; and cholesterol; the majority are non-smokers and two thirds are established on RAAS blocking agents. From a renal standpoint, UACR is well within the guideline-defined "normal" range and median eGFR of 90.3ml/min/1.73m^2 reflects normal kidney function according to the current definitions [144] making this population ideal for the purposes of evaluating the prospective performance of a novel early diagnostic biomarker.

The Glasgow sub-population comprised 18% of the PRIORITY cohort at the time of data extraction. Study participants in Glasgow were similar to the complete PRIORITY population in terms of age; use of RAAS blocking medications; smoking status; diastolic blood pressure; cholesterol; UACR and eGFR. Differences in gender; diabetes duration; BMI; systolic blood pressure; and HbA1c did reach statistical significance but were numerically modest and are unlikely to be of real clinical relevance in terms of therapeutic decision making or to introduce any major bias. Prevalence of retinopathy was significantly higher in Glasgow when compared to the rest of the study population (28% vs 15.1%). At baseline 36% of UKPDS participants had diabetic retinopathy [59] and findings locally are certainly more in keeping with this. While local retinopathy data was sourced from annual retinal photography within the Scottish National Retinal Screening Programme, it is not clear how this was collected across other centres and if self-reporting were employed by some this would doubtless result in lower prevalence.

One notable factor for consideration in the Glasgow sub-population is of course the higher median CKD273 classifier score; a difference which did reach statistical significance. The clinical and prognostic significance of this finding is unclear and cannot be inferred from this descriptive analysis of baseline characteristics. If considered alongside the apparently increased prevalence of retinopathy in Glasgow, it could be speculated that this may imply an association between CKD273 and microvascular disease in other vascular beds, but as described above the means used to determine retinopathy status in different centres is unknown and this finding should not be over-interpreted. The Glasgow cohort are phenotypically similar to the whole study population with regard to routinely measured clinical parameters and the minor numerical differences shown in Table 5-1 seem unlikely to confer significantly increased risk of DN. Certainly data shown in chapter 4 do not suggest a direct association between these measurements and CKD273 score, albeit in a smaller study population. Furthermore, despite higher median CKD273 score the actual proportion of high risk participants was similar across study sub-populations. It should also be borne in mind that this is not yet a complete baseline dataset, and therefore clinical phenotypes may well change as recruitment progresses. The uncertain implications of this finding along with the minor, yet statistically significant numerical differences in some clinical parameters do indicate that results of analyses performed in the local study population should be interpreted with caution, and cannot be readily generalised to the whole study population at this stage.

Despite being “low risk” according to traditionally accepted clinical parameters, 12.5% of the PRIORITY study cohort (13.7% Glasgow participants) have been categorised as “high risk” for development of DN based on urinary proteomic risk stratification and the trial will determine in due course whether this assumption is correct. What characterises these “high risk” individuals remains unclear. From the clinical data collected at baseline traditional risk factors are relatively well-controlled and renal function well within normal limits. In order to determine the contribution of clinical parameters to CKD273 classifier score cross-sectional analysis was performed using data collected from Glasgow study participants baseline visits between April 2014 and May 2016. Using this cohort allowed access to a clean and up-to-date dataset, avoiding any manipulation or

formal interim analysis of extracted data for the entire PRIORITY cohort which could potentially introduce bias while the recruitment period was ongoing. On univariate analysis only age and UACR were associated with CKD273 classifier score and these parameters, along with BMI, remained significant on multivariate modelling. However, these factors explained only 9% of the variability in CKD273 score, suggesting that other as yet unmeasured and/or unknown variables may play a role in risk of progressive renal disease in T2DM. This in itself adds weight to the argument for novel biomarkers to identify "at risk" individuals. If significant associations were seen between CKD273 and the clinical parameters used to estimate risk, most of which are inexpensive and easy to measure, novel early disease biomarkers would be superfluous.

The particular factors which define "high risk" patients at this early stage in the disease process remain elusive. The results described in chapter 4 could imply a role for altered collagen turnover and matrix-metalloproteinase activity, however whether the differentially regulated collagens contributing to the CKD273 classifier originate from the vasculature or the glomerulus and mesangium is unclear. From the work described in the previous chapter no immediate associations were seen between CKD273 and markers of vascular stiffness and subclinical atherosclerosis, potentially suggesting that the classifier is a more specific marker of early renal disease rather than generalised vascular dysfunction. However, this was cross sectional work in a small cohort across the spectrum of DN and these relationships merit further clarification in a larger, more homogeneous without the confounding influence of pre-existing CKD. This will be further explored in chapter 6.

The work described in this chapter has several limitations. Firstly, this analysis is based on data extracted from the central study database at a pre-specified time point. As recruitment will continue until September 2016 it is possible that the characteristics of the cohort will change. In addition, the agreement with study steering committee required that this should be a descriptive analysis only, focusing on the whole cohort and any differences in the Glasgow subset. For this reason further manipulation of the data provided was avoided and as a result a complete, clean database was not compiled. Similarly, the completeness of the data relies heavily on timely and accurate data entry at

specific study sites, a factor which is outwith the control of the author. Working within the confines of a preliminary incomplete dataset means that these results should be interpreted with caution. There were a number of numerically small yet statistically significant differences between the Glasgow cohort and the whole study population. Although these minor differences may not have any true clinical significance the associations seen between clinical parameters and CKD273 score are not readily generalisable to the complete PRIORITY study cohort.

In summary, the PRIORITY cohort represents a truly low-risk population of T2DM patients with normal renal function; an ideal group to study novel biomarkers for prediction of DN. Factors which characterise high risk individuals remain elusive, and a clean homogeneous cohort of patients with preserved renal function and normoalbuminuria also offers the opportunity to examine the relationship between CKD273 biomarker, vascular and renal phenotypes more closely without these confounding influences.

6. Urinary proteomics for assessment of subclinical vascular and renal disease in diabetic patients with normal renal function

6.1 Introduction

Our understanding of the natural history of DN is evolving and it is generally accepted that the traditional 5-stage progression is not uniformly seen in all patients, with alternative phenotypes such as early GFR decline and nonalbuminuric disease emerging in recent years. Similarly, it is becoming evident that use of an arbitrary diagnostic threshold for MA does not adequately define either cardiovascular or renal risk. There are a wealth of published data which prove that the link between UAE and both cardiovascular and renal outcomes is a continuum rather than a categorical classification, and that even minor elevation of UAE well within the currently accepted "normal range" confers a significant increase in risk. A number of studies have illustrated the potential of the CKD273 urinary proteomic classifier for early diagnosis of DN in advance of any clinically detectable change in eGFR or UAE [268, 295] and the outcome of the PRIORITY trial [380] will determine whether these assumptions are correct.

The Steno Hypothesis has already been described in chapter 4 [97]. In brief, this theory proposes that MA is in fact a marker of generalised inflammation and vascular disease rather than a specific renal biomarker. It seems intuitive, therefore, that the same could be true of the CKD273 classifier: it captures multiple aspects of disease pathogenesis simultaneously; is composed largely of differentially regulated collagen fragments [268] which are key mediators of a number of disease processes including renal and cardiovascular disease; and other proteomic panels which comprise many similar peptide fragments have been described in the literature for prediction of cardiovascular events in different patient populations [283]. The relationship between CKD273 and vascular structure and function in a cohort of patients across the spectrum of DN was examined in chapter 4, and although no association was found between classifier score and clinical vascular phenotypes the confounding effects of

albuminuria and CKD made these data difficult to interpret. The PRIORITY study includes a more homogeneous cohort of patients free from these confounding influences. This is therefore an ideal group of patients in which to evaluate whether the CKD273 classifier is providing information on generalised subclinical vascular damage and inflammation, or rather providing specific information about the kidney.

6.2 Aims

This study aimed to determine the relationship between CKD273 and:

- markers of subclinical vascular damage and inflammation;
- markers of renal function and morphology;

in a cohort of normoalbuminuric patients with type 2 diabetes and preserved renal function.

6.3 Methods

This study was carried out in parallel to the PRIORITY trial. A full description of the PRIORITY study protocol can be found in chapter 2. In addition a number of the clinical and biochemical analyses performed in this study have been detailed in chapter 4. This section will focus primarily on the techniques which have not previously been documented, and will refer to the relevant chapters where necessary to avoid repetition.

6.3.1 Ethical approval

This work was approved by the PRIORITY study steering committee as a sub-study to the main trial following the consortium meeting in January 2014. It was subsequently reviewed and approved by the West of Scotland Research Ethics Committee 1 (ref 13/WS/0284) and NHS Greater Glasgow and Clyde R&D (ref GN12DI096) in May 2014.

6.3.2 Participant selection and recruitment

Recruitment streams into the PRIORITY trial have been extensively described in chapter 2. Inclusion into this aspect of the study was primarily based on willingness to participate - all patients included in the Glasgow arm of the trial were deemed eligible (Figure 6-1).

At the study screening visit a sub-study specific PIS (Appendix 10) was provided, and these investigations were performed at the baseline visit for patients who wished to be involved. This avoided any confounding effect of initiation of study medication on results, and minimised inconvenience to study participants that may have been caused by additional visits. Patients with any condition that would affect interpretation of the vascular studies (any condition that would affect interpretation of clinical vascular studies (e.g. atrial fibrillation; ventricular bigeminy; cardiac pacemaker) were excluded.

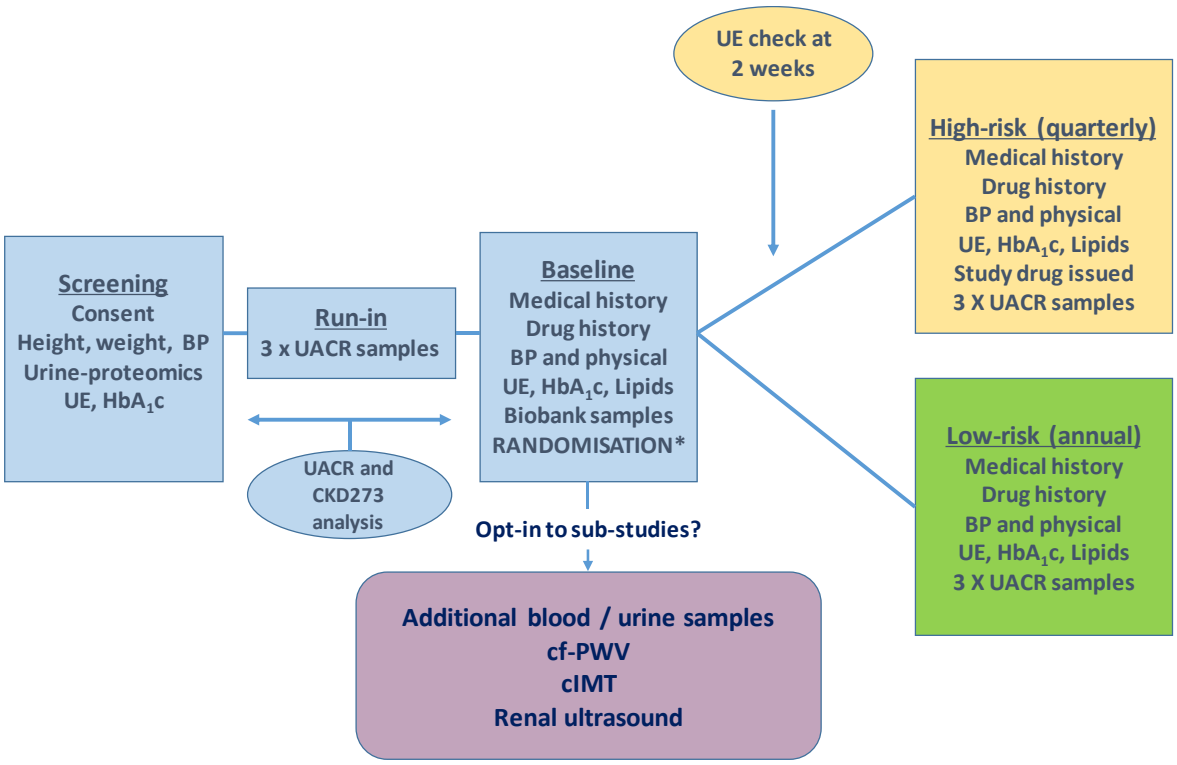

Figure 6-1. PRIORITY study visit schedule illustrating vascular sub-studies.

BP, blood pressure; UE, urea and electrolytes; HbA1c, glycated haemoglobin; UACR, urine albumin: creatinine ratio; cf-PWV, carotid-femoral pulse wave velocity; cIMT, carotid intima-media thickness.*Participants in high-risk group only.

6.3.3 Study visits

Additional investigations were performed at the PRIORITY study baseline visit. Routine study procedures performed at this stage are fully described in chapter 2. All study visits were held at the British Heart Foundation Glasgow Cardiovascular Research Centre and latterly at the Clinical Research Facility on the Queen Elizabeth University Hospital campus in a quiet room, temperature controlled to 22-24˚C. Participants were advised to abstain from caffeinated beverages for at least 6 hours prior to their scheduled appointment. Following routine baseline requirements, the procedures described below were undertaken in participants who gave informed consent (Appendix 11).

6.3.3.1 Pulse wave velocity

Arterial stiffening is the result of arteriosclerosis which affects the vessel media, rather than atherosclerosis, as measured by cIMT for example, which primarily affects the intimal layer. Loss of vascular compliance results in a faster pulse wave transit time. Among the many methods used to evaluate arterial stiffness (cf-PWV; defined as the velocity of the pulse as it travels from heart to carotid and femoral vessels) is considered the "gold standard" [450]. This is most commonly measured by automated devices which record pulse waves from the common carotid and femoral vessels using tonometry probes, subsequently transit time and distance travelled (which is assimilated to the skin distance between the two recording sites) are used to calculate cf-PWV [450]. Arterial stiffness robustly predicts all-cause mortality and cardiovascular outcomes even after adjustment for traditional cardiovascular risk factors [435, 452]. cf-PWV is endorsed by clinical guidelines for reclassification of "intermediate risk" patients [431] and its utility has even been demonstrated in patients with pre-existing CKD [453]. The procedure has the advantage of being non-invasive, automated, reproducible and easily performed [437]. For the purposes of this study the procedure was performed as described below.

Pulse wave velocity was measured with the patient lying in supine position with neck slightly extended using the automated SphygmoCor XCEL system (Atcor Medical, Australia). Peripheral blood pressure was recorded as described in chapter 4, and the result manually input into the SphygmoCor software tool. A blood pressure cuff, connected to the automated software, was placed around the upper thigh. Transit distance was then measured manually. This involves measurement of the distance from carotid pulsation to sternal notch, then from sternal notch to the top of the thigh cuff. These distances were input into the SphygmoCor software tool which used an automated algorithm to determine transit distance where the former is subtracted from the latter. The carotid pulse was palpated digitally, then the applanation tonometer (Millar Instruments, Houston, USA) was applied over the site of maximal pulsation and held in position throughout the procedure. The tonometer was connected to a dedicated laptop computer for data collection and analysis. After a satisfactory series of pulse waves were obtained, the thigh cuff inflated automatically for

detection of femoral pulsation and recording of femoral pulse waves. The software then calculated cf-PWV after a satisfactory series of simultaneous carotid and femoral pulse waves were obtained. The mean of 2 readings meeting quality control checks was recorded (Figure 6-2).

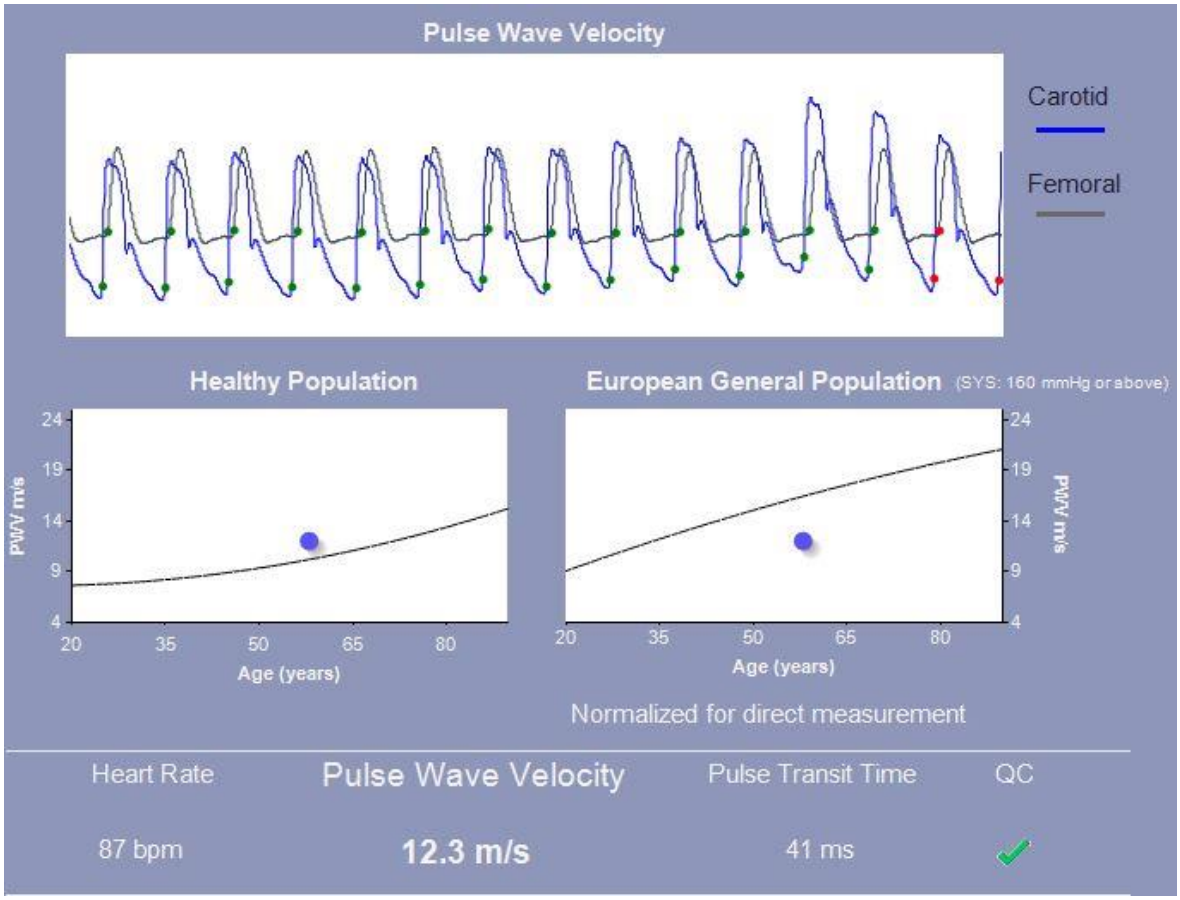

Figure 6-2. Assessment of carotid-femoral pulse wave velocity.

Representative example of carotid-femoral pulse wave velocity output generated using the SphygmoCor XCEL device (Atcor Medical, Australia).

All procedures were performed by the author or by a single trained research nurse for consistency, and the SphygmoCor utilised an internal quality control measure where any readings varying by more than 10% from the previous recording were highlighted to the operator.

6.3.3.2 Carotid intima media thickness

This procedure was described in detail in chapter 4 and the same method was used for the purpose of this study. Scanning was performed by the author or a trained research nurse. Intraobserver coefficient of variation was less than 10% for both individuals. Intraclass correlation between observers was 0.956 (95% CI 0.819-0.990).

6.3.3.3 Renal ultrasound

Early studies by Mogensen and colleagues showed that the first stages of DN were characterised by glomerular hyperfiltration which can be accompanied by kidney hypertrophy [8, 454]. It was therefore decided to perform renal ultrasound in order to determine whether there was any association between CKD273, a proposed marker of early DN, and basic renal morphological parameters: kidney length and cortical thickness.

Renal ultrasonography was performed in a dedicated, temperature controlled room using the Acuson Sequoia 512 scanner and L7 5-12 MHz curved array probe (Siemens, Erlangen, Germany). Participants were asked to remove clothing from their upper body, loosen any waistbands and given a gown to wear in order to optimise exposure, they were then asked to lie in the supine position with the ipsilateral arm behind their head to optimise visualisation. The right kidney was visualised longitudinally, and pole to pole length measured. Transverse images were then obtained to measure cortical thickness, and the mean of 3 measurements from upper, middle and lower poles obtained. The same procedure was then performed for visualisation of the left kidney. All scans

were performed by the author following training by a consultant radiologist at the Western Infirmary in Glasgow.

6.3.3.4 Biomarker studies

STNFR 1 and cystatin C were selected as novel predictors of DN, as described in chapters 1 and 4. Plasma levels were measured by enzyme-linked immunoassay (ELISA) (EKF Diagnostics, Walton on Thames, UK) at the University of Glasgow by Dr Ruth Mackenzie and Elaine Butler.

High-sensitivity CRP was selected as an indicator of systemic inflammation and vascular disease. As opposed to TnT and NT-proBNP which reflect ischaemia and cardiomyocyte damage, CRP is thought to relate more closely to vascular wall biology. It is well documented that CRP is present in atherosclerotic plaques and secreted from injured vascular walls [455] and levels correlate with other cardiovascular risk factors and inflammatory markers [437]. There is robust evidence linking CRP to future cardiovascular risk [456]. In addition, **TnT** and **NT-proBNP** were selected as described in chapter 4. These markers were measured by ELISA (Roche, West Sussex, UK) by Elaine Butler and Sarah-Jane Duffus at the University of Glasgow, using the Elecsys C411 for NT-proBNP and TnT and the Cobas C311 for CRP and Cystatin C.

6.3.4 Statistical analysis

Statistical analyses were performed using SPSS (SPSS, IBM Analytics, New York, USA) and Minitab16 (Pennsylvania, USA) software packages. Normality of data distribution was assessed by manual inspection of histograms and the Kolmogorov-Smirnov test. Normally distributed data were expressed as mean ± standard deviation (SD), while data that were not normally distributed were expressed as median and interquartile range. 2-sample t-tests were used for comparison between “high” and “low” risk groups on raw or transformed data

where appropriately distributed. Pearson's correlation coefficients were used to evaluate determinants of CKD273 classifier score using raw data or transformed data where appropriate depending on distribution. P-values <0.05 were considered statistically significant.

6.4 Results

Between May 2014 and January 2016 144 PRORITY trial participants consented to take part in this study.

6.4.1 Clinical characteristics

The clinical characteristics of the cohort are shown in Table 6-1. Two thirds were male and 62% were currently treated with ACE-I or ARB. Current or previously treated retinopathy was present in 31%. Median BMI was in the obese range and blood pressure was well-controlled although glycaemic control was suboptimal. UACR and eGFR, the guideline-endorsed parameters for diagnosis and monitoring of DN, were well within the currently accepted "normal" range. Despite this, 16 (11%) of participants fell into the "high risk" category for later development of DN according to urinary proteomic classification and the PRIORITY trial will later determine whether this assumption is correct.

Parameter	Mean±SD/Median (range)
Age (years)	61 (24-73)
Gender (M/F)	97/47
Diabetes duration (years)	9.9±6.2
RAAS blockade? (Y/N)	90/54
Retinopathy? (Y/N)	45/99
BMI (kg/m^2)	30.8 (21-49)
SBP (mmHg)	135.2±12.6
DBP (mmHg)	77.9±12
HbA1c (mmol/mol)	62.5 (36-106)
Cholesterol (mmol/l)	4.2 (2.3-7.6)
Triglycerides (mmol/l)	1.8 (0.7-4.8)
ACR (mg/g)	5.2 (1.3-39.1)
eGFR (ml/min/1.73m^2)	88.5±14.5
CKD273 score	-0.256±0.371
Risk category (H/L)	16/128

Table 6-1. Clinical characteristics of cohort end January 2016.

Data are mean±SD or median (range) depending on distribution. eGFR calculated by CKD-EPI formula. 'Retinopathy' encompasses presence of retinal changes on digital retinal photography and participants who have undergone laser therapy. RAAS, renin angiotensin aldosterone system; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; ACR, albumin: creatinine ratio; eGFR, estimated glomerular filtration rate.

Table 6-2 compares the clinical characteristics of "high risk" versus "low risk" participants. There were no differences in clinical parameters between the two groups with the exception of BMI, systolic blood pressure and treatment with RAAS blockade. However, as only 11% of study participants fell into the "high risk" group according to proteomic risk classification these comparisons are difficult to interpret. As an alternative, the cohort were also split by mean CKD273 score for analysis which gave two subgroups of 72 participants each. Based on this analysis again there was no significant difference in clinical parameters between the groups with the exception of age, treatment with RAAS

blockade and UACR. eGFR was numerically lower in participants with CKD273 score above the mean, however this did not reach statistical significance. These data are also shown in Table 6-2.

