The Role of SGLT2 Inhibitors and DPP4 Inhibitors in Preventing Diabetic Nephropathy

by

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Declaration

This thesis is submitted to the University of Sydney in fulfillment of the requirement for the Degree of Doctor of Philosophy.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Authorship Attribution

Chapter 2 of this thesis is published as “Komala MG, Panchapakesan U, Pollock C, Mather A. Sodium glucose cotransporter 2 and the diabetic kidney. Curr Opin Nephrol Hypertens. 2013 Jan;22(1):113-9.”. I searched and reviewed the literature and wrote the manuscript.

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I designed and conducted the experiments with my co-authors, analysed the data and contributed to the draft of the manuscript.


I designed and conducted the experiments with my co-authors, analysed the data and contributed to the draft of the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

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Abstract

Diabetic nephropathy is the most common cause of end stage kidney disease in the world. Newer diabetic medications have arrived over the last few years and promised better outcomes. However there is lack of experimental and clinical evidence in favour of better outcomes.

The two most important drug categories that have emerged in this century are the dipeptidyl peptidase-4 inhibitors DPP4 inhibitors (DPP4i) and sodium glucose cotransporter inhibitors (SGLT2i). Their role in preventing diabetic nephropathy irrespective of their glycaemic benefits is unknown. The aim of our project is to demonstrate the renoprotective benefits of these newer medications.

In this thesis we used endothelial nitric oxide synthase knock out (eNOS -/-) mice and induced type 1 diabetes using streptozotocin (STZ) injection. We studied the changes of diabetic nephropathy in these mice including clinical outcomes, biochemical changes, inflammatory and fibrotic pathways. We determined the outcomes of diabetic mice treated with an SGLT2 inhibitor. This study showed that SGLT2 inhibitors might not have a beneficial role in preventing diabetic nephropathy when blood glucose levels were high. Subsequently we studied the role of DPP4i in preventing the interaction between DPP4 and cation independent mannose-6-phosphate receptor (CIM6PR) in the setting of high glucose in an in vitro model using kidney proximal tubular cells. Our results showed that DPP4i reduced the interaction between DPP4 and CIM6PR possibly resulting in the prevention of activation of latent TGFß. We proved this subsequently in an in vivo model of STZ induced type 1
diabetes in eNOS -/- mice using two different DPP4i. We demonstrated that linagliptin and saxagliptin were able to reduce tubulointerstitial fibronectin deposition in STZ induced type 1 diabetic in eNOS -/- mice. In addition we also showed that saxagliptin reduced markers of inflammation and renal hypertrophy. We showed reduced expression of pSmad2/3, a downstream marker of TGFβ activation with both linagliptin and saxagliptin signifying the pathway, which is targeted by the DPP4i.

Hence our studies have helped in partly identifying the puzzle of diabetic nephropathy and provide some answers on the role of newer anti diabetic agents in preventing it.
List of Publications


Saxagliptin reduces renal tubulointerstitial inflammation, hypertrophy and fibrosis
(Chapter 7)
Abstracts arising from this thesis


4. M Komala, S Gross, K Pegg, A Mather, C Huang, C Pollock and U Panchapakesan Does SGLT2 inhibition protect against diabetic nephropathy. 2013 The 7th World Congress of Nephrology Hong Kong. [Poster Presentation]

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**List of Abbreviations**

ACE: angiotensin converting enzyme  
ACR: albumin creatinine ratio  
ADA: adenosine deaminase  
AMDCC: Animal models of diabetic complications consortium  
ANP: atrial natriuretic peptide  
AP1: activator protein 1  
ARB: angiotensin receptor blockade  
ASO: anti sense oligonucleotide  
ATPase: adenosine triphosphatase  
BMD: bone mineral density  
BNP: brain natriuretic peptide  
cAMP: cyclic adenosine monophosphate  
CCL2: C-C Motif ligand 2  
CD26: cluster differentiation 26  
cDNA: complementary deoxyribonucleic acid  
CIM6PR: cation independent mannose-6-phosphate receptor  
CTGF: connective tissue growth factor  
DILI: drug induced liver injury  
DN: diabetic nephropathy  
DPP: dipeptidyl peptidase  
DPP4i: dipeptidyl peptidase-4 inhibitors  
eGFR: estimated glomerular filtration rate  
EGP: endogenous glucose production
Empa: empagliflozin

eNOS: endothelial nitric oxide synthase

FAP: fibroblast activator protein

FDA: United States Food and Drug Administration

FFPE: formalin fixed paraffin embedded

FGF: fibroblast growth factor

FRG: familial renal glycosuria

GIP: gastric inhibitory polypeptide

GLP-1: glucagon like peptide-1

GLUT-1: glucose transporter 1

GLUT-2: glucose transporter 2

GLUT-9: glucose transporter 9

GSI: glomerulosclerotic index

HD: high density lipoprotein

HK2: human kidney-2

HMGB1: high mobility group box 1

HNF1α: hepatocyte nuclear factor α

HRP: horseradish peroxidase

IC50: half minimal inhibitory concentration

ICAM-1: intracellular adhesion molecule-1

IGF-IIR: insulin like growth factor-II receptor

IGF-1: insulin like growth factor 1

IL-6: interleukin-6

IL-8: interleukin-8

JAK-STAT: Janus Kinase signal transducer and activator of transcription
LAP: latency associated peptide
LDL: low density lipoprotein
Lina: linagliptin
M6P: mannose-6-phosphate
MACE: major adverse cardiovascular effects
MCP-1: macrophage chemotactic protein-1
mRNA: messenger ribonucleic acid
Mydd88: myeloid differentiation factor-88
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NIK: NF-κB inducing kinase
NLR: nucleotide binding oligomerisation domain like receptors
NO: nitric oxide
NOX: NADPH oxidase
PAI-1: plasminogen activator inhibitor-1
PAS: periodic acid Schiff
PBS: phosphate buffered saline
PDGF: platelet derived growth factor
PFA: paraformaldehyde
PKA: protein kinase A
PKC: protein kinase C
PRR: pattern recognition receptors
pSmad2: phosphorylated Smad 2
pSmad2/3: phosphorylated Smad 2/3
PT: proximal tubule
PTC: proximal tubular cell
RAAS: renin angiotensin aldosterone system
RAGE: receptor for advanced glycation end products
ROS: reactive oxygen species
RTPCR: reverse transcription polymerase chain reaction
SDF-1α: stromal derived factor-1α
SGK1: serum and glucocorticoid regulated kinase-1
SGLT1: sodium glucose cotransporter 1
SGLT2: sodium glucose cotransporter 2
SGLT2i: sodium glucose cotransporter 2 inhibitors
SNGFR: single nephron glomerular filtration rate
STZ: streptozotocin
Telmi: telmisartan
TGF: tubuloglomerular feedback
TGFβ: transforming growth factor β
TLR2: toll like receptor 2
TLR4: toll like receptor 4
TNF-α: tumour necrosis factor α
TSP-1: thrombospondin 1
TyK: tyrosine kinase
UKPDS: UK Prospective Diabetes Studies
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
Chapter 1: Introduction
Diabetic nephropathy is the commonest cause of end stage kidney disease. This is responsible for significant morbidity and healthcare cost for the individual and the community. Diabetes causes progressive kidney disease through a number of mechanisms. Diabetes causes progressive glomerulosclerosis and tubulointerstitial fibrosis. The extent of tubulointerstitial fibrosis determines the degree of loss of kidney function.

The management of diabetes has changed over the last decade with newer treatments including dipeptidyl peptidase 4 (DPP4) inhibitors and sodium glucose cotransporter 2 (SGLT2) inhibitors heralding a paradigm shift in therapeutic approach. These drugs are used in association with metformin and insulin to improve glycaemic control. These drugs may have additional benefits in preventing diabetic complications, especially diabetic nephropathy, independent of benefits derived by glucose lowering. The aim of this study was to investigate the renoprotective benefits of these drugs independent of glucose lowering.

The proximal convoluted tubule is a very active part of the nephron and is involved in glucose and sodium reabsorption. Most of the filtered glucose is reabsorbed by sodium glucose cotransporter 2 (SGLT2) with the rest being reabsorbed by sodium glucose cotransporter 1 (SGLT1) [1]. In diabetes, there is increased filtration of glucose into the urine due to hyperglycaemia and a large proportion of this is reabsorbed by SGLT2 and the rest by SGLT1. Blocking of SGLT2 receptors resulted in prevention of glucose reabsorption and improvement in glycaemic parameters. Hyperfiltration is an early phenomenon in diabetic nephropathy. This may be related to increased proximal tubular sodium and glucose reabsorption in diabetes resulting in
tubuloglomerular feedback mediated increase in glomerular filtration [2]. Prevention of glucose reabsorption has also shown improvement in glucose induced hyperfiltration [3]. Hence we wanted to explore the dual benefits of SGLT2 inhibitors, namely reduction of hyperfiltration injury and toxic effects of high glucose in prevention of progression of diabetic nephropathy. The specific aims of this study were

a) To determine the benefits of SGLT2 inhibitor empagliflozin in preventing progression of diabetic nephropathy in an animal model of streptozotocin (STZ) induced Type 1 diabetes independent of glucose lowering.

b) To compare the outcomes with current best practice management of diabetic nephropathy, namely renin angiotensin blockade with telmisartan, an angiotensin receptor blocker.

This study showed that there was no renoprotective benefit in this animal model of type 1 diabetes in the setting of high blood glucose.

Dipeptidyl peptidase peptidase 4 inhibitors (DPP4i) increase meal induced insulin release by preventing glucagon like peptide-1 (GLP-1) degradation by enzyme DPP4. This helps in prolonged duration of action of GLP-1, which stimulates insulin release. However DPP4i have other important roles to play apart form glycaemic benefits. Importantly DPP4 is well expressed in the kidney tubule and possibly plays a role in the progression of diabetic nephropathy through promotion of profibrotic pathways in diabetes. Animal studies have pointed to renoprotective benefits of DPP4i, although these studies have also shown improved glycaemic control making it difficult to determine the specific renoprotective benefits of DPP4i independent of its glycaemic benefits [4]. It has been previously been shown that the DPP4 inhibitor linagliptin...
reduced the conversion of latent to active transforming growth factor β (TGFβ). It has also been shown that cation independent mannose-6-phosphate receptor (CIM6PR) plays an integral role in the activation of TGFβ. We know that CIM6PR and DPP4 colocalise on the cell membrane possibly promoting the activation of TGFβ. We wanted to determine the mechanism by which DPP4i interact with CIM6PR and prevent the activation of TGFβ in an in vitro model. We also wanted to explore the renoprotective benefits in an in vivo model of STZ induced type 1 diabetes. DPP4i are of two types, namely peptidomimetic and non peptidomimetic. Hence we wanted to explore the specific renoprotective benefits of both types of DPP4i independent of glucose lowering. The specific aims of this study were

a) To determine the interaction between the CIM6PR and DPP4 in context of high glucose and to determine if the DPP4 inhibitor linagliptin reduces this high glucose induced interaction

b) To determine the benefits of the non peptidomimetic DPP4 inhibitor linagliptin and the peptidomimetic inhibitor saxagliptin in preventing progression of diabetic nephropathy in an animal model of streptozotocin (STZ) induced Type 1 diabetes independent of glucose lowering.

c) To compare the outcomes with current best practice management of diabetic nephropathy, namely renin angiotensin blockade with telmisartan, an angiotensin receptor blocker.

This study revealed that linagliptin reduced high glucose induced interaction between CIM6PR and DPP4 and downstream markers of the TGFβ pathway. This study also showed that saxagliptin was able to reduce downstream markers of the TGFβ pathway and reduced tubulointerstitial collagen deposition.
This thesis consists of the following eight chapters:

Chapter 1 provides a brief introduction to the research question in this thesis and the aims and objectives of the thesis.

Chapter 2 is a peer reviewed publication in Current Opinion in Nephrology and Hypertension (Sodium glucose cotransporter 2 and the diabetic kidney) [5]. This provides a summary of literature regarding the role of SGLT2 in diabetic nephropathy and the available evidence at that time regarding the renal outcomes of SGLT2 inhibitors.

Chapter 3 is a peer reviewed publication in Expert Review in Clinical Pharmacology (Empagliflozin for the treatment of type 2 diabetes) [6]. This review looked at the pharmacological aspects of the study drug, empagliflozin and the available evidence on renal outcomes from animal based studies and clinical trials.

Chapter 4 is a peer reviewed publication in PLoS One (Inhibition of kidney proximal tubular glucose reabsorption does not prevent against diabetic nephropathy in type 1 diabetic eNOS knock out mice) [7]. The renal outcomes of empagliflozin treatment on STZ induced diabetic endothelial nitric oxide synthase knock out mice (eNOS -/-) were studied. Both clinical and histopathological parameters were determined and the expression of glucose transporters were determined in diabetic and control mice.

Chapter 5 provides a summary of the current literature regarding DPP4i and the role of DPP4 inhibition and GLP-1 agonists on renal outcomes in diabetic nephropathy.