Exploring clinical determinants of CKD273 score, this was associated with age (r=0.171, p=0.040) and BMI (r=0.179, p=0.032); but not with disease duration (r=0.079, p=0.346); blood pressure (r=-0.049, p=0.094 for systolic; r=-0.017, p=0.835 for diastolic); HbA1c (r=0.007; p=0.938) or cholesterol (r=-0.109, p=0.192).

6.4.2 CKD273 and renal parameters

As shown in Table 6-2, there was no significant difference in eGFR or ACR between the "high" and "low" risk groups. When the cohort was divided by mean CKD273 score to account for the uneven distribution of participants between risk groups, UACR was higher among those with CKD273 score above the mean, while remaining well within the low-normal range. In this analysis the difference in eGFR between categories was not statistically significant. In keeping with findings of the pilot study there was no association between CKD273 score and eGFR (r=-0.067, p=0.425). On univariate analysis a weak association with UACR was seen (r=0.176, p=0.034); but this was lost following adjustment for clinical determinants of CKD273 score. These plots are illustrated in Figure 6-3.

	Low risk (n=128)	High risk (n=16)	P-value	CKD273<mean (n=72)	CKD273>mean (n=72)	P-value
Age (years)	61 (24-73)	61 (44-69)	0.656	61 (24-72)	63 (41-73)	*0.008*
Gender (M/F)	87/41	10/6	0.663	52/20	45/27	0.213
Diabetes duration (years)	10.1±6.4	8.6±4.4	0.375	9.3±6.4	10.6±6	0.215
RAAS blockade (Y/N)	76/52	14/2	*0.018*	37/35	53/19	*0.006*
Retinopathy (Y/N)	40/88	5/11	1.000	22/50	23/49	0.857
BMI (kg/m^2)	30.4 (21-48.9)	35.7 (22.8-47)	*0.042*	30.3 (21-43)	31.2 (23-49)	0.176
SBP (mmHg)	134±12	142±13	*0.017*	134±12.1	136±13.2	0.324
DBP (mmHg)	77±9	82±9	0.086	78.38.7	77.4±9.2	0.541
HbA1c (mmol/mol)	62 (36-106)	67 (45-81)	0.743	64 (36-100)	61.5 (43-106)	0.620
Cholesterol (mmol/l)	4.1 (2.3-7.6)	4.3 (2.7-6)	0.584	4.2 (2.3-7)	4.2 (2.4-7.6)	0.459
Triglycerides (mmol/l)	1.8 (0.7-4.8)	1.8 (1-3.1)	0.833	1.7 (0.7-4.6)	1.9 (0.7-4.8)	0.525
ACR (mg/mmol)	5.1 (1.3-39.1)	5.7 (1.9-20.2)	0.988	4.7 (1.3-39.1)	6.4 (1.9-39.5)	*0.007*
eGFR (ml/min/1.73m^2)	88.2±14.8	90.7±11.8	0.524	90.6±14.9	86.5±13.9	0.090
CKD273 score	-0.337±0.302	0.396±0.175	*<0.001*	-0.549±0.216	0.038±0.235	*<0.001*
Risk category (H/L)	NA	NA	NA	0/72	16/56	*<0.001*

Table 6-2. Clinical characteristics according to proteomic risk classification (left hand columns) and mean CKD273 score (right hand columns).

Data are mean±SD and median (range) depending on distribution. eGFR calculated using CKD-EPI formula. Of note, normoalbuminuria was defined as 2 of 3 consecutive spot ACR being <30mg/g. Therefore two participants had median ACR technically within the microalbuminuric range but were still included in the study. Comparisons are by 2-sample t-test, using transformed data where appropriate. * denotes statistical significance. RAAS, renin angiotensin aldosterone system; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; ACR, albumin: creatinine ratio; eGFR, estimated glomerular filtration rate.

STNFR 1, cystatin C and ultrasound measures of renal morphology were also measured in this cohort. Table 6-3 displays these values according to participant risk categories. When analysed according to CKD273 risk status, there was no significant difference in markers of renal morphology, cystatin C or STNFR1 levels between groups. However, when the analysis was performed according to mean CKD273 score in more evenly sized groups, STNFR1 levels were significantly higher in participants with CKD273 above the mean. There was no significant association between CKD273 and kidney size ($r=0.042$, $p=0.491$) or cortical thickness ($r=0.044$, $p=0.673$). Nor was there an association with STNFR1 or cystatin C levels in this cohort of patients with preserved renal function ($r=0.079$, $p=0.435$ for STNFR1; $r=0.053$, $p=0.605$ for Cystatin C). Renal morphological parameters were both closely correlated with eGFR as shown in Figure 6-4.

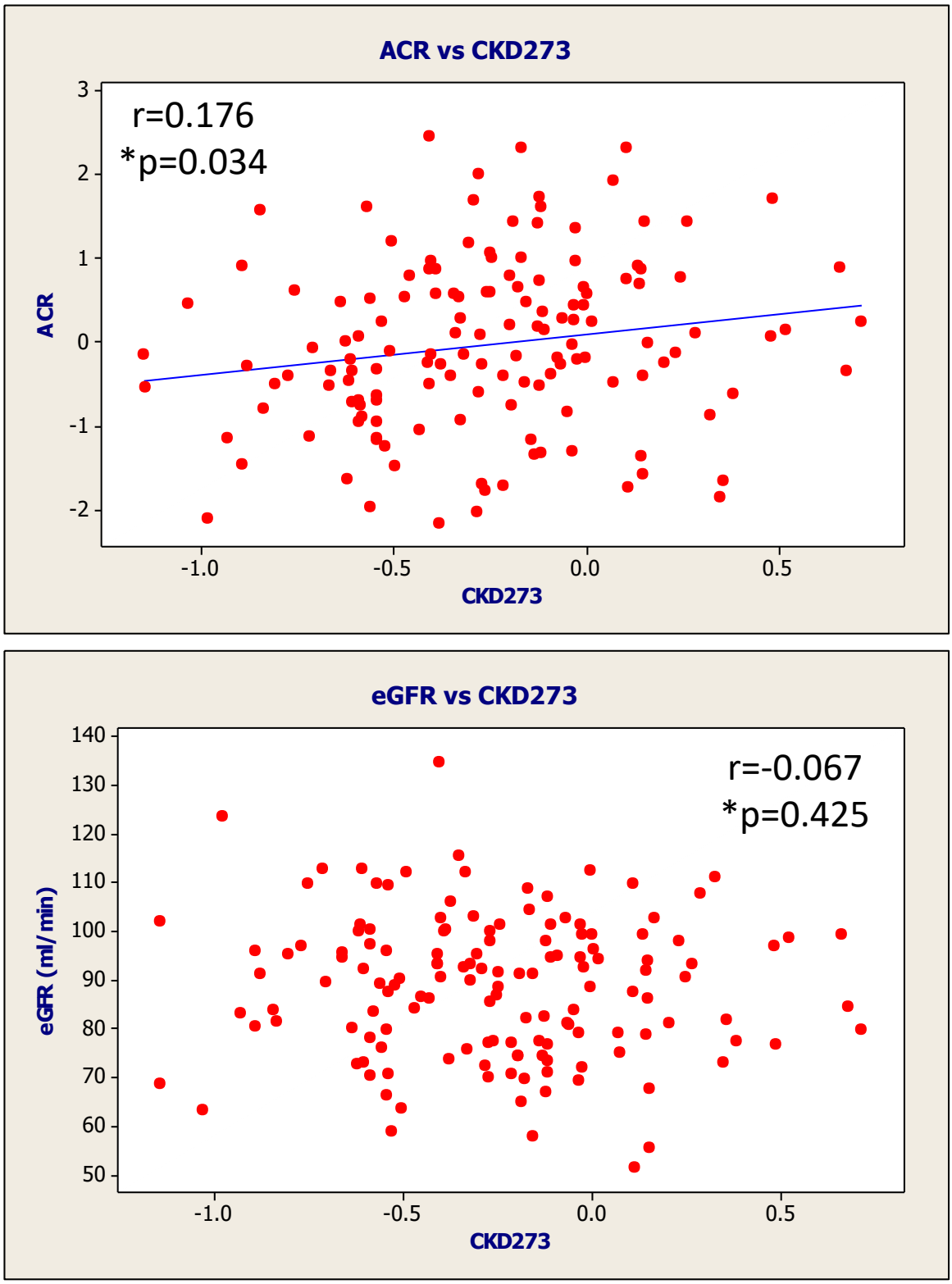

Figure 6-3. Association between CKD273 classifier and clinical indices of DN in normoalbuminuric patients with preserved eGFR.

Correlations by Pearson's method using raw data for eGFR and transformed data for ACR. * denotes statistical significance. Of note, the relationship between CKD273 and ACR was no longer significant on multivariate regression modelling. ACR, albumin:creatinine ratio; eGFR, estimated glomerular filtration rate.

	Low risk (n=128)	High risk (n=16)	P-value	CKD273<mean (n=72)	CKD273>mean (n=72)	P-value
ACR (mg/mmol)	5.1 (1.3-39.1)	5.7 (1.9-20.2)	0.988	4.7 (1.3-39.1)	6.4 (1.9-39.5)	**0.007*
eGFR (ml/min/1.73m^2)	88.2±14.8	90.7±11.8	0.524	90.6±14.9	86.5±13.9	0.090
Cystatin C (mg/l)	0.72±0.21	0.74±0.15	0.828	0.71±0.2	0.74±0.21	0.381
STNFR1 (pg/ml)	149.3 (75.3-294.7)	136.5 (107.1-184.9)	0.306	133.3(75.3-273.4)	155.3 (95.4-294.8)	**0.032*
Kidney size (cm)	10.8±0.97	11.2±0.83	0.154	10.8±0.94	10.8±0.98	0.985
Cortical thickness (cm)	1.73±0.21	1.77±0.22	0.443	1.73±0.22	1.74±0.21	0.780

Table 6-3. Renal parameters according to proteomic risk classification (left hand columns) and mean CKD273 score (right hand columns).

Data are mean±SD and median (range) depending on distribution. eGFR calculated using CKD-EPI formula. Of note, normoalbuminuria was defined as 2 of 3 consecutive spot ACR being <30mg/g. Therefore two participants had median ACR technically within the microalbuminuric range but were still included in the study. Comparisons are by 2-sample t-test, using transformed data where appropriate. * denotes statistical significance. ACR, albumin: creatinine ratio; eGFR, estimated glomerular filtration rate; STNFR1, soluble tumour necrosis factor receptor type 1.

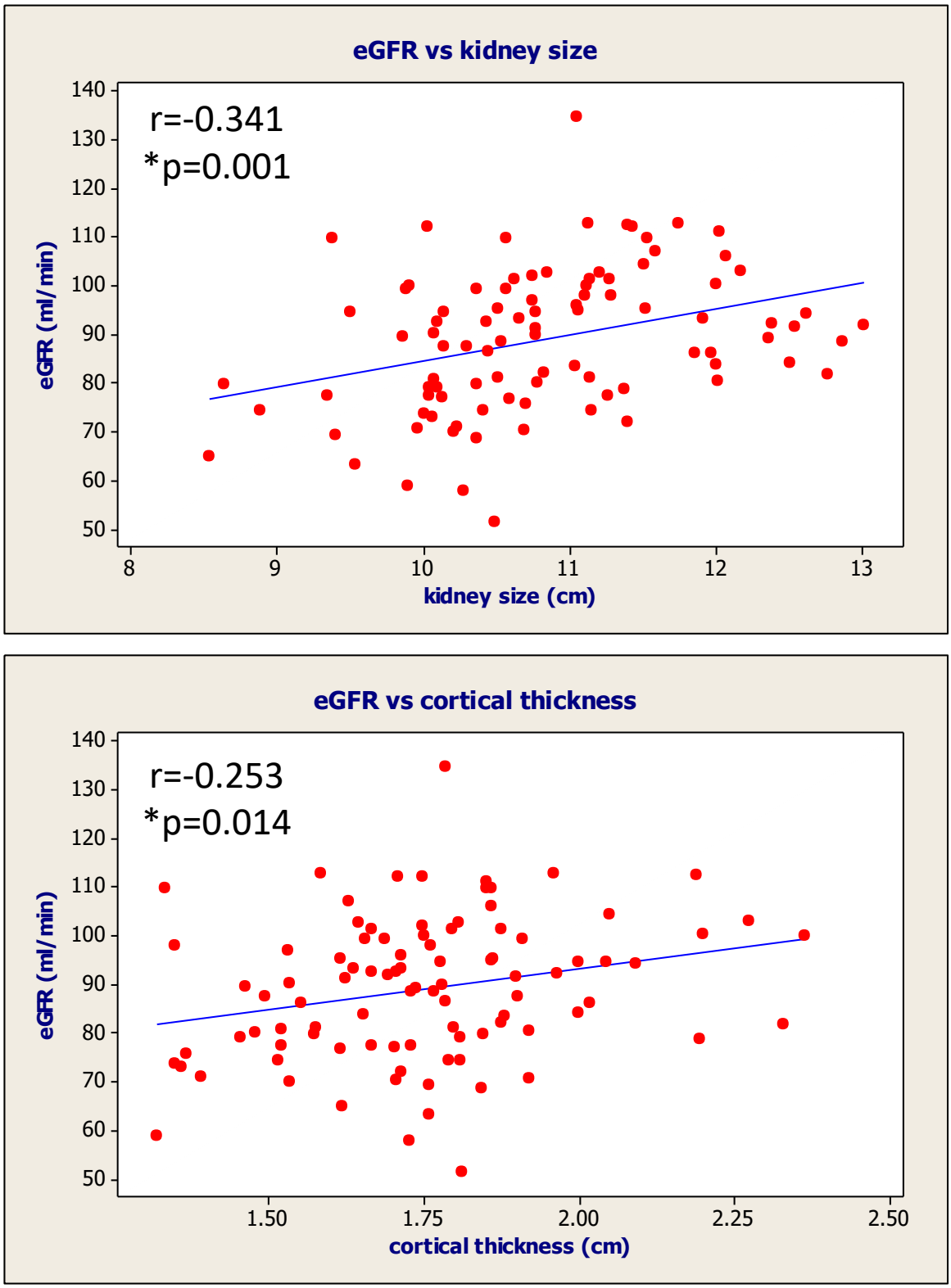

Figure 6-4. Association between eGFR and renal morphology.

Correlations by Pearson's method using raw data * denotes statistical significance. eGFR, estimated glomerular filtration rate.

6.4.3 CKD273 and subclinical vascular damage

Both clinical markers of vascular damage and circulating cardiovascular biomarkers were measured. While 144 participants underwent clinical vascular phenotyping, biomarker studies were performed in a subset of 100 participants as a convenient sample size.

6.4.3.1 Clinical vascular phenotypes

Vascular phenotypes are shown in Table 6-4. There was a spread of measures spanning the continuum from lower to higher levels of cardiovascular risk. Comparing subclinical organ damage between "high" and "low" risk groups; cf-PWV was numerically higher and cIMT numerically lower among high risk patients, but neither of these findings reached statistical significance and their relevance in such disparately-sized groups is questionable. When the cohort was split by mean CKD273 score there were no significant differences in markers of subclinical vascular damage between subgroups. On univariate analysis there was no association between these markers and CKD273 score ($r=0.134$, $p=0.131$ for cf-PWV; $r=-0.051$, $p=0.558$ for cIMT).

Of course DN is generally thought of as a microvascular complication of diabetes and these vascular parameters focus more on larger vessels. For this reason retinopathy status was selected as a crude surrogate for microvascular disease. As shown in Figure 6-5 the prevalence of retinopathy was no different in participants with CKD273 score above or below the mean.

	Low risk (n=128)	High risk (n=16)	P-value	CKD273<mean (n=72)	CKD273>mean (n=72)	P-value
cf-PWV (m/s)	9.2 (6.3-13.9)	9.7 (7.7-12.3)	0.053	9.4 (6.3-13.9)	9.3 (6.5-13.9)	0.600
cIMT (mm)	0.858±0.170	0.766±0.145	0.051	0.860±0.18	0.836±0.16	0.417

Table 6-4. Vascular phenotypes in 144 normoalbuminuric T2DM patients with preserved eGFR.

Data are mean±SD or median (range) depending on distribution. cf-PWV, carotid-femoral pulse wave velocity; cIMT, carotid intima media thickness.

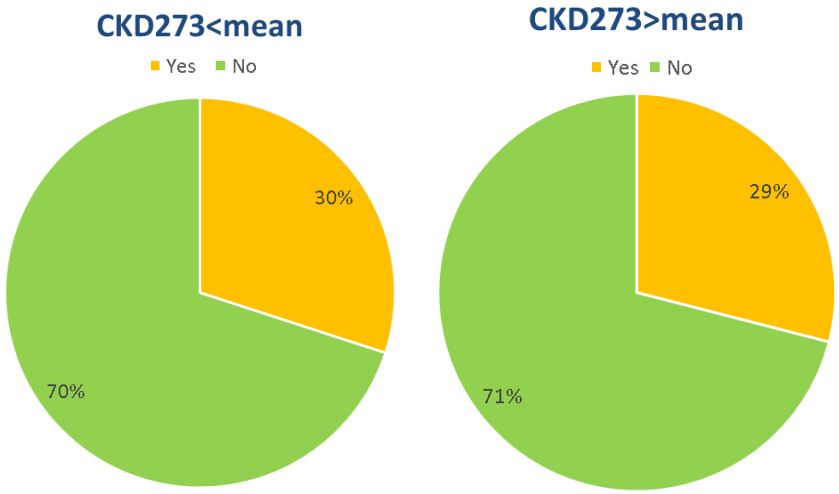

Figure 6-5. Prevalence of retinopathy in participants with CKD273 score above and below the mean.

Green colour indicates absence of retinopathy; orange indicates presence of retinopathy. Percentage of participants in each category as shown. Comparison by Fisher's exact test. P=1.000

6.4.3.2 Circulating cardiovascular biomarkers

Table 6-5 shows circulating cardiovascular biomarker measurements in a subset of 100 study participants. When analysed by proteomic risk classification there were no differences in circulating levels of CRP, NT-proBNP or TnT between "high" and "low" risk groups, although again the difference in size of these groups was marked. When analysed by mean CKD273 score, although numerically higher in participants with classifier score above the mean, these differences did not reach statistical significance. On univariate analysis no associations were demonstrated between CKD273 score and TnT (r=0.054, p=0.595); NT-proBNP (r=0.157, p=0.123); or CRP (r=-0.057, p=0.510).

	Low risk (n=91)	High risk (n=9)	P-value	CKD273<mean (n=49)	CKD273>mean (n=51)	P-value
CRP (mg/L)	1.2 (0-18.5)	1.2 (0.4-3.7)	0.957	1.19 (0.1-18.5)	1.22 (0.03-9.87)	0.597
NT-proBNP (pg/ml)	52.8 (8.7-812.8)	47.3 (23.9-88.9)	0.378	47.7 (8.7-207.1)	56.9 (8.8-812.8)	0.109
TnT (pg/ml)	7.7±5.6	10.4±10.1	0.452	7.4 ± 5.5	8.5 ± 6.7	0.367

Table 6-5. Circulating cardiovascular biomarkers in 100 normoalbuminuric T2DM patients with preserved eGFR.

The table shows vascular phenotypes according to proteomic risk category (left columns) and mean CKD273 classifier score (right columns). Data are mean±SD or median (range) depending on distribution. Comparisons by 2-sample t-test using raw or transformed data where appropriate. cf-PWV, carotid-femoral pulse wave velocity; cIMT, carotid intima media thickness.

6.5 Discussion

Small, retrospective analyses have suggested that the CKD273 urinary proteomic classifier could be a useful early biomarker of DN, identifying "at risk" patients in advance of any clinically detectable albuminuria or decline in eGFR [268, 295, 296]. Validation of this concept requires a prospective study including biomarker-guided clinical intervention, and the PRIORITY trial will in due course determine whether CKD273 can perform adequately as a novel early DN biomarker [380]. In chapter 4 of this thesis the relationship between CKD273 and renal and vascular phenotypes was explored in a small cohort of T2DM patients, spanning the spectrum of DN from normoalbuminuria to macroalbuminuria or reduced eGFR. This cohort was small and the presence of albuminuria and CKD in some patients made it impossible to draw inference from this in terms of the wider T2DM population, particularly those without clinically detectable kidney disease, as these are the individuals in whom the classifier is believed to show true predictive potential. In order to explore the relationship between CKD273 classifier score and vascular and renal phenotypes without the confounding influence of albuminuria, the present study included a larger cohort of T2DM patients with no discernible evidence of DN where guideline-endorsed clinical indices were well within the traditionally accepted "normal" range.

In this study which included 144 participants who underwent detailed renal phenotyping including measurement of traditional clinical indices of kidney disease; novel biomarkers with pathophysiological significance and radiological assessment of renal morphology there was no association between CKD273 classifier and any of these parameters. On univariate analysis a weak correlation with UACR was seen but this was not evident in multivariate analysis. The lack of association with eGFR or UACR is at odds with previously published studies [289, 290] and to a degree also with the pilot work presented in chapter 4. However these studies included patients with established CKD and thus saw a greater spread of data than the current study, in which renal parameters remained well within the expected normal range. In addition, no association was seen with renal morphology or selected novel renal biomarkers. The clear relationship between renal morphological parameters and eGFR suggests that these measures were made correctly, and in fact they were also associated with

STNFR1 and cystatin C. There are no published data exploring relationship of CKD273 classifier to novel renal biomarkers or morphology, but it may be that this small, homogeneous cohort was underpowered to detect an association given the limited spread of data.

Despite the lack of association between CKD273 classifier and renal parameters or discernible differences between "high" and "low" risk groups there was a marked spread of CKD273 scores, and according to proteomic risk status 16 participants (11%) were determined to be at "high risk" of later development of DN. Of course the PRIORITY trial is based on the hypothesis that such "at risk" patients who may benefit from early intensive intervention cannot be identified by traditional clinical parameters, and it is tempting to infer from the lack of association with renal phenotypes that CKD273 is providing additional information on risk that is not conveyed by these other markers. Time will tell whether these 16 individuals truly are at increased risk of progression to DN. This is not to infer that the CKD273 classifier outperforms the other markers evaluated in this study, indeed such assumptions cannot be made from cross-sectional data; and it may well be the case that simply establishing a lower threshold for definition of MA for example could also identify patients at higher risk of progressive renal disease. It does show, however, that a higher CKD273 classifier score does not simply translate into higher UACR; STNFR1; cystatin C; or abnormal renal morphology; and therefore use of these markers to guide therapeutic decision-making may not provide the same information about the preclinical stages of disease i.e. they are not necessarily "interchangeable" with proteomics.

The link between renal disease and cardiovascular risk is well-established and in some ways the kidney can be considered a window to the vasculature, as even low levels of UAE or minimal reductions in eGFR translate into increased risk of events [145]. MA is established not only as a renal marker but also as an indicator of cardiovascular risk. In fact its utility for the latter is probably greater; as it is now generally accepted that not all patients who progress to DN will necessarily develop MA in advance of decline in GFR and MA is not always a key step in the inevitable progression towards ESRD. For a "preclinical" disease marker such as CKD273 which is thought to identify high risk individuals at a very

early stage in disease development, it is unlikely that much association with symptomatic cardiovascular disease will be seen for the simple fact that these patients are at such an early stage in the evolution of CKD that any clinically relevant changes in the vasculature have not yet developed. Similarly it is unlikely that the marker would detect significant cardiovascular disease in this particular study cohort, as symptomatic individuals or those who have suffered recent events were excluded as detailed in chapter 2. However, one concern that remains is the unknown mechanistic significance of the abundant collagen fragments included in the classifier.

Collagen fragments represent a significant proportion of the peptides captured within the CKD273 classifier [268]. Altered collagen turnover is key to a number of disease processes including both renal and cardiovascular disease. In a panel used for early identification of DN it is tempting to speculate that this has mechanistic significance and these fragments are the result of altered GBM composition. However the majority of differentially regulated fragments are cleaved from collagen types 1 and 3, which tend to originate more from the vasculature while collagen type 4 is found more abundantly in the kidney [457, 458]. In addition, other proteomic panels have been evaluated for prediction of cardiovascular events [283] and a significant number of overlapping peptides between these classifiers and CKD273 have been noted. In order to evaluate this in more detail, markers of subclinical organ damage are of some benefit as hard clinical endpoints will take some time to develop in this relatively low-risk cohort. There was no demonstrable association between CKD273 classifier score and vascular stiffness or subclinical atherosclerosis, as assessed by cf-PWV and cIMT; nor with cardiovascular biomarkers including CRP; TnT and NT-proBNP in this study cohort. The clinical vascular phenotype did not differ significantly between “high” and “low” risk patient groups. The trend towards higher cIMT in the low risk patients is unexpected, and could be explained by Type 1 error due to low numbers of high risk patients overall.