Chapter 6 is a peer reviewed publication in PLoS One (Linagliptin limits high glucose induced conversion of latent to active TGFβ through interaction with CIM6PR and limits renal tubulointerstitial fibronectin) [8]. This chapter determined
the role of linagliptin, a non peptidomimetic DPP4 inhibitor, in preventing high glucose induced interaction between membrane bound DPP4 and CIM6PR. This chapter also looked at role of linagliptin on clinical parameters and in reducing tubulointerstitial fibronectin deposition in diabetic nephropathy in STZ induced diabetic eNOS +/- mice.

**Chapter 7** is a peer reviewed publication in Nephrology (Saxagliptin reduces renal tubulointerstitial inflammation, hypertrophy and fibrosis in diabetes) [9]. This chapter determined the effect of saxagliptin, a peptidomimetic DPP4 inhibitor on clinical parameters and in preventing inflammation and fibrosis in STZ induced diabetic eNOS +/- mice.

**Chapter 8** provides a summary and updated clinical evidence regarding the role of SGLT2 inhibitors and DPP4i since the publication of the above mentioned chapters. It also describes the future direction of research with regards to these agents in preventing diabetic nephropathy and improving cardiovascular outcomes.
Chapter 2: SGLT2 and the diabetic kidney

Komala MG, Panchapakesan U, Pollock C, Mather A.

Abstract

Purpose of review
Reabsorption of glucose in the proximal tubule occurs predominantly via the sodium glucose cotransporter 2 (SGLT2). There has been intense interest in this transporter as a number of SGLT2 inhibitors have entered clinical development. SGLT2 inhibitors act to lower plasma glucose by promoting glycosuria and this review aims to outline the effect on the diabetic kidney of this hypoglycaemic agent.

Recent findings
This review provides an overview of recent findings in this area: the transcriptional control of SGLT2 expression in human proximal tubular cells implicates a number of cytokines in the alteration of SGLT2 expression; experimental data show that SGLT2 inhibition may correct early detrimental effects of diabetes by reducing proximal tubular sodium and glucose transport, suggesting a possible renoprotective effect independent of the glucose lowering effects of these agents; and the nonglycaemic effects of SGLT2 inhibitors may have an impact on renal outcomes.

Summary
The available clinical evidence shows consistent reduction in glycaemic parameters and some evidence suggests additional effects including weight loss and mild blood pressure reduction. There are some side effects that warrant further investigation and establishing whether SGLT2 inhibition provides a renal benefit relies on future long-term studies with specific renal end-points.
2.1 Introduction

Diabetic nephropathy is the most common cause of end stage kidney disease in the world. The prevalence of some degree of renal involvement in diabetic patients reaches 40% [10] with significant progression to end stage disease. A treatment gap exists in the current prevention and treatment of diabetic nephropathy and implementing current best practice reduces this gap at best by 30%. Subsequently, there is significant interest in the role of sodium glucose co-transporter 2 (SGLT2) inhibitors with many drugs from this class in phase 3 clinical trials and some awaiting approval for licensing. These drugs act by blocking glucose reabsorption in the proximal tubule, resulting in increased glycosuria and a subsequent reduction in blood sugar levels. Whilst the use of SGLT2 inhibitors has been confirmed in clinical trials [11, 12] to result in improvement in glycaemic control, the renal effects are less well studied. They include potential benefits both from improved glycaemic control and from effects independent of glycaemic control such as, weight reduction, lowering of blood pressure with improved cardiovascular outcomes and unique renoprotective benefits. There are, however, also potential risks related to increased glycosuria including higher frequency of urinary infections, genital fungal infections, volume depletion and off target effects. This review will outline the current evidence in regard to both the renal risks and benefits of SGLT2 inhibitors.
2.2 Glucose and sodium absorption by the kidney

Nearly 180 g of glucose is filtered in the glomerular filtrate everyday. The kidney absorbs most of this glucose with less than 0.5g excreted in the urine daily [13]. The vast majority of this is accomplished in the proximal tubule.

The sodium dependent glucose transporters (SGLT), located on the apical side of the proximal tubule cell, are able to accumulate glucose within the cell against a concentration gradient by transporting glucose concurrently with sodium. A sodium concentration gradient is provided by a Na-K-adenosine triphosphatase (Na-KATPase) pump located on the basolateral side that pumps sodium out of the cell. Glucose is then passively transported across the basolateral side of the cell via facilitative glucose transporters (GLUT) into the interstitium. In the early segments of the proximal tubule, SGLT2 on the apical membrane is coupled with GLUT2 on the basolateral side, and it reabsorbs up to 90% of filtered glucose under normoglycaemic conditions [14]. By blocking the reabsorption of glucose and sodium in the proximal tubule, SGLT2 inhibitors act to reduce blood glucose levels by enhancing glucose excretion, and promote a natriuretic and diuretic action, which should normalise the altered sodium handling seen in diabetes.

To demonstrate the glucose reabsorption capacities of SGLT2 further, SGLT1 gene knockout mice show nearly 97 % reabsorption of filtered glucose compared to wild type mice [15]. By contrast, it has been shown in SGLT2 gene knockout animals that reabsorption of the filtered glucose is incomplete, ranging from 10-60% depending on the amount of filtered glucose. Whilst this demonstrates that SGLT1 transporters are
not able to completely make up for a lack of SGLT2, there is some compensatory uptake by SGLT1 in this situation[1], which has implications for patients treated with SGLT2 inhibitors. In addition, a third type of sodium glucose transporter, SGLT3, has been identified and evaluated over the last few years. It has been recently shown to participate in glucose dependent sodium intake although it does not transport glucose[16].

There has been increasing work done in recent times in the area of SGLT2 expression and function, which may potentially impact on the importance and efficacy of SGLT2 inhibitors in diabetic patients. There is a tubular transport maximum for glucose limiting the reabsorption capacity which in diabetes is increased, suggesting there is increased ability to reabsorb filtered glucose[17]. This is clearly counterproductive for the diabetic patient, both serving to increase plasma glucose levels and exposing the proximal tubular cells to an increased concentration of glucose. It has become clear that this increase in glucose reabsorption relates at least in part to an alteration in SGLT2 expression on exposure to high glucose, and possibly also to a change in SGLT1 expression. Proximal tubular cells obtained from urine of diabetic patients have shown increased expression of SGLT2[14] and studies in obese Zucker rats have shown that diabetes causes increased RNA expression of SGLT2 and SGLT1 in the kidney[18]. A variety of factors have been linked to the alteration in expression of SGLT1 and 2, including HNF1α and SGK1 [19] but more recently our group has confirmed upregulation of expression of SGLT2 when proximal tubular cells are exposed to transforming growth factor β (TGFβ), a pro-fibrotic cytokine [20]. Interleukin-6 and tumour necrosis factor-α have been shown to increase SGLT2 expression in cultured kidney cell lines after exposure for 96-120 hours [21] and a
pathway for high glucose induced increased SGLT2 expression has been demonstrated via Protein Kinase A (PKA) and Protein Kinase C (PKC) dependent pathways [22, 23]. There is also evidence of interaction between the sodium glucose cotransporters and the renin-angiotensin-aldosterone system (RAAS). It has been shown in animal studies that Losartan, an angiotensin receptor blocker (ARB) reduces SGLT2 expression in diabetic rats on normal and high salt diets [24]. This intriguing connection suggests another possible mechanism for the beneficial role of ARBs in diabetic nephropathy.

Finally, evidence also exists in regard to the effect of glucose on the expression and location of the facilitative glucose transporters, which usually reside in the basolateral membrane of the proximal tubular cell. It has been shown in diabetic rats that GLUT2 expression increases in diabetes and translocates to the luminal surface of the proximal tubular cell playing a role in increased glucose reabsorption [25].

Clearly, while SGLT2 is the predominant glucose transporter in the proximal tubule, its expression and that of the other glucose transporters can be altered in the diabetic milieu having implications for the efficacy of selective SGLT2 inhibitors, perhaps evidenced by their lack of complete glucose blockade despite increasing doses. To that end, there is renewed interest in dual SGLT1/SGLT2 blockade with one drug, LX4211 currently in clinical trials [26].
2.3 SGLT2 inhibitors and the pathogenesis of diabetic nephropathy

Diabetic nephropathy results from high glucose mediated inflammation and altered sodium handling that eventually results in fibrosis. There is both glomerular and tubulointerstitial damage although the decline in renal function parallels more closely the degree of tubulointerstitial damage [27]. High glucose is directly responsible for the changes seen in diabetic nephropathy and improved glycemic control has been demonstrated to slow the progression of the disease [28]. However, there are two processes that contribute to the pathology of diabetic nephropathy that may be expected to be altered by SGLT2 inhibitors, independent of their effect on glucose lowering (Figure 2.1).

The proximal tubular cell secretes inflammatory molecules and growth factors in response to high glucose. This results in activation of an inflammatory cascade and recruitment of macrophages with propagation of hypertrophy and interstitial fibrosis [29]. The most important mediator of this pathogenesis is TGFβ which promotes fibrosis and epithelial to mesenchymal transdifferentiation [30]. There is a host of other inflammatory mediators and growth factors involved in this complex process [31]. It is possible that a reduction in glucose transit through the proximal tubular cells (PTC) may reduce PTC induced inflammation and fibrosis in diabetic nephropathy. Indeed, our group has shown that SGLT2 inhibition in immortalized proximal tubular cell lines (HK2 cells) reduced high glucose induced Toll like Receptor 2 and 4 as well as nuclear factor kappa B (NFκB) and activator protein 1 (AP1) expression, which are important inflammatory and fibrotic mediators in diabetic nephropathy [20].
Another important aspect of diabetic nephropathy is hyperfiltration associated renal injury. Glomerular hyperfiltration associated with enhanced sodium and glucose reabsorption in the proximal tubule occurs quite early in the disease process [32] and plays an important role in diabetic nephropathy [33]. Increased proximal tubular sodium reabsorption results in decreased distal delivery of sodium to the macula densa, which regulates tubuloglomerular feedback. This also activates the renin angiotensin system resulting in elevated intraglomerular pressure and increased glomerular filtration rate (GFR) [34]. By inhibiting sodium reabsorption in the proximal tubule and by thereby increasing sodium delivery to the juxtaglomerular apparatus, it might be expected that the glomerular hyperfiltration would be reversed. Indeed, phlorizin, a non selective SGLT inhibitor, has been shown to abrogate the development of hyperfiltration in whole animal studies, and in single nephron studies, animals treated with phlorizin have a reduction in sodium reabsorption and normalisation of glomerular filtration rate, disproportionate to the improvement in plasma glucose [32]. This finding has been recently confirmed using a rat model and a selective SGLT2 inhibitor, and extended to show that the effect is sustained with chronic SGLT2 blockade [3].

2.4 Genetic defects in SGLT2 expression

Familial renal glycosuria (FRG) has been known to exist for a long time and there are several genetic mutations ranging from missense and nonsense mutations to small deletions and splicing mutations that are known to result in loss of SGLT2 function. Their inheritance has variously been described as autosomal recessive [35] or co-dominant inheritance with variable penetrance [36]. To date, 49 different SLC5A2
mutations have been reported in association with FRG but a recent study detailing FRG in 23 unrelated children that were consistent with a codominant inheritance pattern with incomplete penetrance has further extended the allelic heterogeneity [37]. The degree of glycosuria depends on the pattern of gene inheritance, with heterozygotes having far less glycosuria than individuals with homozygous inheritance, and ranges from a few grams to >120 grams per day [38, 39]. This condition seems to be well tolerated by the individuals affected, apart from activation of renin angiotensin system due to volume depletion in some individuals and occasional aminoaciduria. The literature is consistent with FRG being a largely benign condition with no serious adverse consequences.

However, the recent development of the Sweet pee mouse model may be cause for some concern. This model, which carries a nonsense mutation in the Slc5a2 gene, has a loss of function of the SGLT2 protein that mimics patients with similar SLC5A2 mutations. As expected, when diabetes mellitus is induced, these mice demonstrate increases in glycated haemoglobin that are less than their wild type counterparts, but a little unexpectedly, Sweet pee mice suffer from growth retardation, increased infections and mortality [40]. While this might appear to be a cautionary message in regard to the use of SGLT2 inhibitors, it should be remembered that diabetes was induced with streptozotocin, which mimics a type 1 diabetic phenotype, not the type 2 diabetes for which SGLT2 inhibitors are intended. However the sweet pee model has no known relevance for human disease and no known similarity to SGLT2 effect.

Finally, a recent study has explored the impact of common SGLT2 variants on glucose homeostasis in non-diabetic individuals finding alterations in glucose and
insulin concentrations dependent on SGLT2 activity [41]. This concept of genetic variability causing alterations in SGLT2 function within patients receiving SGLT2 inhibitors opens the door for further pharmacogenomic studies to clarify the role of SGLT2 inhibitors in treating diabetic patients.