To summarise, the results of this study suggest that inter-subject variation in CKD273 classifier score cannot be fully explained by renal biomarkers or kidney morphology. In addition the classifier does not seem to simply be an indicator of subclinical vascular disease. However there are a number of limitations to

consider. First, this is a relatively small cohort which may be underpowered to detect small differences in clinical vascular phenotypes. Second, the cross-sectional nature of this project in combination with a lack of correlations cannot be translated into any clinically meaningful predictive information, however this will be addressed by the PRIORITY trial which will collect data not only on renal outcomes but also on cardiovascular events in a much larger study population. Finally, DN is generally considered a microvascular complication of diabetes and the clinical phenotyping methods used in this study focus on larger vessels. Retinopathy status was selected as a crude measure of microvascular disease in this cohort and while no difference in the prevalence of retinopathy was seen between “high” and “low” risk groups it is feasible that studies focusing on the microvasculature may have yielded different results.

7. Utility of the CKD273 classifier for prediction of renal endpoints in a cohort of patients with microalbuminuria

7.1 Introduction

DN and associated comorbid conditions place a significant burden on global healthcare services. As such, recent years have seen an exponential increase in published manuscripts reporting on novel biomarkers for earlier diagnosis of the condition in order to facilitate delivery of targeted preventative therapies towards those individuals who stand to benefit most. It is now understood that DN is a more heterogeneous condition than previously thought, and use of multi-marker proteomic panels could therefore offer increased sensitivity and specificity for early diagnosis by simultaneously capturing alterations in numerous disease pathways. Despite the surge of publications describing novel proteomic biomarkers none have yet reached the stage of clinical implementation as rigorous evaluation procedures are required to justify such transitions. These stages have been described in more detail in chapter 4.

The majority of the work contained within this thesis focusses on the CKD273 urinary proteomic biomarker, which has shown particular promise as a tool for predicting progression of both diabetic and non-diabetic CKD [422]. The panel has been validated in several independent cohorts [289-291, 296, 298] and retrospective work has suggested utility beyond the current guideline-endorsed parameters for early identification of normoalbuminuric patients at risk of progression to DN [296], a hypothesis which is currently being tested in the PRIORITY trial [380]. While enhanced sensitivity and specificity for early, pre-clinical diagnosis and targeted intervention represents the 'holy grail' of personalised medicine, one key step in proteomic biomarker evaluation requires demonstration of either enhanced sole performance or additive value to current diagnostic standards [459]. The PRIORITY trial will indeed evaluate the performance of CKD273 as an early diagnostic tool for DN in a large cohort of normoalbuminuric patients, but data on the performance of the classifier for re-stratifying microalbuminuric patients are lacking. The importance of this

particular issue may be questioned in the face of the drive towards offering early therapy primarily to prevent development of MA, however microalbuminuric patients represent a particularly interesting subpopulation for biomarker studies for a number of reasons.

Firstly, the original prospective studies published in the 1980s which reported progression rates to overt proteinuria and DN of 60-80% in diabetic patients with MA have been followed by contemporary publications describing progression rates closer to 30% [21, 22, 28, 30, 31, 460]. In addition regression to normoalbuminuria is a common finding among this subpopulation and a significant proportion remain microalbuminuric without ever progressing to overt proteinuria or experiencing a GFR decline [19]. Second, many patients with diabetes have been shown to develop a reduction in GFR without ever becoming clinically microalbumin-positive [41, 45]. Thirdly, although development of MA has traditionally been equated with later development of DN it is also an extremely sensitive cardiovascular risk marker both in diabetic and non-diabetic populations. There is currently no available means by which to differentiate MA linked to cardiovascular disease from MA indicative of underlying renal disease and it could therefore be argued that any change in UAE within the microalbuminuric range may reflect alterations relating to a completely different disease process that does not necessarily have a causal relationship to diabetic kidney disease [461]. Taken together, the issues described above may indicate that MA is not a necessary, committed and irreversible step in the progression of DN. Whilst undoubtedly an important indicator of both renal and cardiovascular risk, the presence of MA should perhaps be evaluated as one factor within a broader range of biomarkers and clinical characteristics to accurately predict an individual's risk of progressive renal disease. Current guidelines advocate use of RAAS-blocking agents and more stringent blood pressure targets in MA positive diabetic patients in order to delay disease progression. However, these and other antihypertensive agents are of course associated with side-effects and strict blood pressure targets can be problematic in certain subpopulations. For example, in elderly patients aggressive hypertension management can be associated with falls and symptomatic hypotension [462] which in turn may limit adherence to treatment. However, in the Systolic Blood Pressure Intervention Trial (SPRINT) these patterns were not

seen even with the tight blood pressure targets [463] required and this subject therefore remains controversial. For these reasons, use of additional complementary biomarkers for accurate identification of microalbuminuric patients who are more or less likely to progress to overt DN could also be an attractive clinical tool to guide personalised therapy.

While proteomic biomarker panels represent an exciting avenue for early and accurate diagnosis of a variety of conditions, simultaneously evaluating a large number of differentially regulated peptides means that these approaches can also provide insight into the pathogenic mechanisms underlying these conditions, offering potential to discover alternative novel biomarkers or therapeutic targets. Of course the abundance of a particular peptide fragment in the urine could result from increased excretion or reduced degradation; and whether these peptides originate in the kidneys or bladder, the vasculature or more distal sites is also far from clear. In addition, given that the majority of the peptides comprising the CKD273 classifier are collagen and albumin fragments it seems intuitive to question whether the panel merely represents a more sensitive MA test. For these reasons, more detailed exploration of the abundance of individual peptide fragments and their relationship to patient characteristics is an equally important area for further study. In order to address the issues described above a cohort of T2DM patients with MA, preserved renal excretory function at baseline and several years of follow up data was required. To date, no study has assessed the performance of the CKD273 classifier in this specific patient population.

In 2011, Reinhard et al published the results of a cross-sectional study designed to determine the relationship between albumin excretion and circulating and imaging biomarkers of subclinical vascular disease in a cohort of 200 Danish T2DM patients with varying degrees of albuminuria and preserved renal function [464]. The cohort was subsequently followed up over the next 4 years at the Steno Diabetes Centre in order to determine factors predictive of cardiovascular outcomes [465]. Although the outcome of interest in the context of this particular study was cardiovascular events, follow up data included monitoring of eGFR and UAE. This group therefore seemed ideally suited to evaluate the

predictive performance of the CKD273 classifier for prediction of renal events in patients with a degree of pre-existing early renal disease.

The work described within this chapter was conducted with the collaboration of colleagues at the Steno Diabetes Centre in Denmark, using the cohort described above. Drs Bernt-Johan von Scholten and Morten Lindhart were involved in drafting the ethical approval application, selection and dispatch of urine samples and provided the clinical data corresponding to study subjects. The author was responsible for co-ordinating the conduct of the study, collation of results and statistical analysis.

7.2 Aims

The aims of this study were:

- to explore determinants of the CKD273 classifier in a cohort of Danish T2DM patients with micro- or macroalbuminuria but preserved renal function;
- to determine whether baseline CKD273 score could identify patients with micro- or macroalbuminuria who were more or less likely to experience a decline in eGFR;
- to explore the relationship between individual peptide components of the CKD273 classifier and renal parameters.

7.3 Methods

The work contained within the chapter was designed in collaboration with colleagues at the Steno Diabetes Research Centre, where participants were originally recruited and followed-up as part of the index trial. The design and methodology of both the original cross-sectional and later follow-up studies have been described elsewhere [464, 465]. Where relevant within this section key

elements will be described in brief but the primary focus will be methodology pertaining to the urinary proteomic study.

7.3.1 Ethical approval

The work described by Reinhard [464] and von Scholten [465] was compliant with the Declaration of Helsinki and approved by the local ethics committee. All participants gave written informed consent at the time of inclusion. The work described within this chapter was presented to the local Danish ethics committee as an additional research proposal and received approval in July 2015.

7.3.2 Participant selection and recruitment

Recruitment into the index study has been described elsewhere [464]. In brief, 200 T2DM patients attending the Steno Diabetes Centre secondary care clinic were identified between January 2007 and February 2008. All received intensive multifactorial management including measures to address glycaemic control; lipid profile and blood pressure; as well as antiplatelet agents and lifestyle advice in accordance with the Steno-2 study [303]. Included patients were between 20 and 70 years of age; had the capacity to give informed consent; were diagnosed with T2DM according to the World Health Organization criteria; and had confirmed MA at screening, defined as UAE exceeding 30mg/24 hrs in 2 out of 3 consecutive urine samples. The presence of pre-existing coronary artery disease or other cardiac pathologies based on history, examination and electrocardiographic findings; and eGFR less than 60ml/min/1.73m^2 (as this would preclude contrast imaging of the coronary vasculature) were key exclusion criteria.

7.3.3 Study visits

All study-related procedures were performed at the Steno Diabetes Research Centre in Gentofte, Denmark. In brief, after obtaining informed consent investigators documented participant medical and drug history and measured height, weight, BMI and blood pressure. Peripheral blood samples were then taken for measurement of HbA1c, cholesterol and creatinine, as well as for storage for later biomarker analysis. In addition a spot urine sample was obtained for storage as above. eGFR was calculated using the CKI-EPI formula. Additional clinical measurements included assessment of the carotid, coronary and peripheral arteries. These procedures have been described in detail elsewhere [464] and the data are not relevant to the study described within this chapter.

7.3.4 Follow-up

All study participants were traced through the Danish National Death and Danish National Health registers from January 1st 2014. Information on date and cause of death were obtained for any deceased participants, and details pertaining to any hospitalisations were extracted. As all participants were attending the Steno Diabetes Centre secondary care clinic at the time of recruitment, and many continued to do so following the original study, information on eGFR and UAE was available for the majority.

7.3.5 Urinary proteomic sample selection

After obtaining approval from the local Danish ethics committee, stored baseline urine samples were selected for proteomic analysis. Selection was primarily based on sample availability. Frozen 1ml aliquots of urine were available for 188 participants. These were packaged on dry ice and shipped from the Steno Centre to the University of Glasgow for analysis in July 2015.

7.3.6 Urinary proteomic analysis

The preparation and subsequent CE-MS processing of urine samples were performed at the University of Glasgow as described in Chapter 4. Data analysis and determination of CKD273 classifier score was performed at Mosaiques Diagnostics in Hannover according to the steps described in chapter 2. At the time of processing and analysis no demographic information was available to the Glasgow study team in order to ensure all were blinded to participant demographics, renal function and outcomes.

7.3.7 Data handling

Prior to sample shipment collaborators at the Steno Diabetes Centre and University of Glasgow prepared a predefined data analysis plan. As described above, the Glasgow study team were blinded to participant information including basic demographics; renal parameters; and outcomes until the proteomic analysis was complete. Once CKD273 classifier scoring was available for all analysable samples this information was sent to collaborators at the Steno Diabetes Research Centre who then released individual patient data for statistical analysis. Participants were identifiable only by unique study ID, and the link document was not available to the Glasgow study team.

7.3.8 Endpoints

The primary endpoint was defined as a decline in eGFR of at least 30% at any time point during follow up. Transition in albuminuria status from micro- to macroalbuminuria at any time point during the follow up period was the predefined secondary endpoint. Cardiovascular events were defined as a combination of cardiovascular mortality; non-fatal myocardial infarction; stroke; and heart failure.

7.3.9 Statistical Analysis

Statistical analysis was carried out using SPSS (SPSS, IBM Analytics, New York, USA) and Minitab16 (Pennsylvania, USA) software packages. Normality of data distribution was assessed by manual inspection of histograms and the Kolmogorov-Smirnov test. Normally distributed data were expressed as mean ± SD, while data that were not normally distributed were expressed as median and interquartile range. Correlations were determined by Pearson's method on normally distributed or appropriately transformed data. In the event that data were zero-inflated normalisation was deemed inappropriate and correlations were therefore determined by Spearman's rank method. Kaplan-Meier plots and Cox regression models were used to determine the ability of CKD273 to predict progressive renal disease and mortality.

7.4 Results

Of the 188 urine samples received 31 failed quality control assessment following CE-MS analysis, meaning that CKD273 scores were available for 157 individual participants.

7.4.1 Baseline characteristics

Baseline characteristics of the cohort are shown in Table 7-1. Of the 157 study participants with proteomic scores available 120 (76%) were male and 37 (24%) were female. Median age was 60 years and participants had been diagnosed with diabetes for 12 years. Median BMI was within the obese range and 27% of patients were smokers, but other risk factors including blood pressure; HbA1c; and cholesterol were well-controlled. 60% of study participants were affected by diabetic retinopathy. All participants were microalbumin-positive on 24 hour urine collection at screening, however due to the variability of albuminuria there was a degree of spread at baseline. While median UAE was within the

microalbuminuric range only 99 (63%) participants were microalbuminuric at baseline, 30 (19%) were normoalbuminuric and 28 (18%) had macroalbuminuria. Despite this, renal function was preserved with mean eGFR of 89.5ml/min/1.73m^2 and all participants had eGFR greater than 60 ml/min/1.73m^2 at baseline. Median CKD273 classifier score was 0.271 (-1.078-1.231).

Parameter	Mean±SD/Median (range)
Age (years)	60 (26-71)
Gender (M/F)	120/37
Diabetes duration (years)	12 (1-36)
Retinopathy (Y/N)	93/65
Smokers (Y/N)	42/115
BMI (kg/m^2)	31.4 (21.6-55.6)
SBP (mmHg)	129.6±16.1
DBP (mmHg)	74.8±11.2
HbA1c (mmol/mol)	59 (39-123)
Cholesterol (mmol/l)	3.8 (2-7.6)
UAE (mg/24hrs)	103 (3-1372)
eGFR (ml/min/1.73m^2)	89.5±17.4
CKD273 score	0.271 (-1.078 – 1.231)

Table 7-1. Baseline characteristics of cohort.

Data are mean ± SD or median (range) depending on distribution. eGFR calculated by CKD-EPI formula. 'Retinopathy' encompasses presence of retinal changes on digital retinal photography and participants who have undergone laser therapy. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate.

Previous publications have established a classifier threshold of 0.343 for diagnosis of DN [268]. Taking this threshold as the diagnostic cut-off for the study population, 71 (45%) participants fell within the "high risk" category. A comparison of clinical characteristics between participants with classifier score above and below the diagnostic threshold is shown in Table 7-2. There was a higher proportion of male participants in the "high risk" group and more were smokers. Apart from these characteristics the only parameter differing between the categories was UAE (57mg/24hrs vs 141mg/24hrs). Other clinical parameters including age; blood pressure; BMI; HbA1c; cholesterol; and eGFR were not significantly different in patients with CKD273 score above or below the diagnostic cut-off for DN.

Parameter	CKD273>0.343 (n=71)	CKD273<0.343 (n=86)	P-value
Age (years)	61 (59-71)	61 (60-70)	0.573
Gender (M/F)	61/10	59/27	*0.010
Diabetes duration (years)	10 (1-35)	13 (1-36)	0.153
Retinopathy (Y/N)	42/29	53/33	0.752
Smokers (Y/N)	27/44	16/70	*0.007
BMI (kg/m^2)	31.3 (22.5-55.6)	31.7 (21.6-45.6)	0.662
SBP (mmHg)	130.4±17.4	128.8±15.3	0.547
DBP (mmHg)	75±11.2	73.8±11.3	0.543
HbA1c (mmol/mol)	59 (41-86)	59 (39-123)	0.118
Cholesterol (mmol/l)	3.9 (2-7)	3.8 (2.2-6.1)	0.549
UAE (mg/24hrs)	141 (9-1372)	57 (3-980)	*<0.001
eGFR (ml/min/1.73m^2)	87.6±18	89.6±16	0.452
CKD273 score	0.527 (-1.078 -1.231)	0.140 (-1.004 - 0.780)	*<0.001

Table 7-2. Baseline characteristics of cohort according to CKD273 risk score.

Data are mean±SD or median (range) depending on distribution. eGFR calculated by CKD-EPI formula. 'Retinopathy' encompasses presence of retinal changes on digital retinal photography and participants who have undergone laser therapy. Comparisons are by 2-sample t-test on transformed data where appropriate. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate.

7.4.2 Determinants of CKD273 classifier score

Chapter 5 focused on a cohort of normoalbuminuric T2DM patients and in this population with no evidence of established kidney disease there was a significant association between CKD273 score and UAE while no correlation was found with eGFR. Univariate analysis of classifier score determinants in the current population with varying degrees of albuminuria revealed statistically significant associations with both UAE (r=0.494, p=<0.001) and eGFR (r=-0.208, p=0.009) as shown in Figure 7-1. The only other clinical parameter associated with CKD273 score was age (r=0.182, p=0.023). Other clinical characteristics including disease duration (r=0.086, p=0.285); BMI (r=-0.072, p=0.368); blood pressure (r=0.067, p=0.402 for systolic; r=-0.071, p=0.382 for diastolic); HbA1c (r=-0.039, p=0.625); and cholesterol (r=0.042, p=0.602) were not associated with classifier score in this population.

Multivariate analysis shown in Tables 7-3 and 7-4 demonstrates that when all factors significantly associated with classifier score were included in a regression model the relationship between age and CKD273 lost significance while eGFR and UAE together explain 30% of the variability in CKD273 score. When other variables that could potentially influence DN status were included in the model again only eGFR and UAE remained as significant determinants of classifier score, with R^2 value of 30.92%.

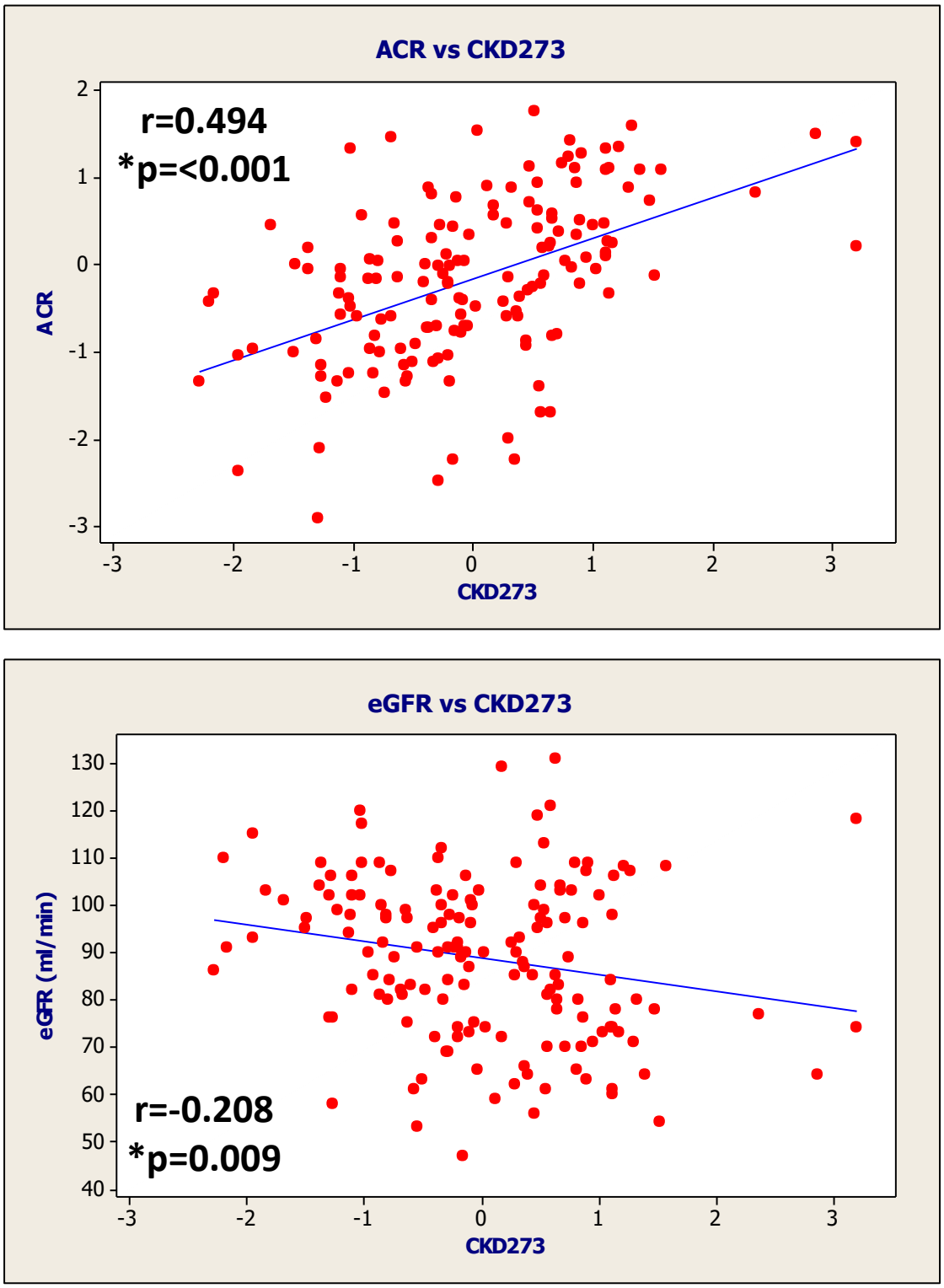

Figure 7-1. Association between CKD273 classifier and clinical indices of DN.

Correlations by Pearson's method using raw data for eGFR and transformed data for ACR. * denotes statistical significance. ACR, albumin: creatinine ratio; eGFR, estimated glomerular filtration rate.

Variable	Coefficient	SE Coefficient	P-value
Constant	1.194	0.417	**0.005*
Age (years)	0.078	0.077	0.313
UAE (mg/24hrs)	0.541	0.071	**<0.001*
eGFR (ml/min/1.73m^2)	-0.013	0.005	**0.007*

Table 7-3. Regression model 1 including all parameters associated with CKD273 on univariate analysis.

Adjusted R^2 30%. SE, standard error; urine albumin excretion; eGFR, estimated glomerular filtration rate.

Variable	Coefficient	SE Coefficient	P-value
Constant	1.676	0.647	**0.011*
UAE (mg/24hrs)	0.570	0.073	**<0.001*
eGFR (ml/min/1.73m^2)	-0.013	0.004	**0.003*
Disease duration (years)	0.010	0.077	0.895
BMI (kg/m^2)	-0.112	0.065	0.088
SBP (mmHg)	0.005	0.005	0.332
HbA1c (mmol/mol)	0.014	0.075	0.855

Table 7-4. Regression model 2 including all parameters associated with CKD273 on univariate analysis as well as factors which could potentially contribute to DN status.

Adjusted R^2 30.92%. SE, standard error; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate; BMI, body mass index; SBP, systolic blood pressure; HbA1c, glycated haemoglobin.

7.4.3 Individual component peptides

The CKD273 classifier includes 273 differentially expressed urinary peptides and these data are assimilated to calculate the overall classifier score using SVM modelling. To determine the relationship between individual peptides and renal parameters the raw peptide expression data were obtained for each study participant. Of all 273 peptides included in the classifier 54 correlated

significantly with UAE on univariate analysis as shown in Table 7-5 and 23 were found to be correlated with eGFR as shown in Table 7-6.

Peptide ID	Correlation coefficient	Peptide ID	Correlation coefficient
p2505	0.192*	**p79136**	0.184*
p24117	0.165*	**p80012**	-0.164*
p30174	0.280**	**p80891**	0.200*
p32470	-0.216**	**p81424**	0.275**
p38011	0.168*	**p82325**	-0.169*
p38879	0.227**	**p82509**	-0.204*
p39163	-0.169*	**p84192**	0.175*
p40645	0.297**	**p86798**	0.260**
p44969	-0.228**	**p89325**	0.250**
p45347	0.189*	**p90344**	-0.235**
p49295	-0.236**	**p92257**	0.405**
p50172	-0.220**	**p92698**	0.297**
p50840	-0.257**	**p96370**	0.269**
p51120	0.294**	**p97301**	0.322**
p55523	-0.225**	**p102392**	0.296**
p55582	-0.174*	**p105105**	-0.158*
p56457	0.243**	**p107929**	0.316**
p60216	0.217**	**p118597**	-0.222**
p62778	-0.167*	**p119026**	0.245**
p67097	-0.172*	**p122400**	-0.159*
p67386	-0.234**	**p125402**	-0.337**
p67462	-0.159*	**p126253**	0.248**
p67632	0.328**	**p130108**	-0.221**
p70413	0.238**	**p130243**	-0.216**
p70911	-0.164*	**p130661**	0.341**
p72533	-0.235**	**p131102**	0.215**
p73697	-0.311**	**p136698**	0.258**

Table 7-5. Correlation coefficients between UAE and the 54 individual peptides which are significantly associated with this parameter on univariate analysis.

* denotes significance at the level of 0.05 (2-tailed). ** denotes significance at the level of 0.01 (2-tailed).

Peptide ID	Correlation coefficient	Peptide ID	Correlation coefficient
p20756	0.211**	p66483	-0.162*
p25053	0.185*	p70911	0.160*
p3508	0.230**	p71312	-0.316**
p35339	0.178*	p7408	0.169*
p37715	0.169*	p76839	0.166*
p39163	0.174*	p97506	-0.203*
p41476	0.157*	p105105	0.199*
p42594	0.215**	p107460	0.235**
p48580	0.205**	p108724	0.210**
p51875	0.184*	p124886	0.173*
p51932	0.194*	p156878	-0.175*
p62778	0.167*		

Table 7-6. Correlation coefficients between eGFR and the 23 individual peptides which are significantly associated with this parameter on univariate analysis.

* denotes significance at the level of 0.05 (2-tailed). ** denotes significance at the level of 0.01 (2-tailed).

Representative examples illustrating these correlations is shown in Figure 7-2, highlighting the fact that some peptides are positively whilst others are negatively correlated with renal parameters. There was virtually no overlap between these peptides as only 4 correlated with both UAE and eGFR (Table 7-7). These were all differentially regulated fragments of collagen α-1 chain and collagen α-2 chain.

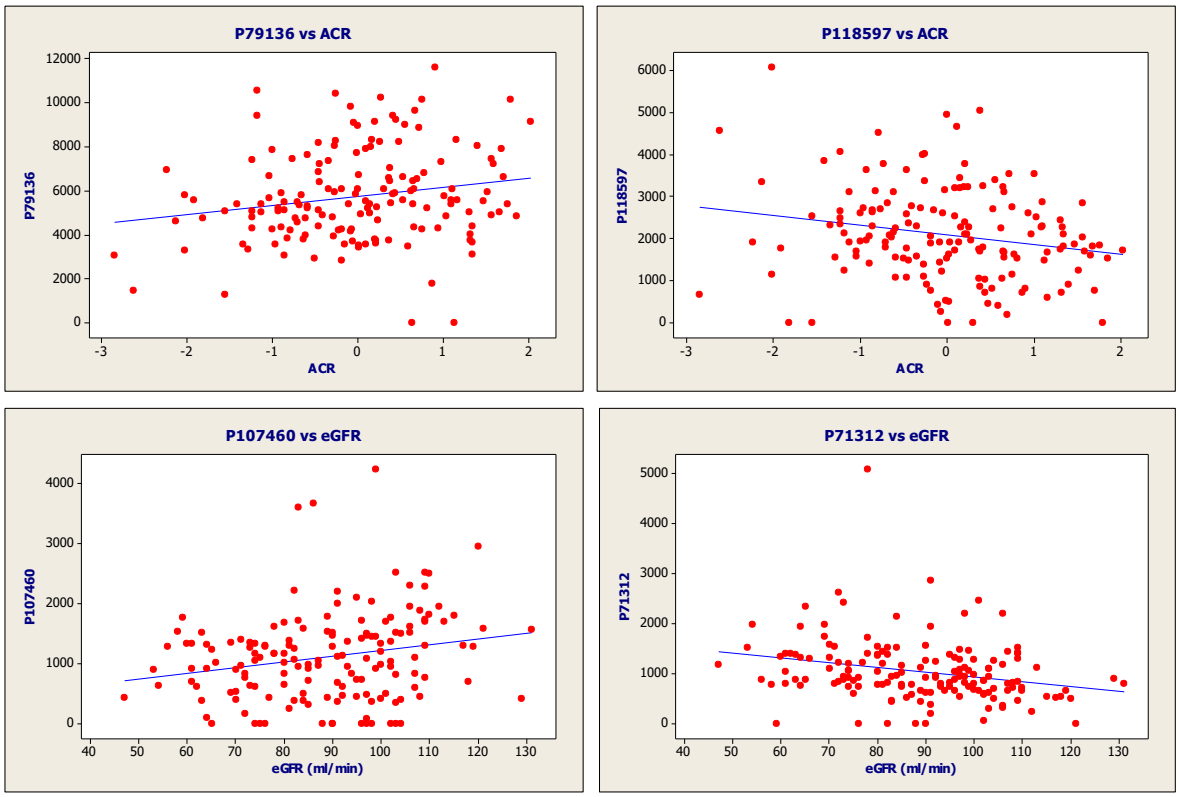

Figure 7-2. Relationships between individual peptides and renal parameters.

eGFR, estimated glomerular filtration rate; ACR, albumin: creatinine ratio.

Peptide ID	Correlation coefficient UAE	Correlation coefficient eGFR
p39163	-0.169*	0.174*
p62778	-0.167*	0.167*
p70911	-0.164*	0.160*
p105105	-0.158*	0.199*

Table 7-7. Correlation coefficients between the 4 peptides which are associated with both UAE and eGFR on univariate analysis.

* denotes significance at the level of 0.05 (2-tailed). UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate. All peptides IDs correspond to fragments of collagen α-1 and collagen α-2.

7.4.4 Renal endpoints

After 4 years of follow up 18 patients (11%) had transitioned to macroalbuminuria while 29 (18%) had experienced a decline in eGFR of at least 30%. Twenty one (13%) participants died during the follow up period and 33 (21%) had been hospitalised as a result of a cardiovascular event.

The limitations of MA as an early renal biomarker have been discussed in detail in chapter 1. A number of authors have shown that MA does not necessarily herald an inevitable decline in renal function and in fact a proportion of patients never progress beyond this stage. In order to determine whether any clinical parameters can be used to differentiate those who are more likely to progress to macroalbuminuria over the 4 year follow up period only the subpopulation of participants who were microalbuminuric at baseline (n=99) were selected for further analysis. A comparison of baseline characteristics between those who remained microalbuminuric (n=81) and those who progressed to macroalbuminuria (n=18, defined as 24hr UAE greater than 300mg) is shown in Table 7-8. In summary, there was no difference in age; gender; disease duration; smoking status; BMI; blood pressure; HbA1c; cholesterol or baseline UAE between progressors and non-progressors. As expected, a greater proportion of progressors had retinopathy at baseline and eGFR was lower in this group, although still well within what is clinically considered to be the “normal” range. CKD273 classifier score at baseline did not differentiate progressors from non-progressors in this sub-population.

Parameter	Non-progressors (n=81)	Progressors (n=18)	P-value
Age (years)	61 (32-70)	62 (41-70)	0.217
Gender (M/F)	60/21	14/4	0.458
Diabetes duration (years)	10 (1-35)	15 (4-36)	0.183
Retinopathy (Y/N)	45/36	14/4	**0.032*
Smokers (Y/N)	17/64	6/12	0.222
BMI (kg/m^2)	31.2 (24.3-55.6)	31.1 (21.6-45.4)	0.464
SBP (mmHg)	129±16	128±14	0.844
DBP (mmHg)	74±11	71±10	0.120
HbA1c (mmol/mol)	61 (39-110)	71 (44-112)	0.077
Cholesterol (mmol/l)	3.8 (2-6.1)	3.8 (2.7-4.8)	0.705
UAE (mg/24hrs)	79 (31-290)	103 (30-276)	0.141
eGFR (ml/min/1.73m^2)	92±17	82±11	**0.005*
CKD273 score	0.275 (-1.00 -1.231)	0.436 (-0.044-0.668)	0.108

Table 7-8. Clinical features progressors vs non-progressors (UAE).

Data are median (range) or mean ± SD depending on distribution. eGFR calculated by CKD-EPI formula. Comparisons are by 2-sample t-test on transformed data where appropriate. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate.

Of course progression to macroalbuminuria does not necessarily imply decline in eGFR. In order to explore the role of clinical parameters in predicting eGFR decline the whole cohort (n=157) was split into progressors (n=29), defined as a decline in eGFR of more than 30% at any time during follow up in keeping with the pre-determined primary endpoint, and non-progressors (n=112) whose eGFR did not decline significantly. This comparison is shown in Table 7-9. Only differences in age and baseline eGFR were statistically significant between these subgroups, although once again baseline eGFR remained well within what is clinically considered the “normal” range. Baseline CKD273 classifier score was not significantly different between those who experienced a decline in eGFR and those who did not.

Parameter	Non-progressors (n=112)	Progressors (n=29)	P-value
Age (years)	61 (29-71)	62 (43-71)	**0.021*
Gender (M/F)	86/26	23/6	0.771
Diabetes duration (years)	12 (1-36)	13 (3-22)	0.970
Retinopathy (Y/N)	65/47	19/10	0.461
Smokers (Y/N)	31/81	6/23	0.437
BMI (kg/m^2)	32 (23-56)	31 (22-45)	0.582
SBP (mmHg)	129±16	132±14	0.355
DBP (mmHg)	75±11	74±12	0.871
HbA1c (mmol/mol)	59 (39-123)	58 (42-112)	0.848
Cholesterol (mmol/l)	3.9 (2-6.6)	3.8 (2.9-6)	0.466
UAE (mg/24hrs)	80 (3-1073)	96 (7-1372)	0.358
eGFR (ml/min/1.73m^2)	91±17	82±14	**0.009*
CKD273 score	0.283 (-1.078-1.231)	0.261 (-0.972-0.774)	0.992

Table 7-9. Clinical features progressors vs non-progressors (eGFR).

Data are median (range) or mean ± SD depending on distribution. eGFR calculated by CKD-EPI formula. Comparisons are by 2-sample t-test on transformed data where appropriate. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate.

7.4.5 Survival analysis

Of course cross-sectional analysis cannot be used to infer any predictive value of clinical or laboratory parameters. In order to determine whether baseline CKD273 classifier score is predictive of later decline in renal function Kaplan-Meier analysis was performed.

The pre-defined primary endpoint for this analysis was occurrence of a decline in eGFR of at least 30% at any time point during the follow-up period. When the cohort were divided into subgroups with CKD273 scores above and below the published threshold for diagnosis of DN (0.343) there was no significant

difference in incidence of the primary endpoint (Log Rank (Mantel Cox) p=0.598). This is illustrated by the Kaplan-Meier plot in Figure 7-3.

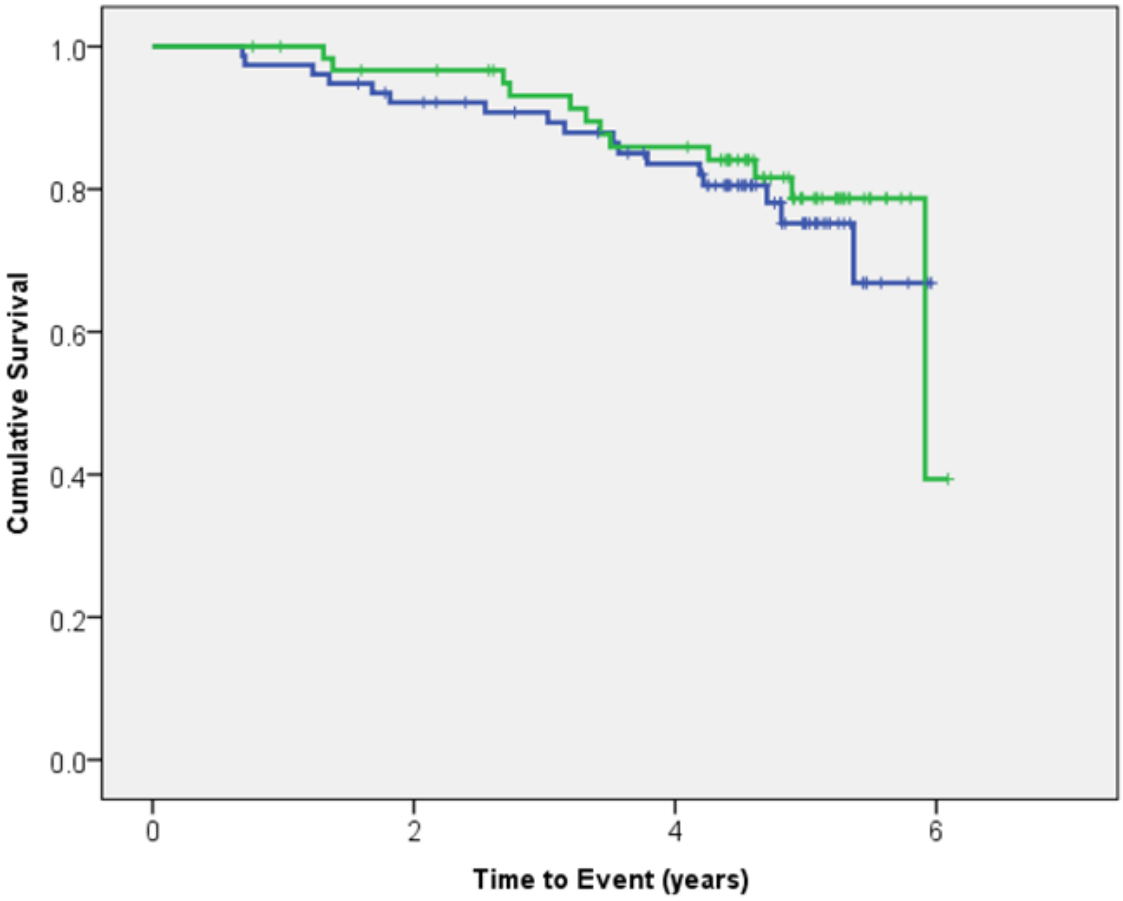

Figure 7.3. Kaplan Meier plot illustrating renal survival in patients with CKD273 score above and below threshold for diagnosis of DN.

Green line represents CKD273 score above 0.343; **blue** line represents CKD273 score less than 0.343. Vertical marks on each curve represent censored data. There was no significant difference in incidence of the primary renal endpoint in patients with CK273 above threshold for diagnosis of DN. Log Rank (Mantel Cox) p=0.598.

In order to adjust renal survival as categorised by CKD273 score for other determinants of renal function baseline UAE and eGFR were included in a Cox regression model. When these 3 predictors of renal function decline were considered, only UAE and eGFR at baseline were significant predictors of a later decline in eGFR as shown in Figure 7-4 and Table 7-9. When CKD273 classifier

score was considered as a continuous rather than categorical variable in this model the significance level for the variable "CKD273" was improved (p=0.096 vs p=0.168) as shown in Table 7-10. For this reason the cohort was also divided into tertiles of CKD273 score for survival analysis. However, CKD273 classifier score was not a significant predictor of renal survival when the cohort was considered in 3 distinct groups according to CKD273 tertile (Log Rank (Mantel Cox) p=0.864), nor when tertile 1 was compared with tertiles 2 and 3 in combination (Log Rank (Mantel Cox) p=0.862) as shown in Figures 7-5 and 7-6.

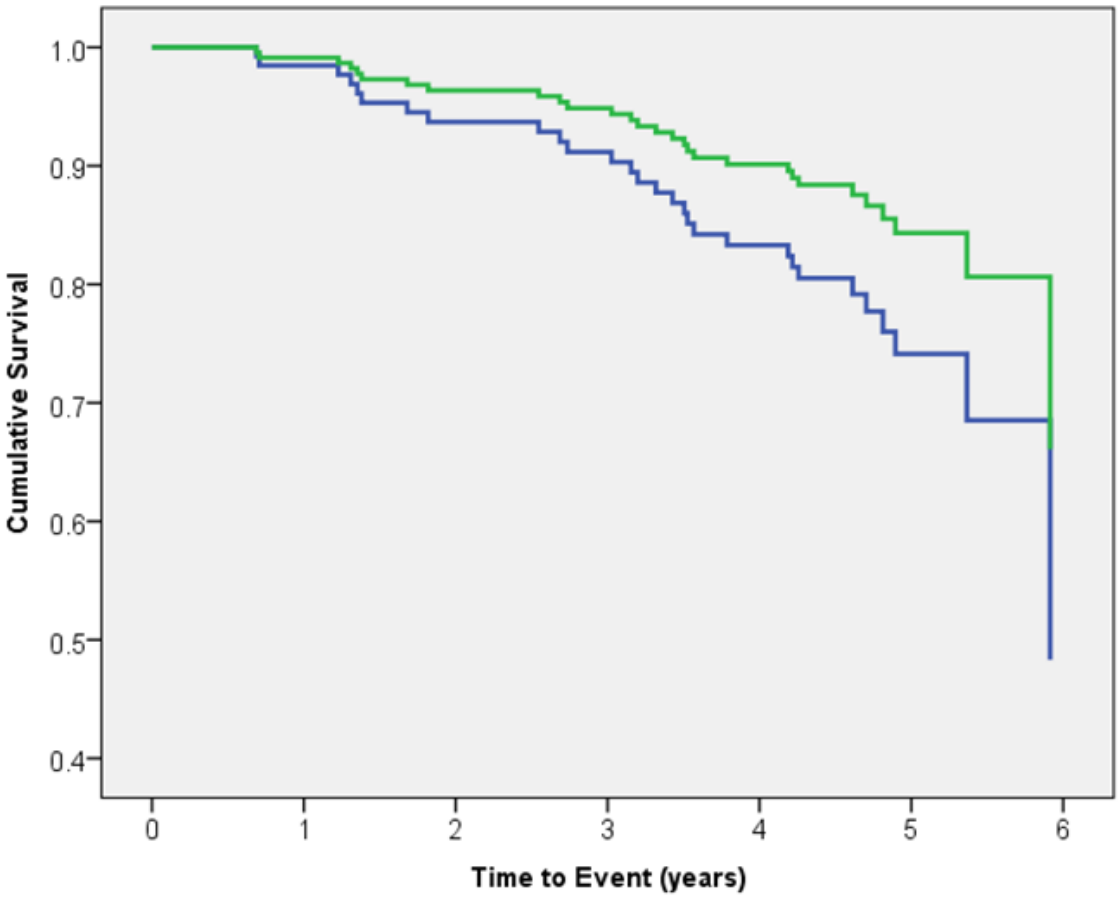

Figure 7-4. Survival plot illustrating renal survival in patients with CKD273 score above and below threshold for diagnosis of DN, adjusted for baseline UAE and eGFR.

Green line represents CKD273 score above 0.343; **blue** line represents CKD273 score less than 0.343. Cox regression analysis confirms that there is no significant difference in renal survival between the two groups (p=0.168). The covariables UAE and eGFR at baseline contribute significantly to incidence of the primary renal endpoint (p=0.016 for UAE; p=0.007 for eGFR).

	B	SE	Wald	df	Sig.	Exp (B)
CKD273 Category	.563	.408	1.903	1	.168	1.756
Baseline UAE (mg/24hrs)	.002	.001	5.826	1	.016	1.002
Baseline eGFR (ml/min/1.73m^2)	-.031	.011	7.294	1	.007	.970

Table 7-9. Cox regression model for renal event including CKD273 risk category and baseline renal parameters UAE and eGFR.

CKD273 threshold for diagnosis of DN is 0.343. B, regression coefficient; SE, standard error of regression coefficient; Wald, wald statistic (b/SE)2; df, degree of freedom; Sig, significance level; Exp (B), odds ratio; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate.

	B	SE	Wald	df	Sig.	Exp (B)
CKD273 score	-.942	.566	2.769	1	.096	.390
Baseline UAE (mg/24hrs)	.002	.001	6.590	1	.010	1.002
Baseline eGFR (ml/min/1.73m^2)	-.035	.012	8.609	1	.003	.966

Table 7-10. Cox regression model for renal event including CKD273 score as a continuous variable and baseline renal parameters UAE and eGFR.

B, regression coefficient; SE, standard error of regression coefficient; Wald, wald statistic (b/SE)2; df, degree of freedom; Sig, significance level; Exp (B), odds ratio; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate.

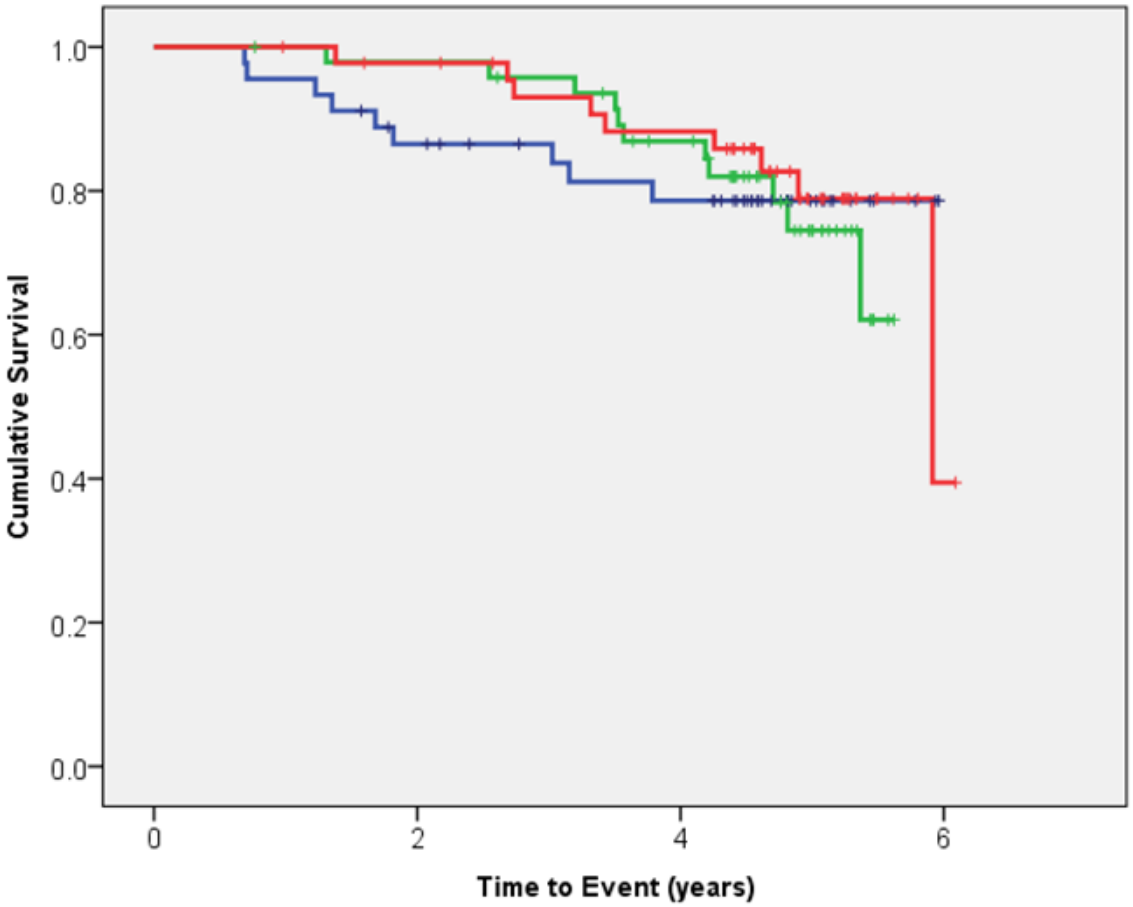

Figure 7-5. Kaplan Meier plot illustrating renal survival according to CKD273 tertile.

Blue line represents tertile 1 (53 patients; CKD273 score -1.078 to 0.143; median -0.165); **green** represents tertile 2 (52 patients; CKD273 score 0.150 to 0.472; median 0.267); **red** represents tertile 3 (52 patients; CKD273 score 0.480 to 1.231; median 0.594). Vertical marks on each curve represent censored data. There was no significant difference in incidence of the primary renal endpoint based on CKD273 tertile at baseline. Log Rank (Mantel Cox) p=0.864.