2.5 SGLT2 inhibitors and renal endpoints

There are at least 12 SGLT2 inhibitors in various stages of clinical development. Some of these are in Phase III trials and two drugs are awaiting approval from licensing authorities. The most advanced among the SGLT2 inhibitors are Dapagliflozin and Canagliflozin. The other prominent drugs being developed under this category include empagliflozin, sergliflozin, ipragliflozin, tofogliflozin and luseogliflozin. These drugs are attractive as antidiabetic agents because of their insulin independent action and a reduced incidence of hypoglycaemia.

The SGLT2 inhibitors have been shown to reduce fasting plasma glucose and HbA1c levels in treatment naïve diabetic patients as monotherapy [12] and also as add on therapy to patients already on insulin [11, 42] and metformin [43, 44]. The degree of drop in HbA1Cc varies from 0.58 to 1% in these trials. Tighter control of diabetes has been shown to reduce the incidence of diabetic nephropathy on long term follow up of the intensive glucose control cohort in UK Prospective Diabetes Studies (UKPDS). Intensive blood glucose control has also been shown to reduce the incidence of diabetic nephropathy in a cohort of patients with type 2 diabetes over a 5-year period in the Action in diabetes and vascular disease: Preterax and Diamicron controlled evaluation trial [45]. Hence SGLT2 inhibitors should have a direct renal benefit due
to their glycaemic effects [28]. Ideally, hard end points of renoprotection should be demonstrated by longterm preservation of glomerular filtration rate (GFR) and prevention of albuminuria, but to date those data are not available. A non selective SGLT inhibitor, T-1095 has been shown to reduce HbA1C and reduce the degree of microalbuminuria in yellow KK mice, an obese type 2 model with insulin resistance [46]. Streptozotocin induced diabetic rats treated with T-1095 showed no increase in urinary albumin levels and kidney weight, when compared with diabetic control rats [47]. This drug has also been shown to preserve glomerular structure and reduce mesangial expansion in diabetic animals [48].

In clinical trials, dapagliflozin has shown transient decline in the GFR on initiation of treatment, which normalises in patients who have normal baseline renal function [49]. However in a 52 week placebo controlled trial in patients with moderate renal dysfunction (GFR >30 and <60), this initial drop remains stable without correction during the duration of treatment although there was improvement in albuminuria [50]. In a 26 week, Phase III placebo controlled trial in patients with moderate renal dysfunction, canagliflozin at varying doses showed mild worsening of creatinine levels (9% and 10% vs 4%) although the albumin creatinine ratio (ACR) and HbA1c showed improvement [51]. Finally, dapagliflozin has been reported to be less effective in lowering HbA1C in patients with a reduction in renal function as might be expected given that the effect of the drug requires ample GFR [50].

Further long term studies with hard renal endpoints are required to establish the renal benefits of SGLT2 inhibition.
2.5.1 Non glycaemic benefits

Whilst improved glycaemic control is likely to translate into improvement in renal outcomes, the SGLT2 inhibitors also have a number of nonglycaemic effects that may contribute to renal benefit, including effects on weight, blood pressure, lipids and uric acid.

Patients receiving SGLT2 inhibition undergo weight loss [11, 52], that initially may represent loss of fluid due to the drugs’ diuretic effect but in the long term is caused by a loss of subcutaneous fat secondary to urinary loss of calories [43]. Body weight loss appears to plateau after several months of treatment, perhaps due to a compensatory increase in calorie intake [12, 43, 53].

As outlined above, SGLT2 inhibition is likely to cause natriuresis and diuresis that would likely be associated with a reduction in blood pressure. SGLT2 inhibition has been shown to prevent blood pressure rises in diabetic rats maintained on a high salt diet [54] and there has been a reduction in seated systolic BP of 4.4 mm Hg and diastolic BP of 2.1 mm Hg among pooled data from dapagliflozin’s clinical trials, without any significant increase in postural hypotension [42, 43].

Canagliflozin has been noted to raise both HDL and LDL in one of the trials [44] and dapagliflozin has been noted to raise HDL [55]. The dual SGLT1/SGLT2 blockers, LX4211 has been shown to reduce triglyceride levels in a 28 day study in diabetic patients and may be related to an increase in glucose like peptide-1 (GLP-1) release
Whilst these results are interesting, they may reflect the associated weight loss these agents induce.

Finally, dapagliflozin has been shown to reduce serum uric acid levels with no significant change in electrolytes [56]. This may occur due to an increase in uric acid excretion via one of two mechanisms: increased flux of sodium through sodium-dependent phosphate transporters that simultaneously serve as urate transporters into the urine; or increased reabsorption of glucose via GLUT9 that exchanges glucose for uric acid [57]. Regardless, with elevated levels of uric acid being linked to progression of chronic kidney disease [58], reduction of levels may serve to improve renal outcomes in diabetic patients.

2.5.2 Renal risks

Most of the clinical trials show increased incidence of genital fungal infections [43], although urinary infections were only minimally different [42, 43] and were largely managed with conventional medications and did not require cessation of SGLT2 inhibitors. Interestingly, in studies where dapagliflozin was added to metformin, there was no difference in incidence of urinary tract infections between the two arms [43], while in those patients where the drug was supplementary to insulin, there was an increase in infection rate [42]. This difference may reflect differences in stage of the disease where insulin is likely to be prescribed and at which patients are more vulnerable to infection.
Given that osmotic diuresis accompanies the glycosuria and natriuresis, there have always been concerns that treatment with SGLT2 inhibition may result in symptomatic volume depletion. Indeed, an increase in urine volume is noted with both with acute and chronic SGLT2 inhibition that at most represents an increase of 400 ml/day [49]. Whilst this tends to translate into mild haematocrit increases, clinical signs of volume depletion are unusual and have generally only been noted among patients on Dapagliflozin who were also being treated with loop diuretics [49].

The major concerns raised by the FDA regarding dapagliflozin, however, were the higher incidence of bladder and breast cancer among patients receiving this medication and one probable case of drug induced hepatitis [49]. The FDA has recommended further trials to evaluate these specific concerns before approval. However dapagliflozin has received a favourable decision from the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency and is awaiting approval. A pharmacovigilance plan for this drug will be implemented as part of the marketing authorisation. Canagliflozin has submitted an application for approval to the FDA and also to the European Medicines Agency.

2.6 Conclusion

The role of SGLT2 in glucose and sodium transport has increased in the last two years. There is increasing knowledge of this transporter and the mechanisms of pharmacological inhibition of both SGLT2 and SGLT1 [59]. A large amount of data from clinical trials is available in the public domain and the drugs will soon enter the pharmacological market. These trials have confirmed glycaemic benefits and minor
adverse effects, which were expected, based on animal studies and early clinical trials. However they have also brought to focus other concerns regarding malignancy and drug reactions. These concerns will only be allayed by longer clinical trials and post marketing monitoring.

Most of the trials have focused on the classic outcome measures of anti diabetic drugs, namely improved glycaemia, weight loss, cardiovascular benefit including blood pressure and lipid profile. Renal disease is one of the major microvascular complications of diabetes and to date, while short term studies have been reassuring in regard to renal safety with these drugs, there has been a lack of long term data clarifying renal benefit. Given the glycaemic benefit of these agents and the other non-glycaemic effects, such as weight loss, blood pressure control and reduced uric acid levels, in combination with the long term effects of reduced glucose reabsorption through the proximal tubular cell and alteration in sodium handling, it would be anticipated that long term trials will yield a benefit.
Figure 2.1: Potential role of SGLT2 inhibition in renoprotection

SGLT2 inhibitors prevent increased transport of glucose through the proximal tubular cell and hence theoretically will prevent hypertrophy, hyperplasia with inflammation and eventual fibrosis. Simultaneously, they reduce the proximal tubular fractional reabsorption of sodium with increased distal delivery of sodium and chloride to the macula densa resulting in activation of tubuloglomerular feedback (TGF) with consequent reduction in single nephron glomerular filtration rate (SNGFR) and prevention of hyperfiltration injury.
Chapter 3: Empagliflozin for the treatment of Type 2 diabetes.

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Abstract

Diabetes and its complications account for a significant healthcare burden. There is increasing prevalence of diabetes and newer drugs are being investigated to improve outcomes. Sodium glucose cotransporter inhibitors (SGLT2 inhibitors) are a newer class of medications, which prevent renal reabsorption of glucose and hence help in glycaemic control without significant risk of hypoglycaemia. Two drugs, namely dapagliflozin and canagliflozin have gained approval and empagliflozin is one of the advanced agents of this class. Early trials with empagliflozin have shown a stable pharmacokinetic profile and pharmacodynamic effects with significant SGLT2 selectivity. Clinical trials have shown improvement in glycaemic control and other benefits including weight loss and lowering of blood pressure. Ongoing trials and surveillance will provide answers about cardiovascular benefits, risk of osteoporosis and cancer.
3.1 Background

Diabetes mellitus is a chronic disease with multisystem involvement leading to microvascular and macrovascular complications. The current worldwide prevalence of diabetes is estimated to be 6.4 % and expected to climb to 7.7 % by 2030 [60]. Type II diabetes constitutes more than 90 % of overall prevalence. The treatment of diabetes has come a long way over the last few decades with a range of medications including insulin, insulin secretagogues and drugs that increase insulin sensitivity. These medications have increased the survival of patients with diabetes over the years in developed countries. However the global mortality due to diabetes has increased by 20 % since 1990 [61]. Moreover the prevalence of microvascular complications like diabetic kidney disease has not improved over the last decade despite an increasing use of anti diabetic medication [62]. With the looming prospect of increasing worldwide prevalence of diabetes, it is important to identify newer drugs that improve management of diabetes and reduce the morbidity and mortality associated with this. This review discusses a new class of agents designed for the treatment of diabetes, the sodium glucose cotransporter inhibitors, with particular reference to empagliflozin, one of its members.

3.2 Sodium glucose cotransporter inhibitors

Most of the glucose filtered by the glomeruli is reabsorbed by two glucose transporters located in the renal proximal tubule, namely sodium glucose cotransporter 2 (SGLT2) and sodium glucose cotransporter 1 (SGLT1). The combination of these two transporters reabsorbs the majority of a large load of filtered
glucose, in the order of 180g glucose/day, such that less than 0.5 g/day of glucose is lost in the urine [13]. SGLT2 is the primary glucose transporter [1], accounting for 90% of glucose reabsorption, and only becomes saturated when the glucose concentration increases above 35 mM [63]. SGLT1 is present in the gut and in the kidneys and is primarily involved in glucose reabsorption from the gut. It plays a minor role in glucose reabsorption in the kidney. Sodium glucose cotransporter inhibitors (SGLT2i) are newer agents in the anti-diabetic armamentarium that act by blocking this glucose reabsorption in the proximal tubule, thereby lowering plasma glucose concentrations by increasing glycosuria. Phlorizin was the first SGLT inhibitor, which was extracted from the bark of the apple tree in 1835. Its ability to induce glycosuria was noted nearly 50 years after its discovery. Phlorizin is a non-specific SGLT inhibitor and blocks both SGLT2 and SGLT1 and has poor oral absorption. Hence it is not suitable for preventing glucose reabsorption due to associated gastrointestinal side effects, namely diarrhea [64]. The current focus has been on developing phlorizin analogues, which selectively inhibit SGLT2 mediated glucose transport at the renal proximal tubule. However it is to be noted that dual blockade of SGLT1 and SGLT2 prevents renal and intestinal glucose absorption and hence dual blockade agents, which produce lesser gastrointestinal side effects are also under development [65]. The most advanced selective SGLT2 inhibitors are dapagliflozin, canagliflozin and empagliflozin. Canagliflozin and dapagliflozin has been approved by FDA and the European Drugs agency for management of type 2 diabetes. Both drugs have also received approval from the Therapeutic Goods Administration in Australia.
A recent meta analysis incorporating available clinical data on SGLT2i shows consistent glycaemic benefits from the use of SGLT2i with improvement in HbA1c by up to 0.66% [66]. These agents have been shown to improve HbA1c significantly as single agent and as add on agent to other therapy including metformin and insulin. This analysis also shows improvement in systolic BP by 3.77 mm Hg and weight loss of 1.77 kg compared to placebo. This lowering of blood pressure is likely to be contributed by osmotic diuresis associated with glycosuria. There are other factors including renin angiotensin aldosterone inhibition due to increased sodium delivery to the juxtaglomerular apparatus [67]. Similarly, weight loss is due to a combination of diuresis and fat reduction and has been noted to be sustained during the course of clinical trials [43]. The SGLT2i are most effective in patients with eGFR> 60 ml/minute/1.73m² with decreasing efficacy as the kidney function is reduced. This is due to the poor filtration of the drug into the proximal tubule as the GFR worsens. It has been also noted that the incidence of urinary tract and genital infections were higher in diabetic patients treated with SGLT2i [66]. The exact cause for the increased incidence of urinary tract infections and genital infections have not been evaluated although it is presumed to be likely due to the increased glucose in the urine. The trials with dapagliflozin have also raised safety concerns with inexplicable, although marginally higher incidence of bladder and breast cancers in the dapagliflozin treated patients [68].