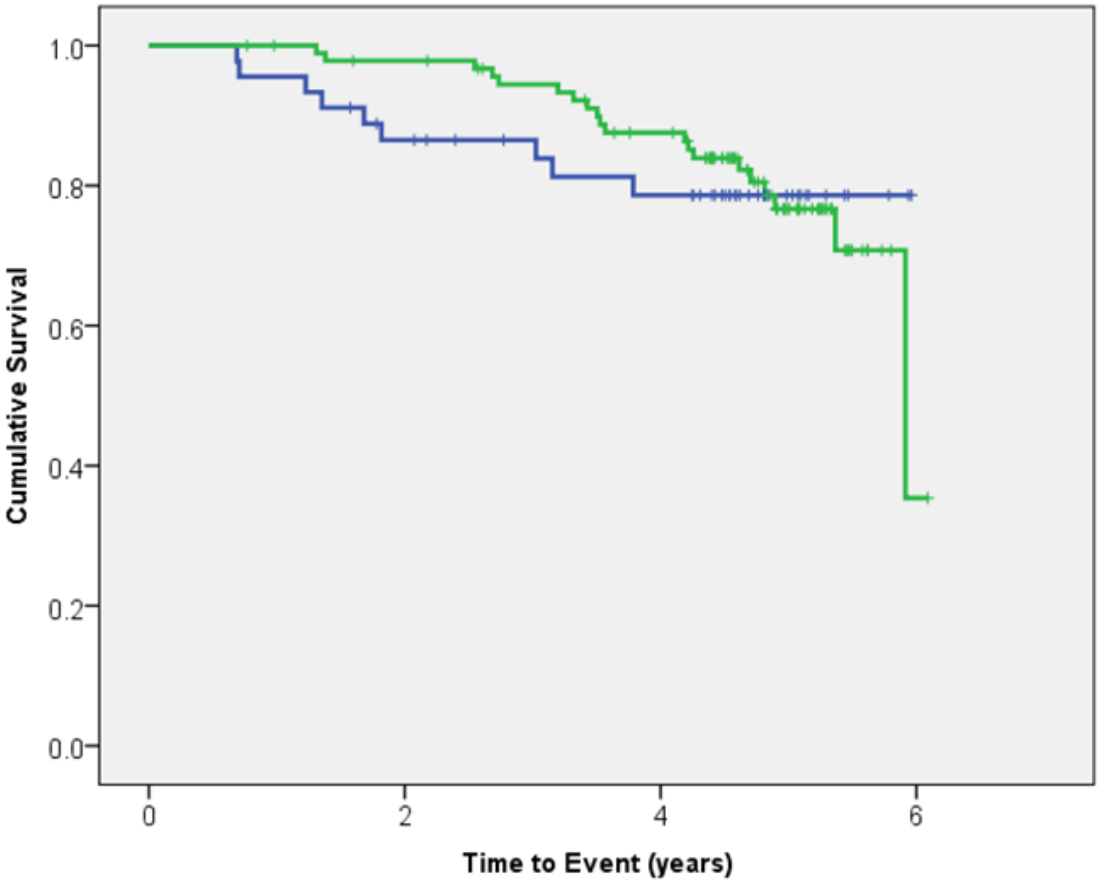

Figure 7-6. Kaplan Meier plot illustrating renal survival according to CKD273 tertile.

Blue line represents tertile 1; **green** represents tertiles 2 and 3. Vertical marks on each curve represent censored data. There was no significant difference in incidence of the primary renal endpoint based on CKD273 tertile at baseline. Log Rank (Mantel Cox) p=0.862.

As the majority of the study cohort had some degree of albuminuria at baseline, many individuals had high cardiovascular risk and may have died from this cause before reaching a renal endpoint. There was no significant difference in cardiovascular events between patients with CKD273 score above and below the pre-defined diagnostic threshold (Log Rank (Mantel Cox) p=0.598) as shown in Figure 7-7. However, analysis of all-cause mortality revealed that CKD273 score above 0.343 was a significant predictor of death in this cohort (Log Rank (Mantel Cox) p=0.004) as shown in Figure 7-8.

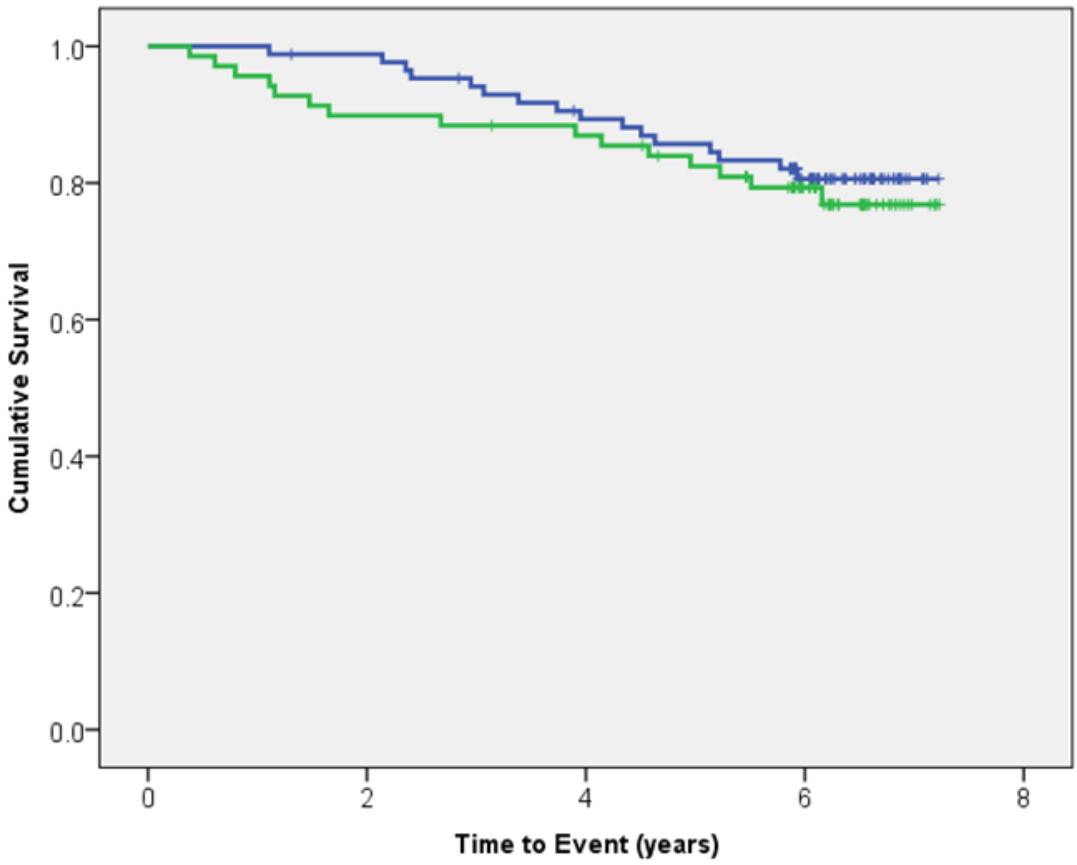

Figure 7-7. Kaplan Meier plot illustrating cardiovascular events in patients with CKD273 score above and below threshold for diagnosis of DN.

Green line represents CKD273 score above 0.343; **blue** line represents CKD273 score less than 0.343. Vertical marks on each curve represent censored data. There was no significant difference in incidence of cardiovascular events in patients with CK273 above threshold for diagnosis of DN. Log Rank (Mantel Cox) p=0.598.

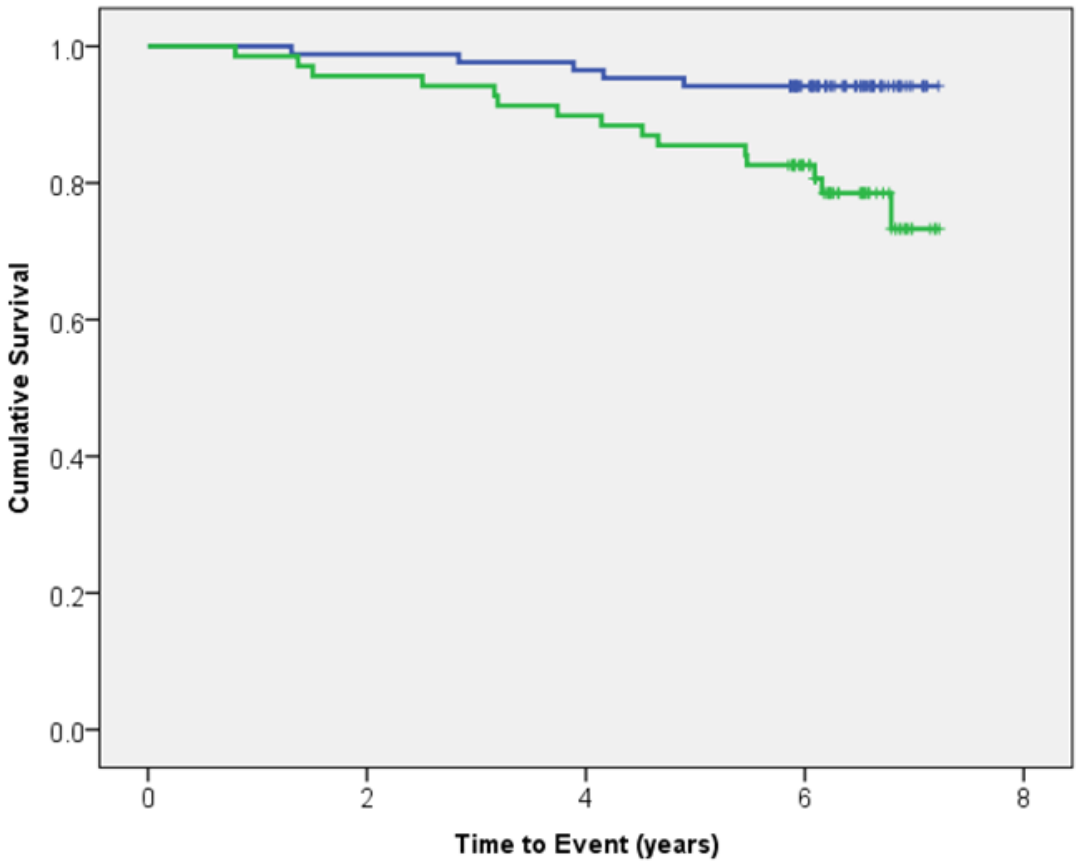

Figure 7-8. Kaplan Meier plot illustrating all-cause mortality in patients with CKD273 score above and below threshold for diagnosis of DN.

Green line represents CKD273 score above 0.343; **blue** line represents CKD273 score less than 0.343. Vertical marks on each curve represent censored data. CKD273 classifier score above the threshold for diagnosis of DN was a significant predictor of mortality. Log Rank (Mantel Cox) p=0.004.

Table 7-11 shows a comparison of baseline characteristics between participants who died during follow up (n=21) and those who survived (n=136). In summary, participants who died during follow up were significantly older and there was a higher proportion of smokers in this subpopulation. All other clinical parameters including gender; diabetes duration; blood pressure; BMI; HbA1c; cholesterol; retinopathy status; and UAE were not found to be significantly different. Median CKD273 classifier score at baseline was higher in participants who died during follow up and this difference did reach statistical significance. Inclusion of age,

gender and CKD273 category in a Cox regression model revealed that both age and CKD273 were significant predictors of mortality as shown in Table 7-12 and Figure 7-9. These characteristics remained the only significant predictors of mortality in a more comprehensive model including additional covariates as shown in Table 7-13 and also when adjusted for smoking (data not shown).

Parameter	Survivors (n=136)	Death during follow-up (n=21)	P-value
Age (years)	60 (29-71)	66 (50-70)	**0.003*
Gender (M/F)	101/35	19/2	0.077
Diabetes duration (years)	12 (1-35)	14 (4-36)	0.135
Retinopathy (Y/N)	79/57	16/5	0.104
Smokers (Y/N)	32/104	11/10	**0.009*
BMI (kg/m^2)	29 (22-56)	27 (24-44)	0.608
SBP (mmHg)	129±16	132±19	0.571
DBP (mmHg)	75±11	70±11	0.087
HbA1c (mmol/mol)	52 (39-123)	51 (43-91)	0.211
Cholesterol (mmol/l)	3.8 (2-6.6)	4 (2.4-7)	0.682
UAE (mg/24hrs)	78 (3-1372)	196 (9-864)	0.116
eGFR (ml/min/1.73m^2)	89±17	87±16	0.581
CKD273 score	0.249 (-1.078-1.133)	0.502 (-0.229-1.231)	**0.013*

Table 7-11. Baseline characteristics of cohort according to mortality during follow up. Data are mean±SD or median (range) depending on distribution.

eGFR calculated by CKD-EPI formula. 'Retinopathy' encompasses presence of retinal changes on digital retinal photography and participants who have undergone laser therapy. Comparisons are by 2-sample t-test on transformed data where appropriate and * denotes statistical significance. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate.

	B	SE	Wald	df	Sig.	Exp (B)
Sex	-.567	.751	.569	1	.451	.567
Age at baseline	.125	.047	7.196	1	.007	1.133
CKD273 category	-1.189	.520	5.221	1	.022	.304

Table 7-12. Cox regression model for mortality including CKD273 risk category, age and gender.

CKD273 threshold for diagnosis of DN is 0.343. B, regression coefficient; SE, standard error of regression coefficient; Wald, wald statistic (b/SE)2; df, degree of freedom; Sig, significance level; Exp (B), odds ratio.

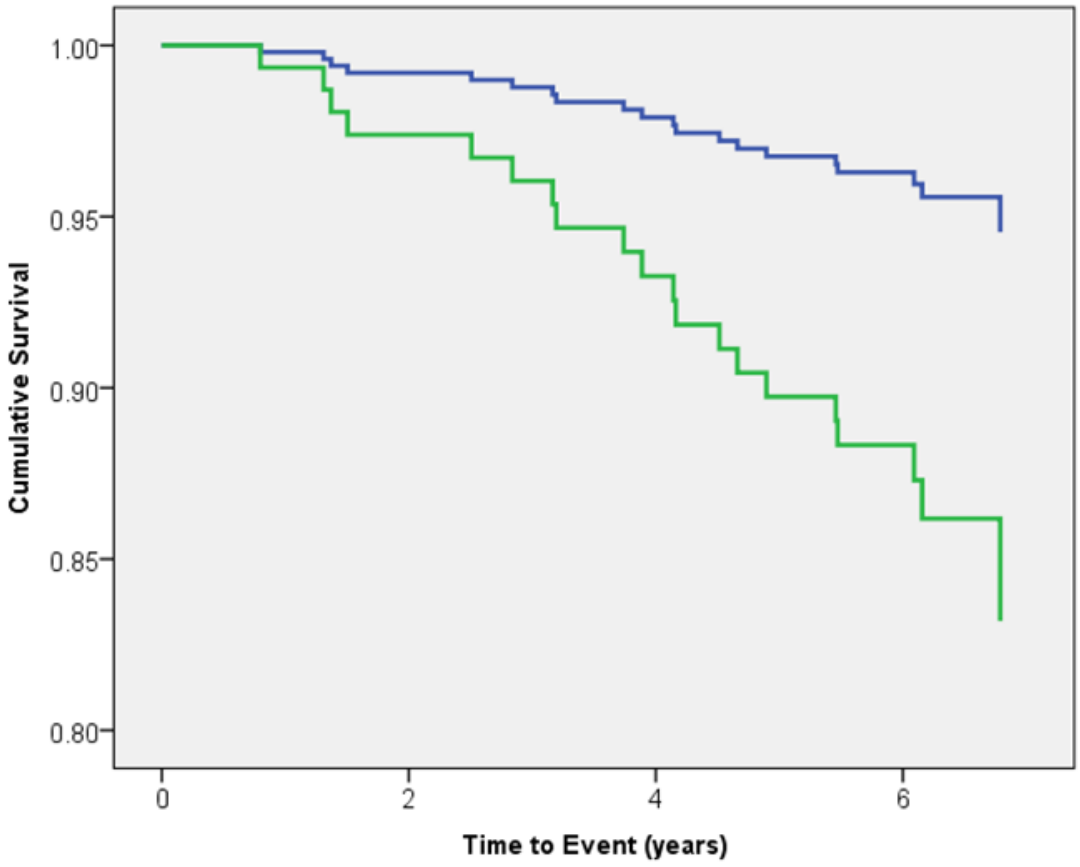

Figure 7-9. Survival plot illustrating all-cause mortality in patients with CKD273 score above and below threshold for diagnosis of DN adjusted for age and gender.

Green line represents CKD273 score above 0.343; **blue** line represents CKD273 score less than 0.343. CKD273 classifier score above the threshold for diagnosis of DN (p=0.022) and age (p=0.007) were significant predictors of mortality.

	B	SE	Wald	df	Sig.	Exp (B)
Sex	-.718	.774	.861	1	.353	.488
Age at baseline (yrs)	.162	.056	8.373	1	.004	1.176
CKD273 category	-1.311	.535	6.002	1	.014	.269
HbA1c at baseline (mmol/mol)	-.110	.230	.227	1	.634	.896
BMI at baseline (kg/m²)	.079	.050	2.507	1	.113	1.082
SBP at baseline (mmHg)	-.001	.015	.005	1	.943	.999
Retinopathy status at baseline	.651	.551	1.396	1	.237	1.917
Cholesterol at baseline (mmol/L)	.238	.282	.709	1	.400	1.268
UAE at baseline (mg/24hrs)	-.001	.001	.921	1	.337	.999
eGFR at baseline (ml/min/1.73m²)	.018	.018	1.008	1	.315	1.018

Table 7-13. Cox regression model for mortality including CKD273 category and additional baseline covariates.

CKD273 threshold for diagnosis of DN is 0.343. HbA1c, glycated haemoglobin; BMI, body mass index; SBP, systolic blood pressure; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate. B, regression coefficient; SE, standard error of regression coefficient; Wald, wald statistic (b/SE)2; df, degree of freedom; Sig, significance level; Exp (B), odds ratio.

From this analysis it appears therefore that the CKD273 classifier is not a predictor of decline in renal function or cardiovascular events in T2DM patients with established early stage kidney disease as indicated by the presence of micro- or macroalbuminuria and preserved eGFR. The classifier does, however, appear to predict mortality in this population which could therefore compete with risk of progressive CKD.

7.5 Discussion

The CKD273 urinary proteomic biomarker is currently being evaluated as a tool for early detection of DN in advance of MA reaching levels in the urine which are deemed significant by current clinical thresholds. Validation studies in a number of different cohorts have been promising [289-292, 295, 296] and the prospective PRIORITY trial will ultimately determine whether CKD273 is a useful guide to early preventative therapy [380]. Trials such as ROADMAP and BENEDICT [373, 376] have provided evidence that such early intervention can potentially delay or prevent the onset of MA, and targeting therapy towards individuals at highest risk could minimise the unwanted side-effects and adverse events associated with these agents if prescribed in an unselected, low-risk population.

Current clinical guidelines advocate prescription of RAAS blocking agents to patients who are persistently microalbuminuric. While the evidence for this intervention is certainly compelling [314, 315] such individuals are not necessarily all at equally high risk of progression to ESRD. As discussed in chapter 1, it is now accepted that MA does not herald an inevitable progression towards decline in eGFR in all patients. In fact, a significant proportion of patients will regress to normoalbuminuria over time [31, 33] and a number will remain within the MA stage without ever progressing further [30]. It could be argued therefore that strategies to identify individuals with persistent MA who are less likely to progress to ESRD may also be associated with some benefits, for example in patients who are intolerant of RAAS blocking agents, or who experience symptomatic hypotension while clinicians strive to achieve strict blood pressure targets.

Previous publications have established a classifier threshold of 0.343 for diagnosis of DN [268]. The cohort included in this work had varying levels of albuminuria (median 103mg/24hrs) and by current clinical standards can therefore be assumed to have a degree of established underlying kidney disease despite preserved eGFR. It is well known however that not all MA-positive patients will progress towards a decline in eGFR, and indeed 54% of the cohort described above had CKD273 score below the diagnostic cut-off for DN.

Prognostic potential cannot be inferred from cross-sectional analysis. Previously published work where the classifier has been retrospectively applied to baseline samples from longitudinal studies has suggested that CKD273 could be a useful tool for prediction of later decline in eGFR; transition in albuminuria status; and even hard clinical outcomes such as progression to ESRD and mortality [289-292, 296, 422]. The majority of these studies were relatively small, and included populations with notable diversity in terms of range of renal excretory function; UAE and aetiology of underlying CKD. Moreover the findings have not been consistent and in healthier cohorts only small numbers of events were seen [291].

Work described in this chapter showed that the CKD273 classifier did not predict the primary endpoint of 30% reduction in eGFR in a cohort composed entirely of T2DM patients with median UAE in the microalbuminuric range but preserved renal function at baseline. This finding was consistent when CKD273 was considered both as a categorical and continuous variable. Traditional renal risk markers UAE and eGFR at baseline were, however, significantly associated with renal outcomes during follow up. This finding is at odds with previously published work by Schanstra et al, who found that the classifier significantly improved prediction of CKD progression in a larger cohort of patients with similar UAE and eGFR at baseline [290]. However, this large cohort included patients with CKD of mixed aetiology and the investigators selected 5% decline in eGFR per year as the primary endpoint so it is not unexpected that more events were seen. Furthermore this study reported receiver operating curve statistics and net reclassification indices but no formal survival analysis was carried out. More recently Pontillo et al reported data from 2672 patients with CKD held in Human Urinary Proteome database in order to assess the utility of the classifier for identification of rapid progressors (defined as decline in eGFR of greater than 5ml/min/1.73m^2 per year) at different disease stages. This cohort also had UAE within the microalbuminuric range but preserved eGFR and were therefore clinically similar to the group described in this chapter. Their analysis showed that the classifier outperformed UAE for identification of rapid progressors in the subpopulation with eGFR greater than 70ml/min/1.73m^2; but its utility declined as disease stages advanced [292]. Once again, this was not a clinically homogeneous group as the underlying aetiology of CKD was varied and in

addition the results were based on receiver operating curve statistics rather than formal survival analysis.

Although published work at a general population level has assessed the predictive performance of the classifier using Cox regression modelling [291] the study described within this chapter is the first to formally evaluate the predictive ability of CKD273 in a homogeneous cohort of T2DM patients using this statistical methodology. The finding that UAE and eGFR at baseline were stronger predictors of renal survival is perhaps not surprising given that these patients had a degree of pre-existing renal disease and the power of the classifier appears to lie more with prediction of DN in lower-risk patients. However, in view of the fact that the majority of the subjects included in this work had micro- or macroalbuminuria the competing effects of cardiovascular events and mortality must also be considered, as it is often the case that individuals with DN die from cardiovascular events before ever progressing to ESRD [285, 385]. The work described within this chapter showed that CKD273 is not a predictor of a composite endpoint of cardiovascular events in T2DM patients with elevated UAE but preserved eGFR, however the classifier did specifically predict all-cause mortality. The explanation for this finding is unclear but for a number of subjects the cause of death was unknown and it is therefore plausible that these could be cardiovascular deaths, particularly in view of the association between cardiovascular mortality and CKD, even in its very early stages [145]. Published data concerning the relationship between CKD273 and mortality are conflicting. The results described in this chapter are in agreement with the work published by Argiles et al, who described an association between baseline CKD273 score and later mortality in a small cohort of patients with more advanced CKD of varied aetiology [289]. Conversely, a large general population level study found that CKD273 did not predict all-cause mortality [291]. However, participants included were younger and normoalbuminuric therefore the mean follow up period of 6 years may not have been adequate to address this question in a lower-risk cohort.

Work described in chapter 5 of this thesis showed that in a cohort of normoalbuminuric T2DM patients CKD273 classifier score correlated with age, BMI and ACR but not with other traditional clinical parameters and together

these factors explained only 7.72% of the variability in classifier score. Previous work in patients with more advanced established kidney disease has shown a strong negative association between eGFR and CKD273 [289, 422], a finding that was confirmed in the work described above. Patients included in the current study had evidence of early stage established kidney disease but preserved renal excretory function. In this population, CKD273 score was associated with eGFR, UAE and age. However, these parameters explained only 30% of the variability in classifier score. Taken together these data support the hypothesis that the CKD273 classifier may be providing prognostic information beyond traditional clinical parameters.

Another aim of the study described in this chapter was to explore the relationships between individual peptides and renal parameters. This question is particularly relevant in the context of the association between UAE and CKD273 described both in this chapter and in published studies [289, 290] as this association has led many to speculate that CKD273 is simply a more sensitive albuminuria test. Certainly the fact that a number of peptides captured by the classifier are albumin fragments would seem to support this argument. Work described in this chapter has shown that only 77 of the 273 peptides captured by the classifier are associated with either eGFR or UAE, whilst the remaining 196 are not. Given that the studies contained within this thesis have shown repeatedly that only 10-30% of the variability in classifier score can be explained by traditional parameters, it seems intuitive that the 196 peptides not associated with renal function may indeed be conveying additional information beyond these conventional markers. Furthermore, the patterns of association between individual peptides, UAE and eGFR are varied. If the classifier were simply a more sensitive measure of albuminuria then surely it would be expected that scatterplots for all 54 peptides associated with UAE would show positive relationships. Finally, although 54 individual peptides are associated with UAE and 23 with eGFR, only 4 peptides were found to be associated with both parameters. The fact that there is virtually no overlap between these associations suggests that many individual peptides convey specific information about other pathophysiological mechanisms underlying the development of DN. For example, in the study by Schanstra and colleagues the peptide fragments displaying the strongest correlation with baseline eGFR were fragments of α1-

antitrypsin; β2M; and collagen α1 (I) chain, whilst only α1-antitrypsin and serum albumin fragments were strongly associated with eGFR slope [290].

Strengths of this work include: recruitment of a more clinically homogeneous cohort of patients than many previously published studies; availability of longitudinal follow-up data; selection of a clinically relevant primary endpoint rather than surrogate markers; and determination of the predictive potential of the CKD273 classifier using formal survival analysis. The study described within this chapter also has a number of limitations. First, the original sample size was small and was further limited by sample availability at the time of selection. Second, although included participants were found to be microalbuminuric at screening there was a wide range of UAE at baseline, highlighting the variability of this parameter and meaning that a number of participants (at least 18%) could be considered to have established DN at inclusion as evidenced by UAE within the macroalbuminuric range. Third, the small sample size translated into a low number of events, limiting the applicability of the survival analysis.

In conclusion, the CKD273 urinary proteomic classifier was not a predictor of renal survival in this cohort of T2DM patients with elevated UAE. However, CKD273 did predict mortality in this population and this competing effect may have resulted in a lower number of renal events.

8. Discussion

8.1 The clinical context

The prevalence of diabetes continues to rise at an alarming rate in both developed and developing countries. This, in turn, has impacted on the development of DN which remains both one of the most frequent complications of the disease as well as ranking among the leading causes of ESRD worldwide. The economic impact of DN is staggering; both in terms of provision of RRT and the significant burden of cardiovascular morbidity and mortality associated with CKD and diabetes. Despite devastating effects of DN on patients and their families as well as healthcare budgets and services our understanding of the pathogenesis and progression of the condition remains relatively limited, and standard clinical strategies for both treatment and prevention are largely unchanged since the landmark trials of the early 1990s highlighted the benefits of RAAS blocking agents in patients with MA or overt albuminuria.

Even with widespread use of these agents a significant proportion of patients continue to progress to ESRD, leading some to speculate that earlier intervention in the pre-clinical stages of disease may offer additional benefits. Clinical trials aiming to answer this question have generally shown that untargeted use of RAAS blocking agents in low-risk populations can delay the onset of MA, but with an associated cost in terms of side effects and adverse events meaning that this strategy is not endorsed by current clinical guidelines. However, this then begs the question of whether early identification of “at risk” individuals and subsequently targeting preventative therapy in a personalised medicine approach may redress this balance? Current clinical practice is centred on the detection of MA as an early clinical index of DN [144], however accumulating evidence in recent years has challenged the paradigm of basing practice solely on this single marker. Although there is irrefutable evidence connecting absolute levels of UAE and its rate of change over time with development of DN [155, 165, 300] the specificity and sensitivity of MA as a single biomarker for early detection are limited. The drive away from focussing on the traditional “albuminuric” pathway towards identification of novel disease pathways and

alternative risk markers has stimulated a surge of publications describing novel biomarkers for preclinical detection of DN, although none have yet made the transition into routine practice. Accurate determination of risk is integral to clinical decision-making in order to facilitate formulation of the most appropriate management plan for each individual patient and it is therefore essential to critically evaluate all novel biomarkers according to predetermined standards if these are intended for use in the clinical setting [379, 459]. The work contained within this thesis therefore focussed on applying these predetermined evaluation standards to CKD273, a novel urinary proteomic biomarker which has shown potential as an early diagnostic indicator of DN.

8.2 Unmet clinical need and potential novel therapeutic strategy

The preliminary step in appraising a novel biomarker requires demonstration of an unmet clinical need and the availability of a suitable intervention. The underpinning supporting evidence is outlined in the introduction to this thesis. As described in chapter 1, MA is often the first clinical marker to indicate early DN. Despite being the current gold standard tool for monitoring response to treatment and determining prognosis, its sensitivity and specificity for early disease detection are limited. Novel biomarkers are therefore required in order to facilitate identification of diabetic patients in the preliminary stages of DN development and thereby target preventative intervention towards those who stand to benefit most. The search for clinically useful early markers of DN is hampered to a degree by the heterogeneity of the condition and its complex pathogenesis, likely involving simultaneous alterations in multiple mechanisms. As such, single biomarkers may be inadequate for precise recognition of at risk patients and exploration of multimarker strategies such as proteomics may prove more effective.

Another key factor for consideration in biomarker development is the availability of effective therapeutic interventions to prevent or delay progression of the condition in question. In the case of DN agents which act on the RAAS are the

current guideline-endorsed strategy for patients with clinically detectable MA [144] but despite their widespread use a proportion of patients continue to progress towards ESRD. Recent years have seen a renewed interest in the role of the non-genomic effects of aldosterone in the initiation and progression of cardiovascular and renal disease through a variety of mechanisms including endothelial dysfunction; oxidative stress; fibrosis; and inflammation, with some suggesting that MRAs should be prescribed to all CKD patients akin to the prescribing of aspirin in ischaemic heart disease [466]. There remains a reluctance among clinicians to use these drugs in CKD patients in combination with ACE-I or ARB because of perceived risks of hyperkalaemia and decline in GFR [392, 393] and the combination is not currently endorsed by clinical guidelines. The meta-analysis presented in chapter 3 served to determine whether these agents are a safe and effective therapeutic option for patients with CKD, in particular to more precisely determine effects on urinary protein or albumin excretion and to quantify the risk of clinically significant hyperkalaemia. Using both published data and unpublished summary data obtained from authors of 19 trials the work described in chapter 3 is the largest and most comprehensive meta-analysis on this matter to date. This work confirms that combining MRA with other RAAS blocking agents can offer substantial reductions of up to 31% in any measure of urinary protein or albumin excretion at the cost of small reduction in GFR and a three-fold increased risk of development of hyperkalaemia. Although this seems clinically intolerable at first glance, the definition of hyperkalaemia in the majority of included studies was 5.5mmol/L, a value which would not trigger therapeutic intervention in routine clinical practice. The incidence of clinically significant hyperkalaemia necessitating treatment was far lower. When interpreted in the context of recent meta-analysis evidence that a 30% reduction in urinary protein or albumin excretion has the potential to confer a 23.7% reduction in risk of ESRD [156] this quantifiable risk of hyperkalaemia could be acceptable in certain circumstances. Furthermore, the recently published Mineralocorticoid Receptor Antagonist Tolerability Study in Diabetic Nephropathy (ARTS-DN) confirmed that use of the nonsteroidal MRA Finerenone offers significant reductions in albuminuria with a lower incidence of hyperkalaemia [415]. In addition, the relationship between proteinuria and cardiovascular outcomes must be considered. While there is little published evidence that reductions in urinary protein or albumin excretion

translate into improved cardiovascular outcomes in patients with CKD and longterm studies focussing on hard clinical endpoints are lacking; a number of small studies, mainly in dialysis dependent patients, have shown beneficial effects on surrogate cardiovascular markers including cIMT [401], left ventricular mass [335, 399] and arterial stiffness [335, 340]. When considered in the context of the multitude of adverse effects of aldosterone on the cardiovascular system; the importance of minimising proteinuria to delay or prevent progression to ESRD; the potential clinical impact of novel agents such as Finerenone and aldosterone synthase inhibitors [467] ; and the direct relationship between proteinuria and cardiovascular risk; the evidence presented in chapter 3 certainly justifies further evaluation of the longer term risks and benefits of MRA treatment in CKD patients. Chapters 1 and 3 of this thesis therefore illustrate the preliminary stages of biomarker evaluation; demonstration of unmet clinical need and availability of a suitable intervention.

8.3 Proof of concept and validation

The second phase in biomarker evaluation requires demonstration that the biomarker in question can accurately identify the condition under study [379] and the pilot work described in chapter 4 of this thesis was designed in order to meet this criterion. The study confirmed that the CKD273 urinary proteomic biomarker was indeed able to reliably distinguish normoalbuminuric T2DM patients from those with MA and overt DN. Application of the predefined classifier threshold of 0.343 to the local test cohort resulted in identification of DN with sensitivity of 80% and specificity of 77%. This work served to solidify the biological foundation for the CKD273 test as a DN risk marker and confirmed the critical diagnostic threshold, factors which underpin the conduct of the large scale multicentre prospective study described in chapter 5. Moreover, such local level validation is essential to ensure that there has been no “overfitting” of the initial training set [268] and reproducibility of results is a key requirement for successful biomarker implementation. The majority of statistical approaches used in biomarker discovery assume that findings can be generalised and that the biomarker is only associated with the condition under investigation. As

these assumptions are not generally reflected in a “real world” setting, validation in independent cohorts is essential and the work presented in chapter 4 served to ascertain the general clinical applicability of CKD273.

The findings illustrated in chapter 4 are consistent with early published discovery data. The first paper to describe the CKD273 biomarker highlighted its utility for diagnosis of CKD with sensitivity of 85.5% and specificity of 100% [268], a degree of accuracy that could not be fully replicated in the local validation study. However, the work was based on samples from a heterogeneous group of patients with CKD of varying aetiology rather than pure diabetes-related disease and it is therefore not unexpected that diagnostic accuracy will vary in a more homogeneous cohort. One key argument emerging from this discrepancy is of course that CKD273 may therefore not be the optimal biomarker for diagnosis of DN specifically. This is undoubtedly true and the PRIORITY study [380] will serve to answer this question in due course. It should be borne in mind, however, that DN itself is a heterogeneous condition and many patients with diabetes and CKD may have mixed aetiological factors including diabetes, hypertension and vascular disease contributing to renal dysfunction. In addition, the multiple interacting pathophysiological mechanisms underpinning development of DN may limit the applicability of a single biomarker for diagnosis of such a complex condition. As such, the concept of a multimarker “omics”-based strategy is appealing [468].

While CKD273 may indeed not be the ideal DN biomarker, the work outlined in chapter 4 is consistent with a 2014 multicentre validation study where application of the pre-determined classifier threshold of 0.343 accurately distinguished DN from diabetic controls with AUC varying between 0.9 and 1.0 across 9 different centres [298] irrespective of patient age, gender or race. These results further support the stability of the classifier for identification of DN. Of course both this study and the local validation work outlined in chapter 4 identified a number of “false” positives. Given what is known about the predictive potential of the classifier it could be argued that these false positives may in fact be true positives but simply at a very early, preclinical stage of disease detectable only by more sensitive means. Such judgements cannot be

inferred from cross sectional work alone and biomarker evaluation also requires proof of incremental value beyond the current gold standard [379].

8.4 Incremental value beyond established risk markers

The third phase in biomarker evaluation involves establishing whether additive information is conveyed by the biomarker in question over and above that obtained using the current guideline-endorsed parameters [379]. This point was addressed by the results set out in chapter 7, where the effectiveness of CKD273 for prediction of hard clinical endpoints was determined in an independent cohort with a degree of established kidney disease as evidenced by albuminuria levels above the currently accepted "normal" range. Although there is a small body of evidence which suggests that CKD273 may have predictive potential beyond traditional parameters such as UAE and eGFR the majority of these studies have been carried out in heterogeneous cohorts including CKD of varying aetiology as well as with a broad range of baseline renal function. The study described in chapter 7 was the first to assess the predictive power of CKD273 in a cleaner cohort of patients with T2DM and elevated UAE. This choice of cohort seemed a particularly relevant progression as it is well-established that not all individuals with MA progress to develop a decline in eGFR or reach ESRD, therefore if a novel biomarker could be used to identify individuals who are more or less likely to progress this information would have a great deal of clinical relevance in terms of directing preventative therapy. Although survival analysis did not support the use of CKD273 to predict 30% reduction in eGFR over a 4 year follow up period the biomarker does appear to predict all-cause mortality in this sub-population. The mechanisms underpinning this relationship are unclear but this finding is in keeping with other published studies in patients with more advanced kidney disease [289].

The results laid out in chapter 7 suggest that in T2DM patients with established kidney disease the CKD273 proteomic classifier does not perform well as a predictor of decline in eGFR. The published literature evaluating the potential of CKD273 in that regard is limited; at the time the study described in chapter 7

was carried out only 2 small studies had evaluated the predictive capabilities of CKD273 in a purely diabetic population[295, 296]. Both these studies were small and focussed on surrogate endpoints rather than hard clinical outcomes. Moreover, neither used formal survival analysis to determine the predictive capabilities of the classifier. Several additional studies have assessed the value of CKD273 in more heterogeneous populations with CKD of varying aetiology. A small study in 76 patients with more advanced CKD found that classifier score above 0.55 predicted progression to dialysis and death over a 3 year follow up period [289]. In a larger cohort of 522 patients with CKD 1-3 and MA the classifier was shown to perform better than UAE for identification of patients whose kidney disease progressed more rapidly during follow up [290]. These findings were echoed in a recent large study including samples from 2672 patients [292] although in general the predictive power was best in early stage CKD and declined from CKD 3 onwards. Placing the results detailed in chapter 7 in the context of the available literature it seems that if the classifier has value as a DN biomarker, this may be more evident in the earliest stages of disease, rather than in patients with established kidney disease.

A further aspect to consider when evaluating the additive information provided by the CKD273 classifier is its relationship to clinical renal parameters in the regression models presented in chapter 5. In this cohort of normoalbuminuric patients with T2DM there was a relatively large spread of classifier scores and CKD273 was associated with ACR on both univariate and multivariate analysis, yet this factor only explained a small proportion of the variability in CKD273 score. Furthermore, comparative data shown in chapter 6 did not demonstrate any major clinically relevant differences in baseline clinical parameters between “high” and “low” risk patients based on CKD273 classifier score. If the hypothesis that CKD273 is indeed an early indicator of kidney disease proves to be correct, then the data shown in chapters 5 and 6 would seem to suggest that many other as yet unexplained factors contribute significantly to variability in classifier score and in this case it could indeed be argued that CKD273 provides some additional information beyond traditional parameters. The PRIORITY trial [380] will determine in due course whether this hypothesis is indeed correct.

8.5 Clinical utility

In order to make the transition from bench to bedside the clinical utility of a novel biomarker must also be rigorously assessed [379]. MA is the current guideline endorsed tool for determination of DN risk. As well as being a marker of renal risk there is a large body of literature confirming its predictive value for cardiovascular morbidity and mortality across a number of different populations [469]. This could also be viewed as a limitation in terms of specificity, as it is difficult to determine whether an individual with MA will progress to DN, cardiovascular disease or both. In order to be a clinically useful early indicator of DN, the ideal biomarker would have a high degree of specificity for the condition in question. The work described in chapter 6 of this thesis was designed with this particular aspect of biomarker evaluation in mind as this question had not been addressed in previously published studies appraising CKD273. The lack of association with clinical markers of vascular stiffness and subclinical atherosclerosis implies that CKD273 could indeed be a more specific early biomarker of renal disease, although of course it is impossible to disentangle this relationship completely in view of the cardiovascular risk associated with CKD as well as the fact that this was a cross-sectional study and prospective data confirming this conjecture are lacking.

8.6 Clinical outcomes

Definitive judgement of the clinical relevance of a novel biomarker hinges on whether use in clinical decision-making improves patient outcomes. The ultimate test is a prospective, biomarker-guided randomised clinical trial where therapeutic strategy is determined by the biomarker under investigation. The work contained within chapters 2 and 5 of this thesis illustrates the design, rationale and baseline characteristics of patients included in the PRIORITY trial [380]. This innovative prospective, proteomic biomarker-guided clinical trial was designed to determine whether CKD273 truly can outperform MA as an early biomarker for DN, and in addition will determine the impact of early intervention with MRAs on disease progression. Whilst this doubtless represents

a significant step forward in clinical translation of proteomic biomarkers and will provide important data irrespective of the eventual outcome, it remains to be seen whether the relatively short follow up period will allow accumulation of clinically meaningful numbers of endpoints. Furthermore, using development of MA as the primary endpoint requires reliance on surrogate biomarkers rather than hard clinical endpoints. This raises the concept of whether use of surrogate markers is acceptable in prospective studies of conditions such as DN which can take many years to evolve, and would consequently require prolonged follow up in order to achieve the holy grail of a biomarker guided trial based on hard clinical outcomes. Irrespective of its flaws as an early biomarker MA is the current guideline endorsed earliest clinical index of DN and remains the pivotal moment in an individual's clinical course which results in the prescription of renoprotective therapy. To that end, detection of MA is a clinically relevant surrogate endpoint which impacts on patient treatment in routine clinical practice. It could be argued that the clinical need for early biomarkers is of more pressing importance that achieving flawless study design in the context of conditions such as DN with far-reaching clinical, social and economic implications. Moreover, if the CKD273 panel does prove to be an effective early biomarker for DN this could itself have implications for the conduct of future clinical trials through identification of a higher-risk population for inclusion in studies, meaning smaller sample sizes and shorter durations of follow up would be required in order to accumulate endpoints.

8.7 Unanswered questions

Whilst CKD273 is undoubtedly the proteomic biomarker which so far has progressed furthest in the pathway to clinical implementation, a number of unanswered questions remain. For instance, the cost effectiveness of routine population-level proteomic testing has yet to be determined. At the time of writing proteomics is an expensive technique using specialised equipment which is not widely available and limited personnel are trained in is operation. Whether these costs are acceptable as a trade off with the financial burden associated with renal replacement therapy or hospitalisations for cardiovascular

disease in DN patients has yet to be formally evaluated. Proteomics has potential not only as a diagnostic tool, but also as a means to improve our understanding of the pathophysiology of complex disease. However, at present the significance of the appearance of individual peptides in the urine is not known; i.e. does the source protein itself play a pivotal role in the progression of DN, or is the particular peptide fragment and the means by which it is cleaved from the source protein by specific proteases of greater relevance? Surely proteomic technologies cannot transition into routine clinical practice until the wealth of data generated by these techniques can be appropriately interpreted for maximum clinical exploitation. Similarly, if the CKD273 biomarker is ultimately shown to identify patients who are at high risk of progression to DN, this begs the question of which phenotypic features differentiate these high risk individuals from the rest of the normoalbuminuric T2DM population. From the work contained within this thesis neither traditional clinical parameters nor intermediate vascular phenotypes appear to be strikingly different between high and low risk groups according to proteomic classification. Further work is therefore required in order to address this issue.

8.8 Future perspectives

A number of the findings described within this thesis merit further dissection. Firstly, the genetics of DN remain largely unexplored. It is well known that diabetic complications cluster in families and shared phenotypes have been demonstrated in both twin and offspring studies, as such the importance of family history cannot be underplayed. At present, details of family history are not collected as part of the PRIORITY study baseline visit however collecting these data retrospectively at subsequent visits would add a further interesting dimension to the trial. Second, the majority of peptides captured in the CKD273 classifier are differentially regulated collagen fragments; mainly collagens type I and III. Although in the case of CKD273 the classifier was developed purely as a biomarker rather than a tool to delve into disease mechanisms, it is attractive to speculate that these data highlight the key role of collagen metabolism in the early genesis of DN. It is well known of course that thickening of the GBM is one

of the earliest histological signs of DN, however the GBM is largely comprised of collagen type IV, with types I and III found in higher quantities in the vasculature. Identifying the source of these fragments of collagens type I and III could therefore offer novel mechanistic insights into DN development, as well the link between renal disease and the vasculature. The wealth of peptide data obtained from a CE-MS run can also be exploited to explore protease activity in greater details and quantifying changes in MMP activity in early disease could translate into identification of novel therapeutic targets for DN prevention. Thirdly, although few statistically significant differences were identified between "high" and "low risk" subjects in the analysis presented in chapter 6, a number of numerical differences would be considered clinically significant. For example BMI, systolic and diastolic BP and cholesterol were numerically higher in patients with CKD273 score above the threshold for identification of DN suggesting a possible link between DN risk and features of the metabolic syndrome. This finding merits longitudinal examination when data are available upon completion of the trial. Finally, as detailed in chapter 7, CKD273 was not found to be predictive of a 30% GFR decline in T2DM patients with elevated UAE. This was perhaps not unexpected in view of the small number of events and short follow up duration, and is in support of the use of CKD273 as biomarker in early, preclinical DN. However, exploration of the relationship between CKD273 and alternative renal endpoints such as change in GFR may be of interest. Furthermore, including intermediate vascular phenotypes in the analysis may help to tease out the interesting mortality finding further.

8.9 Conclusion

Translation of any novel biomarker or panel of biomarkers from discovery in the laboratory into routine clinical practice is a prolonged process requiring a number of necessary validation and evaluation steps. Not only should the biomarker's clinical worth be rigorously tested, it is imperative that appropriate treatment strategies can be offered to those patients deemed "at risk", otherwise the ethics of pre-clinical diagnosis could be questioned if no specific intervention is available. The work contained within this thesis takes the

CKD273 biomarker from discovery, through validation steps which ultimately informed the implementation of a large-scale biomarker guided prospective clinical trial. The studies described herein have confirmed the availability of a treatment which could be effective in reducing risk of progression to overt DN if used in selected patients, and demonstrated that the CKD273 biomarker may indeed have potential to identify these patients at an early stage. Moreover these are the first studies to evaluate the relationship between CKD273 and intermediate vascular phenotypes, an intuitive question given that much of the panel is composed of differentially regulated collagens, the origins of which are not yet clear. Time, and the PRIORITY trial, will tell whether CKD273 truly is a clinically useful early DN biomarker which could be a game-changer in clinical management of normoalbuminuric T2DM patients who are traditionally considered to be at low risk of progressive renal disease and not routinely offered preventative therapies. Regardless of the eventual outcome of the trial, PRIORITY represents a unique and innovative concept for evaluation of a proteomic biomarker and highlights the far-reaching future potential of “omics” technologies in offering truly personalised medicine.

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Appendix 1

PRIORITY Study: Invitation Letter

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

Dear _____

As you attend a Type 2 diabetes clinic in Glasgow, we would like to invite you to participate in the above research study which is being carried out at Glasgow University.

You may know that some people with diabetes are at risk of developing diabetic kidney disease. Diabetic kidney disease increases the risk of heart disease and may lead to kidney failure requiring dialysis or transplantation. In the clinic we use a blood test and a test for traces of protein in the urine to detect early stages of diabetic kidney disease so that we can prevent or delay its progression. In this research study we would like to use a new urine test called "CKD273" that has been found to detect diabetic kidney disease earlier than the standard test. The new test looks at traces of a large number of small proteins in urine and is therefore thought to be more accurate than the standard test.

Most patients are expected to have a normal CKD273 test. They will continue to receive their usual diabetes care, but we would like to look at the CKD273 test in the urine once a year for 3 years. Patients with a positive test result may be at greater risk of developing advanced stages of diabetic kidney disease compared to those with a negative test result. We will therefore offer patients with a positive test result the chance to take part in a 3 year drug trial to investigate whether taking a medicine called Spironolactone can prevent or delay the development of diabetic kidney disease.

If you are interested in taking part or would like to ask any questions about the study, please contact Dr Gemma Currie by email at gemma.currie@glasgow.ac.uk or by telephone on **0141 330 5189**. Alternatively, you may wish to complete the reply slip overleaf.

Many thanks for taking the time to read this information.

Best wishes,

Dr Christian Delles
Principal Investigator

BHF Glasgow Cardiovascular Research Centre
126 University Place
Glasgow
G12 8TA
Gemma.currie@glasgow.ac.uk
Phone: 0141 330 5189
Fax: 0141 330 2711

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

I am / am not interested in taking part in the above study. (Circle as appropriate).

Signed: _____

Print name: _____

Address: _____

_____

_____

_____

Telephone number: _____

Date: __ __ / __ __ / __ __ __ __

Upon completion, please return this slip to:

Dr Gemma Currie
Clinical Research Fellow
University of Glasgow
BHF Glasgow Cardiovascular Research Centre
126 University Place
Glasgow
G12 8TA.
Email: gemma.currie@glasgow.ac.uk
Phone: 0141 330 5189
Fax: 0141 330 2711

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

Dear _____,

You may know that our practice works with the NHS Greater Glasgow and Clyde Glasgow Clinical Research Facility to collaborate in research studies. We have recently agreed to take part in a project which you may be interested in and enclose some information about it for you.

Some people with diabetes are at risk of developing diabetic kidney disease. Diabetic kidney disease increases the risk of heart disease and may lead to kidney failure requiring dialysis or transplantation. In the clinic we use a blood test and a test for traces of protein in the urine to detect early stages of diabetic kidney disease so that we can prevent or delay its progression. In this research study we would like to use a new urine test called "CKD273" that has been found to detect diabetic kidney disease earlier than the standard test. The new test looks at traces of a large number of small proteins in urine and is therefore thought to be more accurate than the standard test.

Most patients are expected to have a normal CKD273 test. They will continue to receive their usual diabetes care, but we would like to look at the CKD273 test in the urine once a year for 3 years. Patients with a positive test result may be at greater risk of developing advanced stages of diabetic kidney disease compared to those with a negative test result. We will therefore offer patients with a positive test result the chance to take part in a 3 year drug trial to investigate whether taking a medicine called Spironolactone can prevent or delay the development of diabetic kidney disease.