Hence these drugs have established themselves as good oral agents for diabetes with demonstrated benefits in reducing HbA1c in addition to other benefits including weight loss and improving blood pressure. The dapagliflozin trials have not shown an increase in cardiovascular events when compared to placebo [69]. However the
incidence of major adverse cardiovascular events (MACE-plus) defined as cardiovascular death, non-fatal myocardial infarction, non-fatal stroke and hospitalization for unstable angina was increased in the first 30 days after initiation of treatment with canagliflozin [70]. The incidence of MACE-plus episodes was similar to placebo after the first 30 days. The canagliflozin trials have shown a dose dependent increase in LDL cholesterol [70]. However, it is unclear if they will have an impact on the cardiovascular risk in diabetic patients in the long term. Bone safety is another area of concern regarding these agents. There was an increased incidence of fractures in patients treated with canagliflozin [70]. Treatment with dapagliflozin was noted to increase fracture incidence in patients with moderate renal dysfunction [71]. The pooled placebo controlled trials of canagliflozin have shown minimal increases in serum calcium of 0.6-1.1% and serum phosphorus of 5.1% with 300 mg canagliflozin. In patients with moderate renal impairment mean serum calcium and phosphorus increased by 1.3 and 7.6% with canagliflozin at a dose of 300 mg. Mean serum PTH declined by 16.1% compared to placebo and there were moderate increase in 25-OH-vitamin D and slight decrease in 1,25-OH-vitamin D although these changes were not statistically significant. However, bone turnover markers were significantly increased with canagliflozin compared to placebo. The total hip bone mineral density (BMD) showed a significant decline of 0.4 and 0.6% with 100 mg and 300 mg of canagliflozin respectively when compared to placebo. However, BMD changes were not significant at the lumbar spine and 1/3 radius. The incidence of fractures seems to be increased in patients on canagliflozin, especially low trauma and upper extremity fractures although they do not reach statistical significance. Results from ongoing longer duration studies are awaited before the effects of canagliflozin on bone health can be fully determined. The proposed mechanisms for increased bone
resorption include weight loss without an increase in physical activity although dapagliflozin showed no increase in fractures with a similar degree of weight loss in patients with normal renal function. There has been no increase in urinary calcium excretion with only small increases in urinary phosphate excretion in 12-week studies. Hence the etiology of increased bone turnover, low BMD and increased risk of fractures remains to be evaluated. One patient was noted to have probable drug induced liver injury (DILI) in an analysis of all the dapagliflozin trials presented to the FDA for approval although canagliflozin does not seem to have had any episode of DILI [69, 70]. These trials have also shown a small decrease in eGFR after initiation of SGLT2i although this seems to correct after discontinuation of the medication.

SGLT2i have been shown to reduce glomerular hyperfiltration very early after initiation and this was expected to improve the incidence of diabetic nephropathy. However it is now emerging that the renal benefits are commensurate with improved glycaemia rather than the ability of SGLT2i to reduce hyperfiltration injury[72]. It has been demonstrated that high glucose mediated toxicity to the β cells of pancreas is ameliorated by early initiation of SGLT2i resulting in preservation of these cells [53]. Hence the beneficial effects of SGLT2i in preventing diabetic nephropathy and improved diabetic control seem to stem from improved glycaemia rather than any additional physiological benefit. This could be true for all other complications of diabetes.
3.3 Pharmacokinetics, pharmacodynamics, potency and selectivity of empagliflozin

The pharmacokinetics of empagliflozin is similar following single dose and multiple doses at steady state. Peak levels are attained 1.5-3 hours after oral administration and half-life ranges from 10-19 hours. This pattern has been suggested to signify linear pharmacokinetics. Population pharmacokinetics were studied in 974 patients with Type 2 diabetes using pooled data from five randomized controlled trials with multiple oral doses of empagliflozin [73]. The steady state exposure was shown to be dependent on weight with no relation to sex or race demonstrated. Additionally, empagliflozin pharmacodynamics were studied in 48 healthy male Japanese patients [74]. This study demonstrated increased urinary glucose excretion between 5-7 hours after drug administration with the degree of glucose excretion being proportional to the dose. Most of the drug effect was attained by 24 hours, although at a higher dose of 100 mg the glycosuric effect was observed for up to 72 hours. It is to be noted that the maximum 24 hour urinary glucose excretion attained by empagliflozin is approximately 89 g in diabetic patients which is about half of the total glucose filtered into the proximal tubule [75]. This finding is similar to trials with other SGLT2i wherein filtered glucose is only partially prevented from being reabsorbed. There are multiple possible reasons that have been proposed for this including competitive antagonism with high glucose for drug binding to SGLT2, upregulation of other transporters which compensate for SGLT2 inhibition and high plasma protein binding which prevents drug delivery to the proximal tubule [76].
Empagliflozin has been shown in in vitro studies to inhibit the uptake of $[^{14}\text{C}]-\text{alpha methyl glucopyranoside (AMG)}$ via hSGLT2 in a dose dependent manner with an IC$_{50}$ of 3.1nM [77]. This was similar to other C-glucoside SGLT2 inhibitors namely dapagliflozin, canagliflozin, ipragliflozin and tofogliflozin. Empagliflozin however displayed the highest selectivity for SGLT2 over SGLT1 among all C-glucoside SGLT2 inhibitors. Empagliflozin displayed high affinity for SGLT2 with a K$_d$ of 57 ± 37 nM in the absence of glucose. However the affinity decreased to a mean K$_d$ of 194 ± 99 nM in the presence of glucose at a concentration of 20 mM. This displays the competitive nature of SGLT2 binding between empagliflozin and glucose. The half life of the empagliflozin and SGLT2 bond was shown to be 59 ± 5 minutes, which was unaffected by high glucose concentration. This extended binding could explain the duration of action of empagliflozin that extends beyond the point of falling serum concentration.

No significant interaction was noted when empagliflozin was coadministered with warfarin [78], verapamil, ramipril, digoxin [79] or ethinyl estradiol and levonorgestrol [80] suggesting that empagliflozin does not interfere with coadministration of other common medications.

Empagliflozin is eliminated by glucuronidation and nearly 19% is excreted unchanged in the urine [81]. Empagliflozin showed increased absorption and higher peak levels along with increased area under the curve in patients with hepatic impairment [82]. These changes in pharmacokinetic parameters were proportional to the degree of hepatic impairment. However the half-life was unaffected and the adverse effect profile was similar among all groups. In this study, a dose of 50 mg
was well tolerated by all groups suggesting that dose modification may not be necessary in patients with hepatic impairment. It has been proposed by the study authors that the increased peak levels in hepatic impairment are due to reduced hepatic enzyme activity suggesting reduced first pass metabolism in hepatic impairment. Renal clearance of unchanged drug is an important pathway for drug elimination. Moreover, the pharmacodynamics of empagliflozin depends on renal function, as the site of action is the renal proximal tubules. As expected, empagliflozin was shown to have slower absorption in patients with renal dysfunction [81], the exact mechanism for which has not been clearly elucidated. However, the clearance rate was reduced and the area under the curve was increased with increasing renal dysfunction. The fractional excretion of empagliflozin also declined with declining renal function from 16.1% in patients with normal renal function to 0.3% in patients with end stage kidney disease (ESKD). A change in pharmacodynamics was noted in parallel with progressive renal impairment. The urinary glucose excretion increased 97.64 g/24 hours from baseline in patients with normal renal function with empagliflozin, whereas there was an increase of just 0.78 g/24 hours from baseline in patients with ESRD.

In summary, empagliflozin has a peak onset of action at 5-7 hours after oral administration with a dose dependent glycosuric response. There is no significant drug interaction with the common medications that have been examined. It displays significant selectivity for SGLT2 in comparison to other SGLTs but its affinity is affected by the glucose concentration. Empagliflozin pharmacokinetics is affected by hepatic and renal impairment, although dose modification has not been recommended
based on available evidence [81]. However, progressive renal dysfunction impacts on the clinical efficacy of empagliflozin due to its mode of action.

3.4 Clinical efficacy of empagliflozin

There have been five published randomised clinical trials on empagliflozin (Table 1). Empagliflozin has been shown to be beneficial in improving glycaemic control as a single agent. In a Phase II trial, empagliflozin has shown reduction in HbA1c by 0.5-0.7% with increasing doses from 5-25 mg qd, compared to placebo in patients with Type 2 diabetes [83]. These results were similar to open label metformin at a dose of 1000-2000 mg daily. Similarly, in a recent Phase III trial in patients with type 2 diabetes, compared with placebo, adjusted mean differences from baseline HbA1c at week 24 were 0.74% at a daily dose of 10 mg and 0.85% at a dose of 25 mg similar to 0.73% seen with sitagliptin at a dose of 100 mg [84].

Empagliflozin has also been shown to have cumulative glycaemic benefits as add on therapy. Empagliflozin, as an add on therapy to metformin, in patients with mild hyperglycaemia, has been shown to improve HbA1c by 0.24-0.71% compared with placebo after 12 weeks of treatment[85]. In the EMPA-REG METSU trial, a Phase III randomised, double blinded, placebo controlled trial in patients with suboptimal control of type 2 diabetes on sulfonylureas and metformin, empagliflozin, as an add on agent, was able to reduce HbA1c significantly by up to 0.82% at a dose of 10 mg and 0.77% at a dose of 25 mg compared to 0.17% with placebo after 24 weeks of treatment, showing the additive benefits of this medication [86]. Similarly, empagliflozin was beneficial as an add on agent to insulin in a double blinded study.
with a significant reduction in HbA1c as early as 18 weeks after initiation of this medication, when compared to placebo. In addition it was also shown to reduce insulin requirements by 6.66 units, compared to placebo after 78 weeks of treatment [87]. Empagliflozin, as an add on agent to pioglitazone with or without metformin, has also been shown in a phase III trial to improve HbA1c significantly after 24 weeks of treatment [88].

In addition to improved glycaemic control, empagliflozin has also been shown to induce weight loss and improve blood pressure. Empagliflozin as an add on to insulin therapy was able to reduce body weight by up to 2.2 kg and improve systolic blood pressure by up to 4 mm Hg after 78 weeks of treatment at a dose of 10 mg daily [87]. A pooled analysis of four randomised, placebo controlled Phase III trials with empagliflozin as either a single agent or as add on therapy to sulphonylureas, metformin or pioglitazone has shown additional improvements in cardiovascular risk factors [89]. In addition to an improvement in HbA1c by 0.68%, weight loss of 2.25 kg and an improvement in both systolic and diastolic BP, empagliflozin also increased HDL cholesterol by 0.07 mmol/L, LDL cholesterol by 0.1 mmol/L, reduced triglycerides by 0.11 mmol/L and serum uric acid by 29 mmol/L after 24 weeks of treatment.

Empagliflozin and other SGLT2i act in an insulin independent manner and hence would be expected to provide glycaemic control irrespective of the stage of type 2 diabetes. This was indeed shown in a recent study where oral glucose tolerance of empagliflozin remained high even at 27 weeks of age in Zucker diabetic fatty rats. This was comparable to metformin and significantly higher than that of glipizide [90].
SGLT2i induce glycosuria and this can have physiological consequences. In a small study of 66 patients with type 2 diabetes, it was shown that empagliflozin increased endogenous glucose production in the fasting state to match glucose loss in the urine. However the systemic exposure to high glucose was reduced after a meal in spite of an increase in endogenous glucose production. Moreover it was noted that glucose oxidation was reduced and lipid oxidation increased suggesting a shift in substrate utilization from glucose to lipids [91]. This could be one of the reasons for the long-term weight loss associated with SGLT2i usage.

Empagliflozin and other SGLT2i act on renal proximal tubules and have reduced efficacy with worsening renal function. In a Phase III study in patients with mild (eGFR 60-90) and moderate (eGFR 30-60) renal dysfunction, it was noted that after 24 weeks of treatment, HbA1c was reduced by 0.68 % and 0.42% in patients with mild and moderate renal dysfunction respectively. This shows the even though there is a reduction in clinical efficacy of empagliflozin with renal dysfunction, it is still able to show benefits compared to untreated patients [92].

3.5 Safety profile and adverse effects

3.5.1 Urinary complications

The adverse effect profile of empagliflozin is similar to other drugs in this category. The incidence of genital infections has been shown to be increased when compared to placebo in a pooled analysis of four major phase III clinical trials. However the incidence of urinary tract infections has not been shown to be substantially increased
Empagliflozin has also been shown to induce frequent daytime urination in some studies [83].

### 3.5.2 Metabolic adverse effects

Empagliflozin has not been found to increase the incidence of hypoglycemia in a phase III trial comparing empagliflozin as add on therapy to pioglitazone or pioglitazone with metformin [94]. Similar to other SGLT2i, empagliflozin has been shown to cause a small drop in eGFR in a randomized open label extension study with empagliflozin or metformin as monotherapy and empagliflozin or sitagliptin as add on therapy to metformin, although this has been reported to be small and comparable among all groups [95]. Volume depletion is again a class effect and has been shown to develop in 3% of patients on a dose of 10 mg or 25 mg of empagliflozin during a 52 week Phase II trial in Japanese patients with type 2 diabetes [96].