If you are interested in taking part or would like to ask any questions about the study, please contact Dr Gemma Currie by email at gemma.currie@glasgow.ac.uk or by telephone on **0141 330 5189**. Alternatively, you may wish to complete the reply slip overleaf.

Many thanks for taking the time to read this information.

Best wishes,

GP Name/signature

BHF Glasgow Cardiovascular Research Centre
126 University Place
Glasgow
G12 8TA
Gemma.currie@glasgow.ac.uk
Phone: 0141 330 5189
Fax: 0141 330 2711

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

I am / am not interested in taking part in the above study. (Circle as appropriate).

Signed: _____

Print name: _____

Address: _____

_____

_____

_____

Telephone number: _____

Date: __ __ / __ __ / __ __ __ __

Upon completion, please return this slip to:

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G12 8TA.
Email: gemma.currie@glasgow.ac.uk
Phone: 0141 330 5189
Fax: 0141 330 2711

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

Do you have Type 2 diabetes?

If so, we would like to invite you to participate in the above research study which is being carried out at Glasgow University.

You may know that some people with diabetes are at risk of developing diabetic kidney disease. Diabetic kidney disease increases the risk of heart disease and may lead to kidney failure requiring dialysis or transplantation. In the clinic we use a blood test and a test for traces of protein in the urine to detect early stages of diabetic kidney disease so that we can prevent or delay its progression. In this research study we would like to use a new urine test called "CKD273" that has been found to detect diabetic kidney disease earlier than the standard test. The new test looks at traces of a large number of small proteins in urine and is therefore thought to be more accurate than the standard test.

Most patients are expected to have a normal CKD273 test. They will continue to receive their usual diabetes care, but we would like to look at the CKD273 test in the urine once a year for 3 years. Patients with a positive test result may be at greater risk of developing advanced stages of diabetic kidney disease compared to those with a negative test result. We will therefore offer patients with a positive test result the chance to take part in a 3 year drug trial to investigate whether taking a medicine called Spironolactone can prevent or delay the development of diabetic kidney disease.

If you are interested in taking part or would like to ask any questions about the study, please contact Dr Gemma Currie by email at gemma.currie@glasgow.ac.uk or by telephone on **0141 330 5189**. Alternatively, you may wish to complete the reply slip overleaf.

Many thanks for taking the time to read this information.

Best wishes,

Dr Christian Delles
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Phone: 0141 330 5189
Fax: 0141 330 2711

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

I am / am not interested in taking part in the above study. (Circle as appropriate).

Signed: _____

Print name: _____

Address: _____

_____

_____

_____

Telephone number: _____

Date: __ __ / __ __ / __ __ __ __

Upon completion, please return this slip to:

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Phone: 0141 330 5189
Fax: 0141 330 2711

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

Dear _____,

As your details are registered with the Scottish Diabetes Research Network we are writing to inform you about another diabetes research project that you may be interested in.

Some people with diabetes are at risk of developing diabetic kidney disease. Diabetic kidney disease increases the risk of heart disease and may lead to kidney failure requiring dialysis or transplantation. In the clinic we use a blood test and a test for traces of protein in the urine to detect early stages of diabetic kidney disease so that we can prevent or delay its progression. In this research study we would like to use a new urine test called "CKD273" that has been found to detect diabetic kidney disease earlier than the standard test. The new test looks at traces of a large number of small proteins in urine and is therefore thought to be more accurate than the standard test.

Most patients are expected to have a normal CKD273 test. They will continue to receive their usual diabetes care, but we would like to look at the CKD273 test in the urine once a year for 3 years. Patients with a positive test result may be at greater risk of developing advanced stages of diabetic kidney disease compared to those with a negative test result. We will therefore offer patients with a positive test result the chance to take part in a 3 year drug trial to investigate whether taking a medicine called Spironolactone can prevent or delay the development of diabetic kidney disease.

If you are interested in taking part or would like to ask any questions about the study, please contact Dr Gemma Currie by email at gemma.currie@glasgow.ac.uk or by telephone on **0141 330 5189**. Alternatively, you may wish to complete the reply slip overleaf.

Many thanks for taking the time to read this information.

Best wishes,

Dr Christian Delles
Principal Investigator

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Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

I am / am not interested in taking part in the above study. (Circle as appropriate).

Signed: _____

Print name: _____

Address: _____

_____

_____

_____

Telephone number: _____

Date: __ __ / __ __ / __ __ __ __

Upon completion, please return this slip to:

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Clinical Research Fellow
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Phone: 0141 330 5189
Fax: 0141 330 2711

University of Glasgow | Institute of Cardiovascular & Medical Sciences

EU PRIORITY

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

Dear _____,

We would like to thank you once again for your participation in our recent study of biomarkers for cardiovascular and renal risk in type 2 diabetes and are writing to inform you about another project which you may be interested in.

As you know, in the clinic we use a blood test and a test for traces of protein in the urine to detect early stages of diabetic kidney disease so that we can prevent or delay its progression. In this research study we would like to use the new urine test called "CKD273" that has been found to detect diabetic kidney disease earlier than the standard test. The new test looks at traces of a large number of small proteins in urine and is therefore thought to be more accurate than the standard test.

Most patients are expected to have a normal CKD273 test. They will continue to receive their usual diabetes care, but we would like to look at the CKD273 test in the urine once a year for 3 years. Patients with a positive test result may be at greater risk of developing advanced stages of diabetic kidney disease compared to those with a negative test result. We will therefore offer patients with a positive test result the chance to take part in a 3 year drug trial to investigate whether taking a medicine called Spironolactone can prevent or delay the development of diabetic kidney disease.

We would appreciate it if you could find the time to read the enclosed patient information leaflet which explains a bit more about the project and what it would involve for you. If you are interested in taking part or would like to ask any questions about the study, please contact Dr Gemma Currie by email at gemma.currie@glasgow.ac.uk or by telephone on **0141 330 5189**. Alternatively, you may wish to complete the reply slip overleaf.

Many thanks for taking the time to read this information.

Best wishes,

Gemma Currie
Clinical Research Fellow

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126 University Place
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Phone: 0141 330 5189
Fax: 0141 330 2711

University of Glasgow | Institute of Cardiovascular & Medical Sciences

EU PRIORITY

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

I am / am not interested in taking part in the above study. (Circle as appropriate).

Signed: _____

Print name: _____

Address: _____

_____

_____

_____

Telephone number: _____

Date: __ __ / __ __ / __ __ __ __

Upon completion, please return this slip to:

Dr Gemma Currie
Clinical Research Fellow
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BHF Glasgow Cardiovascular Research Centre
126 University Place
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G12 8TA.
Email: gemma.currie@glasgow.ac.uk
Phone: 0141 330 5189
Fax: 0141 330 2711

Appendix 2

PRIORITY Study: Participant Information Sheet

University of Glasgow | Institute of Cardiovascular & Medical Sciences

EU PRIORITY

Information about the Research:

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)

Version: **1.2 (27/11/2013)**

Protocol Code: **3004**
EudraCT No: **2012-000452-34**

Name of Researcher: Prof J Petrie, Dr C Delles

Invitation

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

What is the purpose of the study?

People with diabetes are at risk of developing diabetic kidney disease. Diabetic kidney disease increases the risk for heart disease and may lead to kidney failure requiring dialysis or transplantation.

In the clinic we use a blood test (creatinine test) and a test for traces of protein in the urine (albuminuria test) to detect early stages of diabetic kidney disease so that we can prevent or delay its progression. In this research study we would like to use a new urine test called "CKD273" that has been found to detect diabetic kidney disease earlier than the standard test that is used in the diabetes clinic. The new test looks at traces of a large number of small proteins in urine and is therefore thought to be more accurate than the standard test.

Most patients are expected to have a normal CKD273 test. They will continue to receive their usual care, but we would like to look at the CKD273 test in the urine once a year. **We explain this part of the study below ("Part 1. Observation study").**

Patients with a positive test result may be at greater risk of developing advanced stages of diabetic kidney disease compared to those with a negative test result. We will therefore offer patients with a positive test result participation in a drug trial. **We explain the drug trial below ("Part 2. Drug trial").**

We will explain further details of the study at the end of this information sheet ("Part 3. What else is important for me?").

Why have I been chosen to take part?

You have been chosen to take part because you have Type 2 diabetes and do not have diabetic kidney disease according to the standard tests (albuminuria test and creatinine test).

Do I have to take part?

No. It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of your medical treatment.

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Part 1. Observation study

What will be involved if I decide to take part?

Screening visit

We will first check if the study is suitable for you. We will therefore arrange an appointment with you which will take about 30 minutes to 1 hour:

- We will take your medical history and look through your health records
- We will measure your height, weight, heart rate and blood pressure, will examine your abdomen and will listen to your heart.
- We will take a blood sample of less than 30 millilitres (2 tablespoonfuls). We will measure routine blood markers of kidney function, diabetes control, and cholesterol levels and will store some of your sample for measurement of other markers related to diabetes and its complication in the future.
- We will ask you to provide a urine sample. We will use this sample for the CKD273 test and also test if you have a urine infection as this may change the test results. If you have a urine infection we will recommend appropriate treatment and invite you for another screening visit once the infection has resolved.
- We will also ask you to collect a urine sample first thing every morning for the next 3 days. You can send these to us in the prepaid envelopes and containers that we will provide.
- In women of childbearing age we will also perform a pregnancy test.

Once we have all 3 of your urine samples we will check to confirm that the standard test for diabetic kidney disease is negative (normal) and we will then perform the new CKD273 test in the first urine sample that you provided.

Wherever possible we will try and arrange the screening visit together with one of your routine appointments to the diabetes clinic or with your GP. If any of the tests at the screening visit shows that this study is not suitable for you we will explain this to you and your consultant or GP.

Result of the CKD273 test

We will inform you and your consultant or GP of the test result which is available after 6 weeks.

If the CKD273 test is negative (normal) nothing will change with your diabetes treatment and you will continue receiving care as usual. Once a year we would like to access your medical records and ask you to provide us again with a

set of three urine samples. We will make sure that these requests coincide with your clinic or GP appointments so that no additional study visits will be required.

After 3 years we will stop collecting urine samples and the study will be finished. We would, however, like to check your health records also in the future to see if the CKD273 test can make a change to prediction and prevention of diabetic kidney disease over a longer period of time. We expect about 80 out of 100 patients to have a negative (normal) test result.

If the CKD273 test is positive (indicating a risk of developing diabetic kidney disease) we will invite you to take part in a drug trial which we will describe below. We expect about 20 out of 100 patients to have a positive test result.

What will I have to do?

All participants should take part in the screening visit and also collect three urine samples at home.

Patients with a negative (normal) CKD273 test and those with a positive CKD273 test who do not wish to take part in the drug trial will have one study visit per year which we will try to arrange together with your routine appointment in the diabetes clinic or with your GP. We will require annual blood and urine samples from these patients.

If your CKD273 test is positive you will be invited to take part in the drug trial. Please read part 2 of this sheet to learn more about the drug trial.

What are the risks of taking part in this part of the research?

We will take blood from the vein in your arm which may result in a bruise. The amount of blood taken for this research does not place you at any risk.

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Part 2. Drug trial.

What is the purpose of the drug trial?

You have read the information about the CKD273 test in the first part of the information sheet.

This part only applies to patients who turn out to have a positive CKD273 test.

If your CKD273 test is positive we will invite you to take part in a drug trial to study if we can prevent or delay the development of diabetic kidney disease. From what we know about the test we expect that the risk of developing diabetic kidney disease is higher than that of patients with a negative (normal) CKD273 test.

It is important to point out that your risk is still very low as all the routine tests that we use in the clinic (creatinine test and albuminuria test) are normal. The CKD273 test detects the disease at very early stages that we cannot detect with the routine tests.

What will be involved if I decide to take part in the drug trial?

We would like to test if treatment of very early stages of diabetic kidney disease can prevent or delay the development of diabetic kidney disease. For this purpose we will invite those **patients who have a positive CKD273 test** to participate in a drug trial for a duration of 3 years.

Your treatment will continue to be in the hands of your GP or consultant. The only difference will be that you will be asked to take an additional tablet once daily on top of your normal medication.

Sometimes we don't know which way of treating patients is best. To find out, we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same to start with, each patient is put into a group by chance (**randomisation**). This is a "**double blind trial**", and neither you nor your doctor will know in which treatment group you are (although, if your doctor needs to find out he/she can do so). In this case you will either be in the group receiving **spironolactone** (25 milligrams) or a similar looking dummy tablet (**placebo**).

For patients taking part in the drug trial we will arrange 4 study visits per year. At each visit you will return any unused study drug and receive sufficient supplies for

the next 3 months. We will also ask you about any side effects. We will not routinely examine your heart, lung or abdomen again unless of course, you have any symptoms. Apart from this the study visits will be similar to the screening visit (see part 1 of the information sheet) including the blood and urine samples.

What will I have to do?

You should take your study medication regularly in the evening. You should also continue taking your usual medication as before. You cannot take part in another drug trial whilst taking part in this study. You should attend the study visits every three months and should report any side effects as soon as possible using the contact information below. Tablets should be stored at room temperature (between 15 and 25 °C) and must be kept out of the reach of children.

What is the drug that is being tested?

Spironolactone has been in clinical use worldwide for more than 50 years. It is prescribed to patients with high blood pressure, heart failure and other conditions. Spironolactone has also been shown to reverse some of the changes to the heart and blood vessels that are common in people with diabetes. We therefore hope it will also act on the small blood vessels in the kidney and thereby prevent or delay the development of diabetic kidney disease.

What are the risks of taking part in the drug trial?

Blood samples. We will take blood from the vein in your arm which may result in a bruise. The amount of blood taken for this research does not place you at any risk.

Study medication. If you are eligible and willing to take part in the drug trial, your study doctor will discuss the risks and benefits of spironolactone with you. During the study you may have side effects from the study drug as described below. You may experience some, none or all of these side effects or risks, and they may be mild, moderate, or severe. In addition there is always the chance of a very rare or previously unknown effect occurring. You will be closely monitored during the study for any sign of side effects. If any undesirable effects occur, you must tell your study doctor. The study doctor will decide if any treatment is needed and may decide to stop the study drug.

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Known side effects of spironolactone.

Frequency	Side effect
Very Common (>1:10)	--
Common (1:10 – 1:100)	Fatigue, changes in blood salts (high potassium and/or low sodium levels. You may not have any specific symptoms but nausea, confusion, palpitations, seizures, muscle weakness or tingling sensations can occur), breast enlargement in males, menstrual disorders in females
Less often (1:100 - 1:1000)	(Extreme) lethargy, headache, diarrhoea, indigestion, confusion, increased levels of acids in the blood, nausea and vomiting, bleeding from the stomach, rash, hives (nettle rash), changes in the blood count (lowered platelet count)
Rare (<1:1000)	Changes in the blood count (lowered white blood cell count), severe allergic reactions, acute kidney failure.
Very rare (<1:10000)	Inflammation of the liver (hepatitis), softening of the bones (osteomalacia)

Risks to the unborn child.

Please share this information with your partner if it's appropriate.

The treatment might harm the unborn child; therefore you should not take part in this study if you are pregnant, breast-feeding or you may become pregnant during the study period. If you could become pregnant, you will be asked to have a pregnancy test before taking part. You must agree to use a reliable form of contraception during the trial, e.g. oral contraceptive and condom, intra-uterine device (IUD) and condom, diaphragm with spermicide and condom.

If you do become pregnant during the course of the study, we would ask you to tell your study doctor immediately so we can help decide appropriate action. We would discuss referral for specialist counselling on the possible risks to your unborn baby and arrangements will be offered to monitor the health of both yourself and your unborn baby. We may also request your consent to collect information about your health and that of the baby.

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Part 3. What else is important for me?

Expenses and payments

You will not receive payments for taking part in this research. We will, however, reimburse your travel expenses.

What are the benefits of taking part?

We cannot promise the study will help you but the information we get might help to better detect and treat people with diabetic kidney disease in the future. We will feed any relevant findings from this study back to your GP or consultant.

What are the alternatives for diagnosis or treatment?

Patients with diabetes and early stages of diabetic kidney disease will receive medication to lower their blood pressure and to control their blood sugar levels. On top of this, certain types of blood pressure tablets (ACE inhibitors and angiotensin receptor blockers) will be prescribed to these patients if required. In the clinic early stages of diabetic kidney disease are routinely detected by protein excretion in the urine (albuminuria test) or changes in certain blood markers (creatinine test).

In this study we hope to detect diabetic kidney disease at a much earlier stage compared to the tests that are being used in the clinic. If the routine tests are normal and the CKD273 test is also normal your risk of developing diabetic kidney disease should be low, and you will continue receiving the best available care through your GP or consultant. If the CKD273 test is positive we will invite you to take part in the drug trial with spironolactone.

What happens if the CKD273 test becomes positive in subsequent years?

It is possible that the test is negative at the screening visit but becomes positive in the samples taken one to three years later. We will, however, not routinely measure these samples but only store them in a freezer and decide at the end of the study if and how many of them we will measure. From what we know about the test a negative test result at the screening visit should be valid for at least three years.

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What happens when the research study stops?

You will continue receiving care from your GP and/or consultant. No further blood and urine samples for this study will then be required, but you will continue giving blood and urine as required for clinical purposes. We will analyse the test results of all study participants to see if in the future the CKD273 test should be recommended to all people with diabetes.

An independent committee will look at the test results throughout the study. They will advise if there are any unexpected risks or benefits that may lead to extension or early termination of the study.

Five years after the end of the clinical study we will access national and local health registers to collect information about your health status regarding diabetes, cardiovascular disease and kidney disease.

Will my taking part in this study be kept confidential?

Your personal information will be kept on a file and stored in a secure place at the BHF Glasgow Cardiovascular Research Centre. All blood and urine samples will be labelled with a code and not with any personal details so that all analyses will be carried out anonymously. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital or the clinical research facility will have your name and address removed so that you cannot be recognised from it.

What if relevant new information becomes available?

Sometimes we get new information about the treatment being studied. If this happens, your research doctor will tell you and discuss whether you should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue. If you decide to continue in the study he/she may ask you to sign an updated consent form.

If the study is stopped for any other reason, we will tell you and arrange your continuing care.

What will happen if I don't want to carry on with the study?

You have a right to withdraw from the study at any time without giving us an explanation. You can also withdraw from treatment but keep in contact with us to let us know your progress. Information collected may still be used. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

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What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (see contact numbers below). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation. As a participant in this study you have statutory insurance protection which covers all damage that could be caused to your life or your health as a consequence of procedures performed on you during the study. The following insurance policy has been arranged for you:

Policy Number:	390-01163278-14053
Company Name:	HDI-Gerling Industrial Insurance Company - UK Branch
Contact Person:	NK
Company Address:	1, Great Tower Street, London, EC3R 5AA
Telephone:	020 7696 8099
Telefax:	020 7696 8444

If you wish, your study doctor will hand out to you a copy of the general insurance terms and conditions. In the event of injury you can contact the insurance company directly and make your claim for damages. If you are unsure about this you can discuss this issue with the study team.

We also advise that if you hold any private medical insurance it would be important to inform your insurer that you are taking part in this study as this may have implications for your policy.

What will happen to any samples I give?

You will donate blood and urine samples for research purposes. Some examinations on these samples will be done straight away in Glasgow. Other examinations will be done at a later stage in Glasgow or in another laboratory in Europe. We will also store some of your samples for up to 20 years to perform additional tests if required. The samples are treated as "gift"; this means you will not be entitled to any future financial reimbursement related to this study and related research. Further tests on stored samples will again require review and approval by the Ethics Committee. For this purpose we may also have to look at your medical records in the future, but again this would be subject to approval by the Ethics Committee.

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Will any genetic tests be done?

The blood and urine samples that you donate may be examined for genes that could be related to diabetes and associated diseases such as diabetic kidney disease. The tests will be performed in a research laboratory on samples that cannot identify you. The genetic tests will not normally be suitable to detect common genetic disorders, and because we will perform the tests on samples with your name removed we cannot feed any results back to you. Accordingly, the genetic tests will not compromise any insurance that you may hold or wish to take up.

What will happen to the results of the research study?

The results of the research study will be stored on a computer database and are likely to be published in medical journals and to inform the design of future studies. Reports or publications resulting from the study will not contain any personal details. The research doctor will provide a copy of the results on request.

Who is organising and funding the research?

The study will be carried out in 13 study centres across a number of European countries. The sponsor of this clinical study is the Steno Diabetes Center A/S, Niels Steensensvej 2-4, 2820 Gentofte, Denmark. The sponsor takes responsibility for the initiation and management of the clinical trial.

The European Commission Seventh Framework Programme (FP7) HEALTH provides funding for the study.

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Who has reviewed the study

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the West of Scotland Research Ethics Committee 1.

The research has also been reviewed by UK and international scientists and by representatives of the funding organisation (European Commission).

Contact for further information

Should you have any further questions please feel free to call Prof. John Petrie or Dr Christian Delles at the BHF Glasgow Cardiovascular Research Centre (0141 330 3325) or if you wish to obtain independent advice, please call Prof. Naveed Sattar at the BHF Glasgow Cardiovascular Research Centre (0141 330 3419).

Appendix 3

PRIORITY Study: Consent Form

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EU PRIORITY

CONSENT FORM PAGE 1

Title of Project:
Prediction and prevention of diabetic kidney disease ("PRIORITY" Study):

Part 1: Observation study

Name of Researcher:
Prof J Petrie, Dr C Delles

Version: 1.1
Date: 06/02/2014

Patient Identification Code for this Study: | | | | | | |

I agree to take part in the above project.

	Please initial box
• I confirm that I have read and understand the information sheet dated 27/11/2013 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
• I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
• I understand that sections of any of my medical notes and data collected during the study may be looked at by members of the research team, where it is relevant to my taking part in this research. This may also involve linkage to the national medical database that is run by NHS Scotland. I give permission for these individuals to have access to my records.	☐

The University of Glasgow, charity number SC004401

BHF Glasgow Cardiovascular Research Centre
University of Glasgow
126 University Avenue
Glasgow G12 8TA
Phone: +44 (0) 141 330 4558

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EU PRIORITY

CONSENT FORM PAGE 2

Title of Project:
Prediction and prevention of diabetic kidney disease ("PRIORITY" Study):

Part 1: Observation study

Name of Researcher:
Prof J Petrie, Dr C Delles

Version: 1.1
Date: 06/02/2014

Patient Identification Code for this Study: | | | | | | |

Please initial box

- I agree to donate samples of blood and urine, for the purposes of the research project. I understand that samples will be examined for genes and proteins that may be related to diabetes and related conditions. ☐
- I agree that my samples will be retained for future use. ☐
- I agree to my GP or consultant being informed of my taking part in this study. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

1 for participant; 1 for researcher; 1 for casenotes

Appendix 4

PRIORITY Study: Alert Card

EudraCT no.: 2012-000452-34

Patient Alert Card

Study title: **P**roteomic prediction and **R**enin angiotensin aldosterone system **I**nhibition prevention **O**f early diabetic neph**R**opathy In **TY**pe 2 diabetic patients with normoalbuminuria.

To the Patient	This card should be carried with you at all times as long as you are participating in the above clinical trial Please show this card to any doctor or other health care professional who is treating you. Remember to bring your study medication with you (used and unused), and three urine samples, to your next appointment.
To the Health care professional	The holder of this card is participating in a double blind clinical trial, and the patient is receiving study medication. Please contact the study doctor in case of emergency or before any change to the patients other medication is advised.

Study medication:
Spironolactone 25mg or placebo tablets

Prohibited medication during the study:
- Mineralocorticoid receptor antagonist treatment, ATC: C03D
- Lithium, ATC: N05AN

Name: _____

Home Address: _____

_____

Telephone no.: _____

Appointments

Visit	Date	Time
Safety		
3 month		
6 month		
9 month		
12 month		
15 month		
18 month		
21 month		
24 month		
27 month		
30 month		
33 month		
End		
Extra		
Extra		
Extra		
Extra		

In case of emergency (office hours) please contact:
University of Glasgow
Dr Gemma Currie
0141 330 5189

Emergency unblinding (24 hours), please contact:
+44 141 337 4170

Site ID: 04

Patient study ID: ☐☐☐☐

Version 1.3 - 14/11/2013

Appendix 5

PRIORITY Study: Information Letter

University of Glasgow | Institute of Cardiovascular & Medical Sciences

EU PRIORITY

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)
Researchers: Dr C Delles, Prof J Petrie, Dr G Currie.

Dear Dr,

We are writing to inform you that your patient _____ has consented to participate in the above study. This prospective, randomised, double-blind, placebo-controlled trial and observational study is being carried out at 13 European centres and is funded by an EU FP7-Health grant.