### 3.5.3 Cardiovascular adverse effects

Cardiovascular safety is an important consideration for all new drugs especially with adverse outcomes associated with anti diabetic medication previously. No significant adverse cardiovascular outcomes have been noted in all the published trials. However these were not dedicated to study cardiovascular outcomes or were powered to do so. Currently a Phase III multicenter, randomised double blind trial (NCT01131676) is underway to look at the cardiovascular outcomes of empagliflozin 10 and 25 mg versus usual care in patients with type 2 diabetes and high cardiovascular risk [97]. A number of drugs can cause QT interval prolongation and hence have arrhythmogenic
potential. Empagliflozin has not been shown to increase the QT interval in a randomised, placebo controlled, single dose, double blinded, five period cross over study in thirty healthy volunteers [98]. It has also been noted in a randomised open label study that empagliflozin has not shown any changes in magnesium or potassium levels [99]. However the long term cardiovascular safety outcomes will depend on the results from the ongoing trials and post marketing monitoring after empagliflozin gains approval.

3.5.4 Malignancy and hepatic complications

Clinical trials with dapagliflozin have raised concerns regarding an unexplained increase in the incidence of prostate and breast malignancy and drug induced liver injury. No clinical data is currently available on the incidence of these complications with empagliflozin. However the large-scale clinical trials currently being conducted will tell us about these specific outcomes.

3.6 Conclusion

Empagliflozin has been shown to be effective in improving glycaemic control in type 2 diabetes with significant improvement in fasting glucose and HbA1c. This has been attained either as a single agent or as an add on treatment to other agents including metformin, sulphonylureas and insulin. A single daily dose has been found to be effective with the drug efficacy increasing with escalating dose strengths with the most commonly tested doses being 10 mg and 25 mg.
In short term studies it appears safe with low risk of hypoglycaemia although genital infections are increased similar to other agents of this class. Long-term outcomes including cardiovascular risk or benefit and other possible adverse effects await evaluation with ongoing studies likely to shed more light.

### 3.7 Expert opinion

Empagliflozin holds potential as an anti diabetic agent and we expect it to be approved and successfully added to the growing number of SGLT2i. It has a long duration of action and hence will improve patient compliance, as only a single daily dose is required. The short-term studies have shown improvement in cardiovascular risk factors, namely lowering of systolic BP and weight loss. However, it remains to be seen if this converts into improvement in hard clinical endpoints namely reduction in cardiovascular mortality and morbidity. Another area of ongoing research is this agent’s potential to reduce microvascular complications, especially nephropathy in view of its unique mechanism of action. Empagliflozin has been associated with a reduction in GFR, which may reflect a reduction in glomerular hyperfiltration. However it remains to be seen if it has any independent renoprotective benefits or indeed harm. Its use is limited to patients with reasonably preserved renal function and has been shown to retain clinical efficacy till a GFR of 30 ml/minute, becoming ineffective with further reduction in the glomerular filtration rate.

In our opinion, SGLT2i as a class are a welcome addition to the anti diabetic armamentarium although their long term risks and benefits have not yet been fully elucidated. Cardiovascular outcomes may not necessarily improve with significant
lowering of HbA1c and very tight blood sugar control may indeed be associated with increased mortality [100] or may not have any demonstrable cardiovascular benefit [101]. The cardiovascular outcomes of anti diabetic drugs became important after a meta analysis on rosiglitazone showed significant adverse cardiovascular outcomes [102]. However the RECORD study has shown that although risk of heart failure was increased, rosiglitazone did not increase the risk of major adverse cardiovascular events [103] with the FDA lifting restrictions on his medication. It has been shown recently that saxagliptin increases risk of hospitalisation for heart failure without an increased risk of mortality [104]. This was not evident in pre approval trials. Hence it is important to have dedicated trials designed and powered to look at the cardiovascular outcomes rather than use results from pooled data from a number of trials, as they may not provide answers due to study heterogeneity.

Empagliflozin is the latest in this class to attempt FDA approval and it is likely that this drug will have a similar role to play as its counterparts. Like dapagliflozin and canagliflozin, it has been shown to be an effective single or add-on agent for the treatment of type 2 diabetes with a low risk of hypoglycaemia and is associated with a positive effect on cardiovascular risk factors. It too will need longer studies to better understand its place in the growing number of options available for the management of type 2 diabetes but it seems likely that it will contribute to better management of the diabetic patient in the future. However it has received a significant setback after the FDA recently rejected its new drug application citing deficiencies in its manufacturing base at Ingelheim. Hence Boehringer Ingelheim and Eli Lilly will have to invest significantly to correct these deficiencies and reapply for FDA approval. This information became available in a press release by Boehringer Ingelheim and Eli
Lilly on the 5th of March 2014. Hence its further development and approval depends on the rectification of the deficiencies that were identified and may delay the reapplication.

3.8 Five-year review

Empagliflozin will clearly be a significant anti-diabetic drug. In the next five years it has the potential to be used as a single agent or as an additional agent in the management of diabetes. However as mentioned earlier, the future of the drug depends on the outcome of corrective measures that are taken by the manufacturer in response to the complete response letter from the FDA. If approved, we expect its use to be most significant in patients with early type 2 diabetes, especially before the onset of any degree of renal dysfunction. It may have a role in obese patients with type 2 diabetes due to the ability of SGLT2i to induce weight loss. The cardiovascular effects both related to and independent of glycaemic benefits will be evident from current trials. It will especially be interesting to look at the outcomes of adding empagliflozin and other SGLT2i to the usual list of cardiovascular medications including angiotensin converting enzyme inhibitors, angiotensin receptor blockers and diuretics as it is expected to increase the risk of hypotension. The risk of mycotic genital infections will also determine physician choice and will require strict compliance with genital hygiene by patients. We expect answers to all these questions in the next five years.
3.9 Key issues

- SGLT2i are newer anti diabetic agents
- Phase III trials have shown glycaemic benefits, improvement in blood pressure and weight loss
- Short term trials reveal no significant safety concerns although genital fungal infections remains a concern
- Cardiovascular outcomes, risk of cancer and effects on bone morphology remain to be answered
- US FDA has rejected empagliflozin new drug application based on deficiencies at its main manufacturing facility
- The success of empagliflozin depends on correction of deficiencies identified by the FDA and successful reapplication
### Published randomised trials of Empagliflozin

<table>
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<tr>
<th>Study</th>
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<th>Number of patients</th>
<th>Duration and Study population</th>
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<td>2013</td>
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<td>12 week study of patients with Type 2 diabetes who were on Metformin with inadequate diabetic control</td>
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Kovacs et al ★★ 2013 498
24 weeks
Patients with Type 2 diabetes with
7%<HbA1c<10% on pioglitazone ± metformin

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FPG (mg/dl)
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<td>Haring et al. ψ</td>
<td>2013</td>
<td>666</td>
<td>24 week study of type 2 diabetic patients on metformin and sulfonylureas with 7%&lt;HbA1c&lt;10%</td>
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<td>-1.05</td>
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<td>Placebo</td>
<td>-0.15</td>
<td>0.03</td>
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<sup>1</sup>Fasting plasma glucose, <sup>2</sup>Mean daily glucose, <sup>3</sup>Systolic blood pressure, <sup>4</sup>Diastolic blood pressure

★ Significant results noted in HbA1c, FPG and body weight in all groups except with Empagliflozin 1 mg

★★ Clinically significant results in all parameters in all dose groups of empagliflozin
Clinically significant results in all parameters obtained at 12 weeks and were maintained after 78 weeks of extension, except improvement in blood pressure with empagliflozin 10 mg.

Clinically significant results with empagliflozin. Metformin not included in primary analysis and compared with placebo separately.

Significant results in all parameters except DBP.
Chapter 4: Inhibition of kidney proximal tubular glucose reabsorption does not prevent against diabetic nephropathy in type 1 diabetic eNOS knockout mice.


Abstract

Background and Objective
Sodium glucose cotransporter 2 (SGLT2) is the main luminal glucose transporter in the kidney. SGLT2 inhibition results in glycosuria and improved glycaemic control. Drugs inhibiting this transporter have recently been approved for clinical use and have been suggested to have potential renoprotective benefits by limiting glycotoxicity in the proximal tubule. We aimed to determine the renoprotective benefits of empagliflozin, an SGLT2i, independent of its glucose lowering effect.

Research Design and Methods
We induced diabetes using a low dose streptozotocin protocol in 7-8 week old endothelial nitric oxide (eNOS) synthase knockout mice. We measured fasting blood glucose on a monthly basis, terminal urinary albumin/creatinine ratio. Renal histology was assessed for inflammatory and fibrotic changes. Renal cortical mRNA transcription of inflammatory and profibrotic cytokines, glucose transporters and protein expression of SGLT2 and GLUT1 were determined. Outcomes were compared to diabetic animals receiving the angiotensin receptor blocker telmisartan (current best practice).

Results
Diabetic mice had high matched blood glucose levels. Empagliflozin did not attenuate diabetes-induced albuminuria, unlike telmisartan. Empagliflozin did not improve glomerulosclerosis, tubular atrophy, tubulointerstitial inflammation or fibrosis, while telmisartan attenuated these. Empagliflozin did not modify tubular
toll-like receptor-2 expression in diabetic mice. Empagliflozin did not reduce the upregulation of macrophage chemoattractant protein-1 (MCP-1), transforming growth factor β1 and fibronectin mRNA observed in the diabetic animals, while telmisartan decreased transcription of MCP-1 and fibronectin. Empagliflozin increased GLUT1 mRNA expression and telmisartan increased SGLT2 mRNA expression in comparison to untreated diabetic mice. However no significant difference was found in protein expression of GLUT1 or SGLT2 among the different groups.

**Conclusion**

Hence SGLT2 inhibition does not have renoprotective benefits independent of glucose lowering.
4.1 Introduction

Diabetic nephropathy is the commonest cause of chronic kidney disease worldwide [105]. Current best practice in the management of diabetic nephropathy involves tight glycaemic and blood pressure control, which includes specific blockade of the renin, angiotensin aldosterone systems [106, 107]. Although treatment options for patients have expanded in recent years, this has not translated to a reduction in the incidence of diabetic nephropathy [62]. Hence there is a need for novel agents that confer renoprotection.

Sodium glucose cotransporter 2 inhibitors (SGLT2i) are novel diabetic agents that block glucose entry into the kidney proximal tubular cell (PTC), resulting in glycosuria and lowering of blood glucose levels and have the added advantage of not inducing weight gain or hypoglycaemia [108, 109].

SGLTs are located on the luminal aspect of the proximal tubule (PT) and able to transport sodium and glucose from the ultrafiltrate into the cell due to a sodium concentration gradient, generated by the basolateral Na, K-ATPase pump [110]. Sodium glucose cotransporter 2 (SGLT2) is the major luminal glucose transporter located in the S1 and S2 segments of the PT, whilst sodium glucose cotransporter 1 (SGLT1) in the S3 segment contributes to less than 10% of total luminal glucose transport [19]. On the basolateral side of the cell, glucose is then passively transported via facilitative glucose transporters (GLUTs) into the vasculature. In the early segments of the kidney PT, SGLT2 on the apical membrane is coupled with
GLUT2 on the basolateral side and together they reabsorb up to 90% of filtered glucose under normoglycaemic conditions [19].

Hyperglycaemia induces activation of various pathways, which stimulates the production of proinflammatory and profibrotic cytokines relevant in diabetic nephropathy including TGFβ. The effects of high glucose are predominantly mediated through the hypertrophic and profibrotic cytokine, TGFβ which is overexpressed in diabetic nephropathy [111]. There is clear evidence of the damaging effects of TGFβ on PTC growth and function. [112-114] We and others have also shown evidence for TGFβ induced activation of the innate immunity pathway in diabetic nephropathy, in particular Toll like receptor 2 (TLR2) and its endogenous ligand High Mobility Group Box 1 (HMGB1) [115, 116]. We have previously defined the effects of high glucose in mediating inflammatory and profibrotic effects in the PTC [117, 118] and the specific effects of increased PTC sodium transport in early diabetes. [119, 120] Hence it is well established that high intracellular glucose alters intracellular metabolism and promotes inflammatory and profibrotic cytokines resulting in the development of diabetic nephropathy [114, 117, 121].

We have previously shown using human kidney PTC in vitro that empagliflozin, an SGLT2i (provided by Boehringer Ingelheim, Germany), was able to reduce high glucose induced tubular expression of inflammatory and fibrotic markers. In the short term, this occurred without a compensatory increase in SGLT1 or GLUT2 expression [122].
This provided proof of concept to extend these studies to a validated small animal model of diabetic nephropathy. An important aspect of our experimental design was to match glucose levels in all diabetic groups, so that any observed renal outcomes could be interpreted independent of the glucose lowering effect of empagliflozin, which has confounded the interpretation of previous studies to date. This was achieved by using a 5 day low dose protocol of intraperitoneal streptozotocin to induce diabetes [123] and long acting insulin in the diabetic mice to match glucose levels among all the experimental limbs.

4.2 Materials and methods

4.2.1 Animal model

Male eNOS knockout mice on a C57BL/6 background were purchased from Jackson laboratory, USA. Mice were housed singly in filter top cages in a pathogen free facility and had free access to standard chow and drinking water. Diabetes was induced by a low-dose streptozotocin (STZ) protocol. Mice received intraperitoneal injections of STZ (55 mg/kg daily for 5 days) at 7 - 8 weeks of age. Control mice received citrate buffer injections (pH 4.5). Blood glucose was tested using a glucometer (Accuchek Nano, Roche) two weeks after STZ through tail vein blood collection. Diabetes was defined by blood glucose greater than 16 mmol/L after a six-hour daytime fast. Mice with levels below 16 mmol/L were excluded from the study. Fasting blood glucose levels were measured monthly. Long acting insulin (Insulin Glargine, Sanofi Aventis, Australia) was initiated as required from 10 weeks of age and was administered thrice weekly if the blood sugar exceeded 28
mmol/L or if they had lost weight greater than 25% from baseline. The study was approved by the Royal North Shore Hospital Ethics Committee (protocol number 1101-003A). The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes was followed in this study. Animals were anaesthetised using short inhalational anaesthesia with 2% isoflurane for minor procedures. Animals were euthanised under 2% isoflurane anaesthesia using cardiac puncture terminally.

4.2.2 Experimental design

The SGLT2i empagliflozin (provided by Boehringer-Ingelheim Germany) was administered by daily oral gavage (Instech Lab, USA) using 1% hydroxyethylcellulose (Sigma Aldrich) as a vehicle. Current best practice is renin-angiotensin-aldosterone blockade. Hence telmisartan (Boehringer-Ingelheim, Germany) at a dose of 3mg/kg /day was administered in drinking water as a comparative limb. Empagliflozin and telmisartan were initiated at 13 weeks of age. Mice were killed at 32 weeks of age. The groups were as below.

1. Control (ctrl, n=12)
2. Control receiving empagliflozin 10mg/kg daily (ctrl+empa, n=8)
3. Diabetic (dm, n=12)
4. Diabetic receiving empagliflozin 10mg/kg daily (dm+empa, n=10)
5. Diabetic receiving telmisartan, 3 mg/kg in drinking water (dm+tel=7)
4.2.3 Measurement of physiological parameters

Body weight was assessed monthly. Blood pressure was measured using a noninvasive tail vein cuff method (CODA BP apparatus, Kent Scientific, USA) preterminally (Table 1).

4.2.4 Urine biochemistry

Urine was collected at three different time points (48 hour post initiation of treatment, 4-6 weeks after initiation of treatment using metabolic cages and terminally using bladder puncture). Urine creatinine was measured using a picric acid method (Creatinine Companion, Exocell Inc., USA). Urinary glucose was measured using Abbot Architect C16000 analyser. Urine albumin was measured using Elisa (Albuwel, Exocell Inc., USA).

4.2.5 Kidney tissue harvest

The unperfused left kidney was harvested and snap frozen after embedding in OCT compound. The right kidney was perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) and subsequently fixed in 10% neutral buffered formalin for 24-48 hours.
4.2.6 Histology

Formalin fixed paraffin embedded (FFPE) kidney sections were stained with Masson’s Trichrome, Sirius Red and Periodic Acid Schiff. Assessment of histological change was done in a blinded manner. The Glomerulosclerotic index (GSI) was calculated based on previously described methodology[124]. Atrophic tubules were defined by dilatation, epithelial shedding and thinning of epithelium. Tubular damage was scored by counting the number of atrophic tubules per 400 tubules at X 200 magnification.

Immunohistochemistry for fibronectin and TLR2 were done on 4 micron paraffin embedded sections using rabbit anti mouse fibronectin (F3468, Sigma Aldrich, USA) at a concentration of 1:2000 and rabbit anti mouse TLR2 (Imgenex, San Diego, California) at a concentration of 1:250. The chromogenic reaction was carried out with 3,3’-diaminobenzidine chromogen (Dako, Australia) solution for 10 minutes. Immunohistochemistry for F4/80 staining was done on 10 micron frozen section slides. They were incubated with rat anti mouse F4/80 (MCA497R, ABD Serotec, USA) at a concentration of 1:100 for one hour followed by HRP tagged goat anti Rat antibody at a concentration of 1:200 (ABD Serotec, USA).

F4/80 positive cells per high power field were counted and averaged for each slide. The degree of interstitial collagen content in Masson’s and Sirius red stains was assessed in a blinded manner using Image J by identifying the percentage of interstitial collagen positive region at X 200 magnification in 5 randomly selected regions. Glomerular fibronectin was quantified using Image J at X 400
magnification after selecting 10-15 glomeruli at random and quantifying the percentage of fibronectin positive area in each glomerulus. Appropriate negative and positive controls were used for all immunohistochemistry stains.

4.2.7 Real time PCR experiments

Total RNA was extracted from kidney tissue using Trizol. cDNA was synthesised using Superscript III (Life Technologies) first strand synthesis. The cDNA was subjected to standard curve measurement to ensure efficiency prior to real time PCR, which was done using SYBR green (Life Technologies, Australia) for MCP-1, fibronectin, TGFβ, collagen IV, GLUT1 and GLUT2 using actin as the endogenous control. PCR for SGLT2 and SGLT1 were done using Taqman PCR Universal Mastermix (Applied Biosystems). The RTPCR was performed on the AB7900 machine (Applied Biosystems, Australia). Gene expression was quantified relative to actin. The primers are listed in Table 2.

4.2.8 Western blot

Frozen tissue was homogenized with Quiagen Tissue Ruptur in 1.5 ml of cold 20mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210mM mannitol, 70mM sucrose and centrifuged at 1,500 x g for 5 min at 4°C. Samples were then analysed by SDS gel electrophoresis (Novex, Life technologies, Australia) and electroblotted to Hybond Nitrocellulose membranes (Amersham Pharmacia Biotech, Bucks, UK). Membranes were then probed with primary antibodies to GLUT1 (ab652, Abcam, Cambridge, UK), SGLT2 (sc98975, Santa Cruz, USA) and actin (Santa Cruz,
USA). Proteins were visualized using Luminata Western HRP Substrate (Millipore) in a LAS 4000 image reader (GE Healthcare Life Sciences). Analysis was performed using Image J software (NIH, USA).

4.2.9 Statistical analysis

Statistical analysis was done using Graph Prism. Data are expressed as mean ± standard error of mean. A P value < 0.05 was considered statistically significant. Significance was assessed using paired t-test for analysing the difference in insulin requirement of diabetic mice before and after initiation of empagliflozin. Blood glucose profile during the study was measured using repeated measures ANOVA. ANOVA with Bonferroni’s correction for multiple comparisons was used for all other statistical analysis.
4.3 Results

4.3.1 Diabetic mice had similar fasting glucose levels

The blood glucose levels were measured monthly, starting two weeks after induction of diabetes and average values were calculated over the duration of the experiment for all groups. The diabetic mice displayed significantly elevated blood glucose levels at 21.4 mmol/L after induction with streptozotocin (Table 4.1) maintained with long acting insulin and empagliflozin treatments, while the glucose level was matched in all the diabetic limbs throughout the experiment (Figure 4.1A) as planned.

4.3.2 Empagliflozin induced glycosuria in non diabetic mice

The control + empagliflozin group displayed nearly 200 fold increase in urinary glucose levels compared to the control mice (Table 4.1). The urinary glucose excretion among all diabetic mice was significantly higher than control mice (Table 4.1).

4.3.3 Empagliflozin reduced insulin requirement and accentuated poor weight gain in diabetic mice

Comparisons were made between the thrice weekly insulin dose of diabetic mice in the diabetic + empagliflozin group, before and after initiation of empagliflozin. The insulin requirements of the diabetic mice were significantly reduced after initiation
of empagliflozin (Figure 4.1B). All diabetic mice had poor weight gain which was pronounced in the diabetic + empagliflozin group (Table 4.1).

4.3.4 Telmisartan but not empagliflozin reduced terminal urinary albumin excretion in diabetic mice

The terminal urinary albumin to creatinine ratio was elevated in the diabetic mice. Treatment with empagliflozin did not improve albuminuria in diabetic mice. This was significantly reduced by treatment with telmisartan in diabetic mice. In the early stages of the illness, SGLT2 inhibition shows a non significant trend towards improving albuminuria in diabetic mice, which is not maintained as the disease progresses (Table 4.1).
Table 4.1 Physical and clinical parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=12)</th>
<th>Control +empagliflozin (n=8)</th>
<th>Diabetic (n=12)</th>
<th>Diabetic +empagliflozin (n=10)</th>
<th>Diabetic +telmisartan (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gain in weight (gram)</td>
<td>5.4 ± 0.5</td>
<td>3.9 ± 1.0</td>
<td>2.4 ± 0.7**</td>
<td>0.8 ± 0.6**</td>
<td>3.1 ± 0.6*</td>
</tr>
<tr>
<td>Left kidney/ body weight ratio</td>
<td>0.77 ± 0.04</td>
<td>0.80 ± 0.03</td>
<td>0.91 ± 0.06</td>
<td>0.82 ± 0.08</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>Average blood sugar (mmol/L)</td>
<td>11.0 ± 0.3</td>
<td>11.5 ± 0.4</td>
<td>21.1 ± 0.4**</td>
<td>22.1 ± 0.6**</td>
<td>22.9 ± 0.7**</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>120 ± 2</td>
<td>128 ± 4</td>
<td>117 ± 2</td>
<td>122 ± 6</td>
<td>118 ± 10</td>
</tr>
<tr>
<td>24 hour urine glucose excretion (µmol/day)</td>
<td>2.6 ± 0.3</td>
<td>456.5 ± 74.4</td>
<td>3038.0 ± 864.8*</td>
<td>3551.0 ± 35.9**</td>
<td>3462.0 ± 320.4**</td>
</tr>
<tr>
<td>Mid experimental albumin/creatinine ratio (µg/mg)</td>
<td>133 ± 25</td>
<td>197 ± 48</td>
<td>987 ± 336*</td>
<td>463 ± 136</td>
<td>180 ± 121</td>
</tr>
<tr>
<td>Terminal albumin/creatinine ratio (µg/mg)</td>
<td>224 ± 36</td>
<td>290 ± 56</td>
<td>1474 ± 388*</td>
<td>1697 ± 596*</td>
<td>80 ± 30#</td>
</tr>
</tbody>
</table>

Data are mean ± standard error of mean.

*=P<0.05 vs control group

**=P<0.001 vs control group

#=P<0.05 vs diabetic group.
Figure 4.1A

Blood glucose (mmol/L)

Months after induction of diabetes
Figure 4.1B

Before empagliflozin

After empagliflozin

Insulin dose (units/thrice a week)
Figure 4.1. Blood glucose levels among diabetic mice were matched throughout the study and showed similar glucose levels among the diabetic mice at 1, 2, 3, 4 and 5 months post diabetes induction and were significantly higher than control mice (A). Empagliflozin reduced insulin requirement in diabetic mice (B). The comparison was made between average insulin requirement per dose administered thrice weekly, before and after initiation of empagliflozin. Data are expressed as mean ± SEM with * = P<0.05 and ** = P<0.001 vs ctrl.
4.3.5 Telmisartan but not empagliflozin reduced the degree of glomerulosclerosis and glomerular fibronectin deposition in diabetic mice

The untreated diabetic mice (Figure 4.2C) developed significant glomerulosclerosis in comparison with control mice (Figure 4.2A). Treatment with empagliflozin showed no improvement in the glomerulosclerotic index in diabetic mice (Figure 4.2D), whereas diabetics treated with telmisartan (Figure 4.2E) had significantly lower glomerulosclerotic scores. The diabetic mice (Figure 4.2I) developed significant glomerular fibronectin deposition in comparison with control mice (Figure 4.2G). There was no improvement with concurrent empagliflozin therapy (Figure 4.2J), while telmisartan was associated with a significant reduction in glomerular fibronectin deposition in diabetic mice (Figure 4.2K).
Figure 4.2 (A-F)
Figure 4.2 (G-L)
Figure 4.2. Diabetic mice demonstrated increased glomerulosclerosis and glomerular fibronectin deposition, which was improved by telmisartan but not by empagliflozin. Representative photographs of PAS stained sections for A) ctrl, B) ctrl + empa, C) dm, D) dm + empa and E) dm + tel groups and quantification of glomerulosclerosis by glomerulosclerotic index (F). Representative photographs of immunohistochemistry for glomerular fibronectin in G) ctrl, H) ctrl + empa, I) dm, J) dm + empa and K) dm + tel groups and quantification of glomerular fibronectin by Image J (L) (Magnification=original X400). Data are expressed as mean ± SEM with **=P<0.001 vs ctrl, #=P<0.05 vs dm and ##=P<0.001 vs dm.
4.3.6 Empagliflozin did not reduce tubulointerstitial inflammation in diabetic mice