We aim to use a urinary proteomic panel (CKD273) to risk stratify a cohort of normoalbuminuric type 2 diabetic individuals into "high" and "low" risk for subsequent development of microalbuminuria. "Low risk" individuals will enter a 3 year observational study while "high risk" individuals will be given the opportunity to participate in a 3 year randomised, placebo-controlled trial to evaluate whether addition of Spironolactone 25mg reduces risk of transition to microalbuminuria. If your patient enters the intervention arm of the study we ask that you avoid prescribing the medications listed below to minimise potential interaction with study medication:

- More than one RAAS blocking agent (**ACE-inhibitor, Angiotensin Receptor Blocker, Renin Inhibitor**);
- **Lithium**;
- Potassium sparing diuretics (**Eplerenone, Spironolactone, Amiloride**).

Should you wish to receive any further information about the study copies of the protocol can be made available to you on request. Alternatively if you have any questions or wish to discuss any aspect of the study, please do not hesitate to contact us at the address below.

Kind regards,

Dr Gemma Currie
Clinical Research Fellow

BHF Glasgow Cardiovascular Research Centre
126 University Place
Glasgow
G12 8TA
Gemma.currie@glasgow.ac.uk
Phone: 0141 330 5189
Fax: 0141 330 2711

Appendix 6

Meta-analysis: Search Strategy

We searched the following databases for relevant clinical studies:

A) EMBASE (1947 – December 2014)

1) Aldo antagonist*.mp
2) Mineralocorticoid receptor blocker*.mp
3) Mineralocorticoid receptor antagonist*.mp
4) Mineralocorticoid antagonist*.mp
5) Spironolactone*.mp
6) Potassium Canrenoate*.mp
7) Canrenone*.mp
8) Aldadiene Potassium*.mp
9) Aldadiene*.mp
10) SC-9376*.mp
11) SC-9420*.mp
12) SC-14266*.mp
13) Eplerenone*.mp
14) Aldactone*.mp
15) Novospiroton*.mp
16) Spiractin*.mp
17) Soldactone*.mp
18) Soludactone*.mp
19) Practon*.mp
20) Phanurane*.mp
21) Luvion*.mp
22) Contaren*.mp

23) Eplecard*.mp

24) Epleran*.mp

25) Eptus*.mp

26) Planep*.mp

1) Chronic renal disease*.mp

2) Chronic renal failure*.mp

3) Chronic kidney insufficiency*.mp

4) Chronic renal insufficiency*.mp

5) Chronic kidney disease*.mp

6) Chronic kidney failure*.mp

7) Proteinuria*.mp

8) Albuminuria*.mp

9) Renal replacement therapy*.mp

10) Dialysis*.mp

11) Haemodialysis*.mp

12) Peritoneal dialysis*.mp

13) Kidney transplant*.mp

14) Renal transplant*.mp

15) Kidney transplantation*.mp

16) Renal transplantation*.mp

SEARCH 1 AND SEARCH 2 = 116

B) PubMed (1966 – December 2014)

1) MeSH Aldosterone antagonist
2) Canrenoate Potassium (tw)
3) Canrenone$(tw)
4) Aldadiene potassium (tw)
5) Aldadiene(tw)
6) Spironolactone(tw)
7) Eplerenone(tw)
8) Aldactone(tw)
9) Aldosterone antagonist(tw)
10) Practon(tw)
11) Phamurane(tw)
12) Soldactone(tw)
13) Soludactone(tw)
14) Spiractin(tw)
15) Novospiroton(tw)
16) SC-9376(tw)
17) SC-9420(tw)
18) SC-14266(tw)
19) Luvion(tw)
20) Contaren(tw)
21) Eplecard(tw)
22) Epleran(tw)
23) Planep(tw)
24) Mineralocorticoid Receptor Antagonist(tw)

25) Mineralocorticoid Receptor Blocker(tw)

1) Chronic kidney disease(tw)
2) Chronic renal disease(tw)
3) Chronic kidney failure(tw)
4) Chronic renal failure(tw)
5) Chronic kidney insufficiency(tw)
6) Chronic renal insufficiency(tw)
7) MeSH chronic renal failure
8) MeSH chronic kidney failure
9) MeSH chronic kidney insufficiency
10) MeSH chronic renal insufficiency
11) MeSH renal replacement therapy
12) MeSH dialysis peritoneal
13) MeSH dialysis renal
14) MeSH proteinuria
15) MeSH albuminuria
16) MeSH renal transplant
17) MeSH kidney transplant
18) Proteinuria$(tw)
19) albuminuria$(tw)
20) renal replacement therapy$(tw)
21) haemodialysis$(tw)
22) dialysis$(tw)
23) peritoneal dialysis$(tw)
24) renal transplantation$(tw)

25) kidney transplant$(tw)

SEARCH 1 AND SEARCH 2 = 57

C) Cochrane (1947 – December 2014)

1) MeSH Aldosterone antagonist
2) Canrenoate Potassium (tw)
3) Canrenone$(tw)
4) Aldadiene potassium (tw)
5) Aldadiene(tw)
6) Spironolactone(tw)
7) Eplerenone(tw)
8) Aldactone(tw)
9) Aldosterone antagonist(tw)
10) Practon(tw)
11) Phamurane(tw)
12) Soldactone(tw)
13) Soludactone(tw)
14) Spiractin(tw)
15) Novospiroton(tw)
16) SC-9376(tw)
17) SC-9420(tw)
18) SC-14266(tw)
19) Luvion(tw)
20) Contaren(tw)

21) Eplecard(tw)

22) Epleran(tw)

23) Planep(tw)

24) Mineralocorticoid Receptor Antagonist(tw)

25) Mineralocorticoid Receptor Blocker(tw)

1) Chronic kidney disease(tw)

2) Chronic renal disease(tw)

3) Chronic kidney failure(tw)

4) Chronic renal failure(tw)

5) Chronic kidney insufficiency(tw)

6) Chronic renal insufficiency(tw)

7) MeSH chronic renal failure

8) MeSH chronic kidney failure

9) MeSH chronic kidney insufficiency

10) MeSH chronic renal insufficiency

11) MeSH renal replacement therapy

12) MeSH dialysis peritoneal

13) MeSH dialysis renal

14) MeSH proteinuria

15) MeSH albuminuria

16) MeSH renal transplant

17) MeSH kidney transplant

18) Proteinuria$(tw)

19) albuminuria$(tw)

20) renal replacement therapy$(tw)

21) haemodialysis$(tw)

22) dialysis$(tw)

23) peritoneal dialysis$(tw)

24) renal transplantation$(tw)

25) kidney transplant$(tw)

SEARCH 1 AND SEARCH 2 = 126

Appendix 7

Meta-analysis: Data Extraction Form

Dear colleague, thank you for considering our proposal to join the above meta-analysis. The following is a list of data required to enable us to incorporate data from your trial in to the meta-analysis.

1. Blood Pressure (office measurement)
    a. Mean systolic BP (±SD) at baseline
        i. Treatment group (±SD) ……
        ii. Control group (±SD) ……
    b. Mean diastolic BP (±SD) at baseline
        i. Treatment group (±SD) ……
        ii. Control group (±SD) ……
    c. Mean systolic BP (±SD) at end of study
        i. Treatment group (±SD) ……
        ii. Control group (±SD) ……
    d. Mean diastolic BP (±SD) at end of study
        i. Treatment group (±SD) ……
        ii. Control group (±SD) ……
    e. Mean change (±SD) in systolic BP from baseline
        i. Treatment group (±SD) ……
        ii. Control group (±SD) ……
    f. Mean change (±SD) in diastolic BP from baseline
        i. Treatment group (±SD) ……
        ii. Control group (±SD) ……

2. Renal Function
    a. Mean serum creatinine (±SD) at baseline
        i. Treatment group (±SD) ……
        ii. Control group (±SD) ……
    b. Mean serum creatinine (±SD) at end of study
        i. Treatment group (±SD) ……
        ii. Control group (±SD) ……

c. Mean change (±SD) in serum creatinine from baseline

   i. Treatment group (±SD) ……

   ii. Control group (±SD) ……

d. Mean GFR (±SD) at baseline (actual or estimated GFR accepted)

   i. Treatment group (±SD) ……

   ii. Control group (±SD) ……

e. Mean GFR (±SD) at end of study (actual or estimated GFR accepted)

   i. Treatment group (±SD) ……

   ii. Control group (±SD) ……

f. Mean change (±SD) in GFR from baseline (actual or estimated GFR accepted)

   i. Treatment group (±SD) ……

   ii. Control group (±SD) ……

3. Renal protein/albumin excretion

   Please specify whether proteinuria or albuminuria has been measured (PCR or ACR).

   a. Mean protein/albumin creatinine ratio (±SD) at baseline

      i. Treatment group (±SD) ……

      ii. Control group (±SD) ……

   b. Mean protein/albumin creatinine ratio (±SD) at end of study

      i. Treatment group (±SD) ……

      ii. Control group (±SD) ……

   c. Mean change in protein/albumin creatinine ratio (±SD) from baseline

      i. Treatment group (±SD) ……

      ii. Control group (±SD) ……

   d. Mean 24 hour protein/albumin excretion (±SD) at baseline

      i. Treatment group (±SD) ……

      ii. Control group (±SD) ……

   e. Mean 24 hour protein/albumin excretion (±SD) at end of study

      i. Treatment group (±SD) ……

      ii. Control group (±SD) ……

f. Mean change in 24 hour protein/albumin excretion (±SD) from baseline

i. Treatment group (±SD) ……

ii. Control group (±SD) ……

4. Serum potassium

a. Mean serum potassium (±SD) at baseline

i. Treatment group (±SD) ……

ii. Control group (±SD) ……

b. Mean serum potassium (±SD) at end of study

i. Treatment group (±SD) ……

ii. Control group (±SD) ……

c. Mean change in serum potassium (±SD) from baseline

i. Treatment group (±SD) ……

ii. Control group (±SD) ……

d. Number of patients who developed Hyperkalaemia (K+>5.5) after baseline visit

i. Treatment group (±SD) ……

ii. Control group (±SD) ……

Appendix 8

Pilot Study: Patient Information Sheet

24/06/2013

Contact: Dr Gemma Currie
BHF Glasgow Cardiovascular research Centre
126 University Place
Glasgow, G12 8TA
0141 330 5189/gemma.currie@glasgow.ac.uk

Version 1.2

Biomarkers of Cardiovascular and Renal Risk in Patients with Type 2 Diabetes at Varying Stages of Diabetic Nephropathy

Information about the research

You have been invited to take part in a research study. Before you decide you need to understand the purpose of the research and what it will involve for you. Please read the following and discuss it with others if you wish. Feel free to ask us if anything is unclear to you or you require more information. You should take time to decide whether you wish to take part.

What is the purpose of the study?

We would like to assess new ways of looking at risk of cardiovascular and kidney disease in patients with Type 2 diabetes using blood and urine tests as well as studying the structure and function of blood vessels.

Why have I been chosen to take part?

We are inviting people with Type 2 diabetes who attend the clinics at Stobhill Hospital to take part in this study.

Do I have to take part?

No, it is up to you to decide whether or not you want to be involved. Participation in the project is voluntary. If you do decide to take part you will sign a consent form confirming this. You will be free to change your mind and withdraw your consent at any time. We will inform your GP and hospital Consultant that you are taking part in this project.

What is involved?

If you decide to take part, you will be invited to attend the BHF Glasgow Cardiovascular Research Centre for a single study visit. We will check that you have read and understood the information leaflet and ask you to sign a consent form agreeing to take part in the study. We will then measure your height, weight and blood pressure before taking a blood and urine sample from you. We will then perform a variety of tests to look at your blood vessels in more detail.

Contact: Dr Gemma Currie
BHF Glasgow Cardiovascular research Centre
126 University Place
Glasgow, G12 8TA
0141 330 5189/gemma.currie@glasgow.ac.uk

We will perform the following tests:

We will record a standard ECG (heart tracing) if this has not been done in the clinic recently.

You will be asked to lie down on a bed and we will perform the following tests and measurements.

1) We will perform **pulse wave analysis** at the radial artery (at your wrist). This involves applying a pencil-shaped probe to your wrist for about a minute. This will help to tell us how stiff your arteries are and provide information about the blood pressure in vessels close to your heart and brain.

2) We will then use the same technique to measure **pulse wave velocity**. Three ECG stickers will be placed on your chest and the pressure probe will be applied to the carotid artery (in your neck) and the femoral artery (in your groin). It can take a few minutes to find the best position in order to get a strong signal.

3) We will then look at the carotid (neck) arteries with an ultrasound scanner. This gives us information about the thickness of the vessel wall (**intima-media thickness**). This is an early marker of changes in blood vessels. Again this scan is performed while you are lying down and will take about 15 minutes.

4) We will then use the ultrasound scanner to look at the brachial artery (in your upper arm). This test (**brachial artery flow mediated dilation**) involves inflating a blood pressure cuff around your lower arm for 5 minutes to prevent blood flowing into the vessel. Using the ultrasound probe we will record the width of the vessel before and for 5 minutes after the cuff has been deflated. This gives us information about the ability of your blood vessels to relax and the test takes up to 20 minutes.

5) Using the same ultrasound machine we will then look at the blood vessels supplying your kidneys (**renal resistance index**). This involves applying the probe to your stomach and sides and will take around 15 minutes.

At the end of the study visit we will ask you to tell us about how you felt the visit went and any ways we could improve things for future participants. We will give you some information about the test results but they are not expected to influence any decisions about your treatment.

What are the risks of taking part?

There are no serious risks involved in taking part in the study. You might experience a bit of discomfort having your blood taken or perhaps develop a small bruise afterwards. Your blood will be taken by an experienced member of staff and we will do our best to keep you as comfortable as possible. The volume taken (20ml, or a tablespoonful) is small, and will not cause you any problems. Measurement of blood flow in your upper arm with the blood pressure cuff inflated can lead to some numbness which will disappear when the cuff is deflated. Again, this test may cause a small bruise to appear on your forearm which will disappear within a day or two.

Contact: Dr Gemma Currie
BHF Glasgow Cardiovascular research Centre
126 University Place
Glasgow, G12 8TA
0141 330 5189/gemma.currie@glasgow.ac.uk

What are the benefits of taking part?

There are no direct benefits to you for taking part but we hope that the results of our study will help in the development of new ways to determine the overall cardiovascular risk of people with Type 2 diabetes in the future.

Will my details be kept confidential?

All information about you will be stored securely at the BHF Glasgow Cardiovascular Research Centre. All samples and test results will be labelled with a code rather than your name and analysed anonymously. Any information about you which leaves the building will have your name and address removed so that you cannot be identified.

What will you do with my samples?

The samples that you give us will be used for research purposes only. They will be prepared and stored for analysis later once we have collected more samples from other participants. The samples you give will be treated as "gifts" – you will not receive any financial reimbursement related to this study. We may also store some of your samples for up to 10 years to perform additional tests if required. Any further tests on stored samples need to be agreed by the local Ethics Committee first.

What will you do with the results?

Results of the study will be stored securely on a computer database. They may also be published in medical journals. No personal details will be included in any publications. Copies of the results will be available at your request.

Many of the results of investigations we carry out will not be relevant to your current clinical care. However, in the event that we do identify anything that requires treatment or follow up we will inform your GP and hospital consultant and arrange appropriate onward referral.

Who is organising and funding the study?

The study is being organised by the BHF Glasgow Cardiovascular Research Centre and the University of Glasgow. It is funded by a grant from the European Union. Research staff will not receive any payment for conducting the study. This work is sponsored by NHS Greater Glasgow and Clyde.

Contact: Dr Gemma Currie
BHF Glasgow Cardiovascular research Centre
126 University Place
Glasgow, G12 8TA
0141 330 5189/gemma.currie@glasgow.ac.uk

Who can I contact for more information?

If you would like more information or have any questions about the study please contact

Dr Gemma Currie

Room 313, BHF Glasgow Cardiovascular Research Centre, 126 University Place, Glasgow, G12 8TA

Phone 0141 330 5189/email gemma.currie@glasgow.ac.uk

Should you wish to obtain independent advice about taking part in research please contact

Dr Marie Freel

Room 210, BHF Glasgow Cardiovascular Research Centre, 126 University Place, Glasgow, G12 8TA

Phone 0141 330 3412/email marie.freel@glasgow.ac.uk

Appendix 9

Pilot Study: Consent Form

Contact: Dr Gemma Currie
BHF Glasgow Cardiovascular Research Centre
126 University Place
Glasgow, G12 8TA
Gemma.currie@glasgow.ac.uk
0141 330 5189

Consent Form

Traditional and novel biomarkers of cardiovascular and renal risk in patients with type 2 diabetes at varying stages of diabetic nephropathy.

Name of researcher: Dr Gemma Currie

Version 1.2

24/06/2013

Patient ID Number: ___ ___ ___ ___ ___ ___ ___ ___ ___

I agree to take part in the above project.

I confirm that I have read and understood the information sheet dated 24/06/2013 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

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

I understand that sections of my medical notes and data collected during the study may be looked at by members of the research team where it is relevant to my taking part in this research, or by responsible persons from NHS Greater Glasgow and Clyde. This may also involve accessing the online medical database that is run by NHS Scotland. I give permission for these individuals to have access to my records. ☐

I agree to donate samples of blood and urine for the purposes of this research project. I understand that the sample will be examined for proteins that may be related to risk of cardiovascular and kidney damage. I understand that these samples may be retained for future use. ☐

I agree that my GP and Consultant may be informed that I am taking part in this study. ☐

_____	_____	_____
(name of patient)	(date)	(signed)
_____	_____	_____
(researcher taking consent)	(date)	(signed)

Appendix 10

Vascular Substudy: Patient Information Sheet

21/07/2014 Contact: Dr Gemma Currie Version 1.1
BHF Glasgow Cardiovascular research Centre
126 University Place
Glasgow, G12 8TA
0141 330 5189/gemma.currie@glasgow.ac.uk

Prediction and Prevention of Diabetic Kidney Disease (PRIORITY study).

Substudy: CKD273 as a biomarker for vascular and kidney disease

Information about the research

You have been invited to take part in an additional set of tests (substudy) as part of the main PRIORITY study. Taking part in these additional studies is optional and will not affect your participation in the PRIORITY trial. Before you decide you need to understand the purpose of the research and what it will involve for you. Please read the following and discuss it with others if you wish. Feel free to ask us if anything is unclear to you or you require more information. You should take time to decide whether you wish to take part.

What is the purpose of the study?

We would like to investigate the relationship between the CKD273 urine test and the general health of your kidneys and blood vessels.

Why have I been chosen to take part?

We are inviting people who are taking part in the main PRIORITY study to be involved.

Do I have to take part?

No, it is up to you to decide whether or not you want to be involved. Participation in this additional study is entirely voluntary and will not affect your involvement in the main PRIORITY study. If you do decide to take part you will sign an additional consent form confirming this. You will be free to change your mind and withdraw your consent at any time.

Contact: Dr Gemma Currie
BHF Glasgow Cardiovascular research Centre
126 University Place
Glasgow, G12 8TA
0141 330 5189/gemma.currie@glasgow.ac.uk

What is involved?

If you decide to take part you will undergo a few extra tests at your next study visit. We will check that you have read and understood this information leaflet and ask you to sign an additional consent form agreeing to take part in the substudy. We will then perform a variety of tests to look at your blood vessels in more detail.

We will perform the following tests:

1) When you are giving us the sample of blood that we need for the main PRIORITY study we will take an extra 2 tubes (14ml or one tablespoonful) which we will store in Glasgow to look at markers of early kidney and blood vessel disease.

2) When you give us your urine sample we will store 3ml of it in Glasgow to look at other markers of early kidney and blood vessel disease that can be detected in your urine.

You will then be asked to lie down on a bed and we will perform the following tests and measurements.

3) We will measure **pulse wave velocity**. Three ECG (heart tracing) stickers will be placed on your chest and a pencil-shaped pressure probe will be applied to the carotid artery (in your neck) and the femoral artery (in your groin). It can take a few minutes to find the best position in order to get a strong signal.

4) We will then look at the carotid (neck) arteries with an ultrasound scanner. This gives us information about the thickness of the vessel wall (**intima-media thickness**). This is an early marker of changes in blood vessels. Again this scan is performed while you are lying down and will take about 15 minutes.

5) Using the same ultrasound machine we will then look at the size of your kidneys. This involves applying the probe to your stomach and sides and will take around 15 minutes.

At the end of the study visit we will ask you to tell us about how you felt the visit went and any ways we could improve things for future participants. We will give

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you some information about the test results but they are not expected to influence any decisions about your treatment.

What are the risks of taking part?

There are no serious risks involved in taking part in the study. You might experience a bit of discomfort having your blood taken or perhaps develop a small bruise afterwards. Your blood will be taken by an experienced member of staff and we will do our best to keep you as comfortable as possible. The extra volume taken (14ml, or a tablespoonful) is small, and will not cause you any problems. The other tests should not cause you any discomfort. There is the chance that we could identify a narrowing in the main artery in your neck (carotid) which can mean that you are at a slightly higher risk of having a stroke; if this is the case we will ask our colleagues in the Stroke department at the Western Infirmary to repeat the scan and see you at their clinic for review. Similarly if we see any abnormalities in your kidney scan we will make sure these are followed up appropriately.

What are the benefits of taking part?

There are no direct benefits to you for taking part but we hope that the results of our study will help in the development of new ways to determine the overall cardiovascular risk of people with Type 2 diabetes in the future.

Will my details be kept confidential?

All information about you will be stored securely at the BHF Glasgow Cardiovascular Research Centre. All samples and test results will be labelled with a code rather than your name and analysed anonymously. Any information about you which leaves the building will have your name and address removed so that you cannot be identified.

What will you do with my samples?

The samples that you give us will be used for research purposes only. They will be prepared and stored for analysis later once we have collected more samples from other participants. The samples you give will be treated as “gifts” – you will not receive any financial reimbursement related to this study. We may also store some of your samples for up to 10 years to perform additional tests if required. Any further tests on stored samples need to be agreed by the local Ethics Committee first.

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What will you do with the results?

Results of the study will be stored securely on a computer database. They may also be published in medical journals and may be included in Dr Currie's PhD thesis. No personal details will be included in any publications. Copies of the results will be available at your request.

Many of the results of investigations we carry out will not be relevant to your current clinical care. However, in the event that we do identify anything that requires treatment or follow up we will inform your GP and hospital consultant and arrange appropriate onward referral.

Who is organising and funding the study?

The study is being organised by the BHF Glasgow Cardiovascular Research Centre and the University of Glasgow. It is funded by a grant from the European Union. Research staff will not receive any payment for conducting the study. This work is sponsored by the Steno Diabetes Research Centre in Denmark.

Who can I contact for more information?

If you would like more information or have any questions about the study please contact

Dr Gemma Currie

Room 313, BHF Glasgow Cardiovascular Research Centre, 126 University Place, Glasgow, G12 8TA

Phone 0141 330 5189/email gemma.currie@glasgow.ac.uk

Should you wish to obtain independent advice about taking part in research please contact

Dr Marie Freel

Room 210, BHF Glasgow Cardiovascular Research Centre, 126 University Place, Glasgow, G12 8TA

Phone 0141 330 3412/email marie.freel@glasgow.ac.uk

Appendix 11

Vascular Substudy: Consent Form

CONSENT FORM – Page 1

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)
Substudy: CKD273 as a biomarker for vascular and kidney disease

Name of Researchers:
Dr G Currie, Dr C Delles

Version: 1.1
Date: 25/09/2014

Patient Identification Code for this Study: | | | | | | |

I agree to take part in the above project.

	Please initial box
• I confirm that I have read and understand the information sheet dated __/__/____ for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
• I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
• I understand that sections of any of my medical notes and data collected during the study may be looked at by members of the research team, where it is relevant to my taking part in this research. This may also involve linkage to the national medical database that is run by NHS Scotland. I give permission for these individuals to have access to my records.	☐

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charity number SC004401

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CONSENT FORM – Page 2

Prediction and prevention of diabetic kidney disease ("PRIORITY" Study)
Substudy: CKD273 as a biomarker for vascular and kidney disease

Name of Researchers:
Dr G Currie, Dr C Delles

Version: 1.1
Date: 25/09/2014

Patient Identification Code for this Study: | | | | | | |

Please initial box

- I agree to donate additional samples of blood and urine for the purposes of the research project. I understand that samples will be examined for genes and proteins that may be related to diabetes and related conditions. ☐
- I agree that my samples will be retained for future use. ☐
- I agree to my GP or consultant being informed of my taking part in this study and of any results that are relevant to my medical care. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

1 for participant; 1 for researcher; 1 for casenotes

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