The diabetic mice (Figure 4.3C) developed significantly increased tubulointerstitial infiltration of activated macrophages, demonstrated by F4/80 staining, compared to controls (Figure 4.3A). Concurrent treatment with empagliflozin did not improve this in diabetic mice (Figure 4.3D). However, telmisartan therapy significantly reduced macrophage infiltration in diabetic (Figure 4.3E). The diabetic mice also displayed increased tubular expression of TLR2 (Figure 4.3I). Empagliflozin did not modify the upregulation of TLR2 while telmisartan treated diabetic mice showed TLR2 expression not significantly different to that in control mice (Figure 4.3J and 4.3K).
Figure 4.3 (A-F)

(A) (B) (C) (D) (E) (F)

Macrophages per HPF

ctrl | ctrl + empa | dm | dm + empa | dm + tel |

** | ** | #
Figure 4.3 (G-L)
Figure 4.3. Diabetic animals demonstrated increased tubulointerstitial inflammation, which was not ameliorated by empagliflozin. Representative photographs of immunohistochemistry for tubulointerstitial F4/80 stain for activated macrophages in A) ctrl, B) ctrl + empa, C) dm, D) dm + empa, E) dm + tel groups (Magnification =original X 400) and quantification of F4/80 positive cells in tubulointerstitium (F) which was done by calculating the average number of F4/80 positive cells per high power field (HPF) in each group. Representative photographs of immunohistochemistry for tubular TLR2 in G) ctrl, H) ctrl + empa, I) dm, J) dm + empa, K) dm + tel groups (Magnification =original X 200) and quantification of tubular TLR2 expression by Image J (L). (Data are expressed as mean ± SEM with *=P<0.05 vs ctrl, **=P<0.001 vs ctrl and #=P<0.05 vs dm).
4.3.7 Telmisartan but not empagliflozin reduced the degree of tubular atrophy and tubulointerstitial fibrosis in diabetic mice

The diabetic mice (Figure 4.4C) developed significant tubular atrophy in comparison with control mice (Figure 4.4A). Empagliflozin did not reduce tubular atrophy (Figure 4.4D), while temisartan treated diabetic mice showed tubular atrophy not significantly different to control mice (Figure 4.4E). The degree of interstitial fibrosis was assessed by Sirius Red sensitive collagen staining (Figures 4.4G-K) and Masson’s staining (Figures 4.4M-Q). The diabetic mice showed a non significant trend towards increased collagen deposition with the sirius red stain. However with the Masson’s stain, the diabetic mice had significantly increased collagen deposition (Figure 4.4O), which was not improved by empagliflozin (Figure 4.4P). Telmisartan treated diabetic mice showed tubulointerstitial fibrosis not significantly different to that of control mice (Figure 4.4Q).
Figure 4.4 (A-F)
Figure 4.4 (G-L)
Figure 4.4 (M-R)
Figure 4.4. Diabetic mice demonstrated tubular atrophy and tubulointerstitial fibrosis, which was not improved by empagliflozin and partially reduced by telmisartan. Representative photographs of PAS stained sections of tubulointerstitium in A) ctrl, B) ctrl + empa, C) dm, D) dm + empa, E) dm + tel groups and F) Quantification of tubular atrophy in all groups was done by counting the number of atrophic tubules per 400 tubule count (Data are expressed as mean ± SEM with *=P<0.05 vs ctrl). Representative photographs of tubulointerstitial picrosirius red stain in G) ctrl, H) ctrl + empa, I) dm, J) dm + empa, K) dm + tel groups and L) Quantification of tubulointerstitial Sirius red positive collagen content by Image J. Representative photographs of Masson’s stain in M) ctrl, N) ctrl + empa, O) dm, P) dm + empa, Q) dm + tel groups and R) Quantification of Masson’s positive collagen content by Image J (Magnification =original X 200). (Data are expressed as mean ± SEM with *=P<0.05 vs ctrl, **=P<0.001 vs ctrl).
4.3.8 Empagliflozin did not improve the renal cortical transcription of inflammatory or profibrotic cytokines in diabetic mice

To determine whether empagliflozin or telmisartan modulated the cortical transcription of MCP-1, collagen IV, fibronectin and TGFβ in diabetes, we performed real time PCR from RNA derived from renal cortical tissue. The transcription of MCP-1, fibronectin and TGFβ was increased in diabetic mice and was unchanged by empagliflozin (Figure 4.5). Diabetic mice treated with telmisartan displayed MCP-1 (Figure 4.5A) and fibronectin (Figure 4.5D) transcription, which was not significantly different to control mice although TGFβ transcription was noted to be increased in this group (Figure 4.5C). No change was noted in the transcription of collagen IV between control and diabetic mice (Figure 4.5B)
Figure 4.5. Diabetic mice showed increased renal cortical transcription of inflammatory and fibrotic cytokines, which was not improved by empagliflozin. Real time PCR results for renal cortical transcription of A) MCP-1, B) Collagen 4, C) TGFβ and D) Fibronectin relative to actin (Data are expressed as mean ± SEM with *=P<0.05 vs ctrl and **=P<0.001 vs ctrl).
4.3.9 Empagliflozin significantly increased GLUT1 transcription in diabetic mice although no difference was noted in GLUT1 protein expression

There was no difference in mRNA transcription of any glucose transporters between diabetic and non diabetic mice (Figure 4.6). Empagliflozin treated diabetic mice showed a significant increase in GLUT1 transcription compared to other diabetic mice (Figure 4.6A) and telmisartan treated diabetic mice showed increased SGLT2 transcription compared to diabetic mice (Figure 4.6D). However there was no difference in either SGLT2 or GLUT1 protein expression among the different groups (Figure 4.6E-H).
Figure 4.6

A: Cortical transcription of GLUT-1 mRNA
B: Cortical transcription of GLUT-2 mRNA
C: Cortical transcription of SGLT-1 mRNA
D: Cortical transcription of SGLT-2 mRNA
E: GLUT-1 Actin
F: Renal GLUT-1 protein expression relative to actin
G: SGLT-2 Actin
H: Renal SGLT-2 protein expression relative to actin
Figure 4.6. Empagliflozin increased renal GLUT1 transcription in comparison with untreated diabetic mice although renal protein expression of GLUT1 and SGLT2 were unchanged. Real time PCR results for A) GLUT1, B) GLUT2, C) SGLT1 and D) SGLT2 expressed relative to actin. Expression levels of renal GLUT1 protein (E and F) and SGLT2 (G and H) relative to actin (Data are expressed as mean ± SEM with #=P<0.05 vs dm)
### Table 4.2. PCR primer sequences

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>GCCTGCTGTCCACAGTTGC</td>
<td>CAGGTGAGTGGGGCGTTA</td>
<td>Sigma</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>TAAAAGGACCTCCAGGGACCAC</td>
<td>CCCACTGAGCCTGACAC</td>
<td>Sigma</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>CACGGAGGCGACCATTACT</td>
<td>CTCCGGCGCAATGACGTAGAT</td>
<td>Sigma</td>
</tr>
<tr>
<td>TGFβ</td>
<td>TCAGACATTTCGGGAAGCAGT</td>
<td>ACGCCAGGAATTTGTCAT</td>
<td>Sigma</td>
</tr>
<tr>
<td>Actin</td>
<td>AAGGCAAGCGTGAAAAGAT</td>
<td>GTGGAAGGAGCCAGAGCATC</td>
<td>Sigma</td>
</tr>
<tr>
<td>GLUT1</td>
<td>AACATGGAACCACCGCTACG</td>
<td>GTGGTAGGAGTTGCTGAGATGG</td>
<td>Sigma</td>
</tr>
<tr>
<td>GLUT2</td>
<td>ATCGCCCTCTGGTCCAGTAC</td>
<td>GAACAGTAAAGGCCCAGA</td>
<td>Sigma</td>
</tr>
<tr>
<td>SGLT1</td>
<td>Mm00451203_m1 (Applied Biosystems)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGLT2</td>
<td>Mm00453831_m1 (Applied Biosystems)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.4 Discussion

To our knowledge this is the first study to evaluate the renoprotective effects of SGLT2 inhibition independent of glucose lowering. We used a type 1 diabetic eNOS knockout mouse model, validated by the Animal Models of Diabetic Complications Consortium (AMDCC) [123], with matched glycaemia across experimental groups. Although we have previously demonstrated that empagliflozin reduced high glucose induced expression of inflammatory and fibrotic markers in in vitro studies of human kidney PTC, our results show that it did not confer renoprotection in this in vivo model. This was in contrast to the renoprotection afforded by the angiotensin receptor blocker (ARB) telmisartan.

The strength of our study is based on the determination of renal parameters in a setting of matched high blood glucose levels among diabetic groups. In our study, empagliflozin caused a 200 fold increase in glycosuria in control mice compared to control mice not receiving the drug, which confirms that empagliflozin was absorbed and achieved sufficient concentration in the lumen to actively inhibit PTC SGLT2. Furthermore the diabetic mice receiving empagliflozin had less insulin requirements and less weight gain reflecting the efficacy of empagliflozin in inducing glycosuria in our model. As the effectiveness of SGLT2 inhibition is dependent on the creatinine clearance with decreased efficiency as creatinine clearance decreases we would expect the efficacy to decrease as the severity of diabetic nephropathy increases [125].
In contrast to our findings, there are recent animal studies wherein SGLT2i have shown significant renal benefit [126, 127]. There are however important differences in these studies from our current study. In the study by Nagata et al, a type 2 diabetic model was used and diabetic mice treated with the SGLT2i tofogliflozin had lower plasma glucose levels, making interpretation of renal benefit independent of glucose lowering difficult. The second study by Kojima et al utilized older (> 1 year old) type 2 diabetic rats with established diabetic nephropathy and showed that although matched for glucose levels the diabetic mice receiving the SGLT2i luseogliflozin had better renal outcomes than the mice receiving insulin. The mice receiving insulin had 10 fold higher plasma insulin levels. Studies have shown that insulin itself can increase TGFβ-1 gene expression by mesangial cells [128] and can promote glomerular and interstitial fibrosis [129]. Furthermore, in a recent publication, Wright et al has described the probable role of insulin as an agonist in stimulating SGLT2 activity through activation of protein kinase A and C [130]. This would explain an increase in glucose reabsorption in type 2 diabetic mice with high insulin levels and provide an explanation for better outcomes with SGLT2 inhibition in this model. Current data available from the registration trials of SGLT2i is limited, but suggests a stable reduction in estimated GFR than reverts to baseline after cessation of the drug. This occurs in association with a reduction in albuminuria. However, renoprotective benefits cannot be concluded from available clinical data.

Our studies are consistent with two recent studies by Vallon et al. The first study used SGLT2 knockout mice showing that although there was an improvement in glucose control and glomerular hyperfiltration, there was no improvement in kidney
growth or injury [72]. An important difference between our own study and this study is that there was an improvement in blood glucose among mice treated with empagliflozin. Moreover it was noted that after 18 weeks of diabetes, no increase in TGFβ expression was observed in the renal cortex by their group. Conversely, we did demonstrate increased transcription of TGFβ in the renal cortex of diabetic animals although there was no statistically significant improvement with empagliflozin. Similar to Vallon’s study, we found a statistically non significant trend towards decreased SGLT2 protein expression in diabetic mice, which may have contributed to reduced renoprotection by empagliflozin. However this cannot explain the significant diabetic changes in empagliflozin treated mice in contrast to improvement in the telmisartan group. The second study by Vallon et al in Akita mice, a type 1 model of diabetes, demonstrated that empagliflozin was effective in reducing GFR independent of blood glucose but the reduction in albuminuria, kidney growth and inflammation was thought to be the result of concomitant glucose lowering [131]. Our study in contrast was designed to evaluate renal outcome independent of glucose levels, which has been a confounding variable in all studies to date.

There are several possible reasons for our findings. Firstly, as the blood glucose levels were similarly elevated among all diabetic mice, this has resulted in glomerular injury, as SGLT2 inhibition does not prevent glucose entry into the glomerular compartment. It is known that progressive glomerular injury can cause tubulointerstitial damage through a number of different mechanisms including misdirected filtration (filtrate leakage external to the tubular lumen), obstructed filtration and proteinuria as a result of damage to the glomerular filtration barrier
This then initiates a cycle of progressive tubulointerstitial injury, which is not prevented by blocking glucose entry into the PTC. Secondly, the lack of renoprotection seen in our study could be the result of incomplete inhibition of glucose reabsorption by empagliflozin. We know that SGLT2 inhibition only prevents reabsorption of 40% of glucose and this could be due to multiple reasons. GLUT1 is a major glucose transporter in mesangial cells and is a facilitative transporter of glucose in the S3 segment of the basolateral aspect of the tubular cell with bidirectional transport properties. It is possible that hyperglycaemia could influence the movement of glucose into the tubular cell from the basolateral aspect and is not modified by empagliflozin. This is not dependent on an increase in GLUT1 protein expression.

The diabetic mice in our model had matched high glucose levels, which is not reflective of the well controlled diabetic patient. However this was necessary in the design of the experiment to test the hypothesis whether SGLT2 inhibition can offer renoprotection independent of glucose lowering. There is evidence to suggest that SGLT2i can reduce diabetic nephropathy when glycaemic control is optimal in a Type 2 diabetic animal model and is even more pronounced when used in combination with an angiotensin receptor blocker. However this could have been due to glucose lowering rather than specific SGLT2 inhibition. In summary, we have shown that although empagliflozin reduces high glucose induced inflammatory and fibrotic markers in vitro; it does not have renoprotective benefits independent of glucose lowering in vivo.
Chapter 5: Dipeptidyl peptidase-4 inhibitors and diabetic nephropathy
5.1 Dipeptidyl peptidase-4

Dipeptidyl peptidase-4 (DPP4), which is also known as CD26, is a member of the serine peptidase/prolyl oligopeptidase gene family that includes the membrane-bound peptidases, fibroblast activation protein (FAP)/separase; the resident cytoplasmic enzymes, DPP8 and DPP9; and the nonenzymatic members, DPP6 and DPP10, which are present in neuronal membranes, and prolyl endopeptidase [135]. DPP4 is a unique protein found in many organ systems and has many functions. It was first identified as an enzyme present in liver and kidneys with the ability to hydrolyse glycine-proline dipeptide from a synthetic substrate glycyl-prolyl-β naphthalamide [136]. DPP4 is one of the membrane bound proteases specific for proline and less commonly alanine as the penultimate amino acid and cleaves the terminal dipeptide. This enzyme has an endless list of possible substrates although short peptides are considered as effective substrates rather than larger cytokines, which have not been shown to be cleaved by DPP4 [137]. DPP4/CD26 has a central cavity, which contains the hydrolase and propeller domains of the proteases. Hence this prevents large peptides from becoming substrates for proteolysis [137]. Moreover, DPP4 also demonstrates substrate specific catalysis where replacement of single amino acid can increase or decrease the efficiency of hydrolysis [137]. The most important substrates for DPP4 are glucagon like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) as they are involved in post meal glycaemic control. Cleavage of intact GLP-1 and GIP by DPP4 results in reduced affinity of cleaved product with its receptor and reduces the glucose regulation potential of the cleaved product [135]. DPP4 has also been shown to act as a receptor whereby engagement by anti CD26 antibodies in human hepatocarcinoma derived-PLC/PRF/5 cells results in tyrosine phosphorylation of several proteins in the
intracellular domain [138]. DPP4 also has a significant co-stimulatory role in T cells and interacts with both CD45 and adenosine deaminase (ADA) through its extracellular domain and participates in signal transduction [139]. DPP4 has been shown to bind to collagen and fibronectin and also participates in apoptosis [137]. In addition, DPP4 exhibits significant protein interaction with other membrane bound proteins, namely cation independent mannose-6-phosphate receptor (CIM6PR) and this interaction has been demonstrated to participate in T cell activation [140]. This interaction has recently been suggested as playing a possible role in activation of TGFβ [8]. It has also been shown that DPP4 interacts with integrin β1, which also plays a role in TGFβ activation and diabetic nephropathy [141]. These interactions play a significant role in the pathogenesis of diabetic nephropathy independent of the glycaemic effects of DPP4.

DPP4 exists in two forms, a membrane bound form and soluble form. The membrane bound form has residues 1-766 and the soluble form has residues 39-766 as it does not contain the trans-membrane and intracellular domains [135]. It is noted to be mostly present as a dimer where its enzymatic activity is maximal [135]. It has been shown that the soluble from is secreted from the membrane bound DPP4 [142].

5.2 The incretin system

The incretin hormones are released by the gut in response to a meal and may be responsible for up to 70% of postprandial insulin secretion. They include glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [143]. Apart from insulin secretion, the incretin hormones perform a number of functions
designed to improve glycaemic control. This includes suppression of glucagon secretion, increase in pancreatic β cell mass and increased differentiation of islet precursor cells into β cells, inhibition of β cell apoptosis, deceleration of gastric emptying to promote slow glucose release, suppression of satiety and weight loss [144]. GLP-1 and GIP have a biphasic secretion and their activity is terminated by DPP4. GLP-1 has a half life of < 2 minutes and GIP has a half life of 5-7 minutes [145]. Meal induced secretion of GLP-1 is diminished in patients with type 2 diabetes and this contributes to poor glycaemic control in these patients [146].

This has led to the development of incretin based therapy for diabetes whereby use of parenteral long acting GLP-1 analogues and oral DPP4 inhibitors (DPP4i) are used to improve diabetic control (Figure 5.1). The two classes of incretin based therapies include GLP-1 receptor agonists and DPP4i. The GLP-1 agonists includes liraglutide, a long acting agent with a half life of 11-13 hours and short acting agent, namely exenatide which has a half life of 2.4 hours [147]. There are several DPP4i in clinical practice. These drugs are divided into peptidomimetics or non-peptidomimetics according to their chemical structure, based on whether they mimic the DPP4 substrate. These drugs have different pharmacodynamic properties with linagliptin having the highest potency. They also display selectivity for DPP4 compared to other members of this family with selectivity ranging from <100 fold for vildagliptin and saxagliptin to >14000 fold for alogliptin [148].
5.3 Pathogenesis of diabetic nephropathy

Diabetic nephropathy is the commonest cause of chronic kidney disease worldwide. The incidence of diabetic nephropathy has been increasing in the developed world although the prevalence as a percentage of diabetic patients is stable [62]. However the incidence and prevalence of diabetes and diabetic nephropathy is predicted to increase in the developing world [149]. This has an impact on national resources and is a huge public health problem. There have been no significant treatment measures to prevent the progression of diabetic nephropathy since the advent of medications blocking the renin angiotensin aldosterone system (RAAS) and hence there remains a large treatment gap.

The pathogenesis of diabetic nephropathy is complex. A very simplistic view of diabetic nephropathy involves hemodynamic and metabolic abnormalities associated with diabetes mellitus. It has been shown that glomerular hyperfiltration occurs early in diabetes and in fact even in prediabetes in proportion with the degree of hyperglycaemia [150]. However the classical concept of hyperfiltration, albuminuria and subsequent loss of glomerular filtration is now not classically observed in patients with Type 2 diabetes mellitus [151]. It has also been shown that mesangial stretch results in GLUT-1 overexpression and increased TGFβ production [152]. Mesangial stretch also results in vascular permeability factor (VPF) or vascular endothelial growth factor (VEGF) production, which may contribute to proteinuria [153].

However the underpinning factor in diabetic complications is the constant exposure to high glucose demonstrated in many studies including the UKPDS and DCCT
The Banting lecture by Brownlee in 2004 integrated the various components involved in the pathobiology of diabetes. These pathogenic processes predominate in cells, which cannot regulate intracellular glucose entry in the setting of hyperglycaemia. During hyperglycaemia, there is increased flux of glucose through the polyol pathway, which reduces intracellular reduced glutathione resulting in increased susceptibility to oxidative stress. There is increased production of advanced glycation end products, which regulates gene transcription. There is increased activation of protein kinase C resulting in gene transcription, which increases profibrotic cytokines, namely (TGFβ and vasoconstrictor agents, namely endothelin-1. Increased glucose flux through hexosaminase pathway again increases transcription of TGFβ and plasminogen activator-1, another profibrotic cytokine [156].

The net result of hyperglycaemia is the perturbation of various physiological processes and activation of pathological processes culminating in glomerular and tubulointerstitial fibrosis. Glomerulosclerosis is related to increased expression of the GLUT-1 transporter in the mesangial cells, leading to activation of all the pathological pathways mentioned previously. In addition there is reduction in endothelial nitric oxide synthase, bradykinin 2 receptor blockade, plasminogen activator inhibitor-1 (PAI-1) activation, micro RNA regulation of TGFβ signaling and activation of JAK-STAT pathway through angiotensin II activation which collectively contribute to diabetic glomerulosclerosis [157]. The proximal tubular cell (PTC) is a very important site and plays an active role in pathogenesis of tubulointerstitial fibrosis in the diabetic kidney. It is the site for reabsorption of almost all of the
filtered glucose and a component of the filtered sodium through sodium glucose cotransport systems.

Diabetes induces early hyperplasia of the proximal tubules and various growth factors have been implicated including but not limited to insulin like growth factor-1 (IGF-1), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and VEGF. These changes induce increased ornithine decarboxylase activity leading to PTC hyperplasia. These growth factors also stimulate protein kinase C activity, which further stimulates cellular proliferation. These processes in addition to proteinuria activate inflammation, which contributes to and culminates in tubulointerstitial fibrosis. In addition, proximal tubular hypertrophy and hyperplasia stimulates further glucose and sodium reabsorption thereby exacerbating hyperglycaemia and potentiating hyperfiltration through inhibition of tubuloglomerular feedback [158].

The RAAS plays an important role in diabetic nephropathy. Hyperglycaemia stimulates increased production of renin and activates RAAS, which increases reactive oxygen species and promotes inflammatory and fibrotic cytokines eventually promoting glomerulosclerosis and tubulointerstitial fibrosis. These pathogenic processes culminate in microvascular complication of diabetes including diabetic nephropathy. TGFβ is a central profibrotic cytokine in the pathogenesis of diabetic nephropathy. It increases PAI-1 synthesis, fibronectin production and connective tissue growth factor (CTGF), which promote extracellular deposition of collagen. TGFβ induces fibrosis mainly through the Smad pathway, especially the downstream signaling molecules Smad2 and Smad3. Eventually this results in increased mesangial
Inflammation plays a significant role in diabetic nephropathy and the role of various aspects of the immune system in the development of diabetic nephropathy is becoming more apparent. Formation of reactive oxygen species (ROS) is a prominent feature of early diabetic nephropathy. It has been shown that endothelial nitric oxide synthase (eNOS) is upregulated in diabetic nephropathy. However in diabetes, there is reduced production of nitric oxide (NO) and increased production of ROS due to uncoupling of eNOS [160]. The lack of NO results in loss of vasodilation, reduced ability to inhibit platelet aggregation and reduced ability to suppress inflammation [161]. It has been shown that macrophage infiltration increases in diabetes and the degree of glomerulosclerosis and tubulointerstitial fibrosis correlates with the degree of infiltration [162]. In addition, a number of inflammatory cytokines are activated in diabetes and form part of a number of pathways including but not limited to Janus Kinase (JAK), signal transducer and activator of transcription (STAT), tyrosine kinase (TyK), myeloid differentiation factor-88 (Myd-88) and NF-κB inducing kinase (NIK) [163]. The profibrotic and proinflammatory cytokines result in activation of resident fibroblasts resulting in transformation into myofibroblasts, which produce a large amount of extracellular matrix [164].

Innate immunity has been shown to play a significant role in diabetic nephropathy. There is increasing evidence that high glucose triggers the increased expression of Toll like receptors 4 (TLR4) in tubular epithelial cells with downstream inflammatory signaling, namely NF-κB which in turn activates inflammatory cytokines IL-6 and
CCL-2 and is associated with increased macrophage infiltration [115]. Similarly, it has been shown that high glucose induces Toll like receptor 2 (TLR2) expression in proximal tubular cells and increases NF-κB activation through High Molecular Group Box-1 (HMGB-1) resulting in downstream activation of inflammatory and chemotactic cytokines [116]. The innate immune system is activated to produce interleukin 8 (IL-8) through a series of germ line coded pattern recognition receptors (PRRs). One of these PRRs is the nucleotide binding oligomerisation domain like receptors (NLRs). Cytosolic NLRs associate with apoptosis associated speck like protein and procaspase-1 to form a large multiprotein complex called the inflammasome, which leads to autocatalytic activation of caspace-1 [165]. It has recently been shown that Nlrp3 expression is increased in diabetic mice even before albuminuria and glomerular mesangial matrix expansion. It was also shown that Nlrp3 deficient mice were protected from albuminuria and mesangial expansion despite high glucose levels, suggesting a strong role for the inflammasome in diabetic nephropathy [166].

The incretin system has a role in modulation of pathogenesis of diabetic nephropathy. GLP-1 agonists and DPP4i have been shown to improve inflammation and fibrosis in vitro and in animal models of diabetes and hold promise for additional renoprotective benefits.

5.4 Role of GLP-1 in diabetic nephropathy

In addition to blood pressure lowering and RAAS blockade, the most important strategy in preventing onset and progression of diabetic nephropathy is excellent
control of blood glucose. DPP4i help prolong the action of GLP-1 resulting in better glycaemic control postprandially. However GLP-1 has been shown to have benefits beyond glucose lowering in preventing micro and macrovascular complications of diabetes, through both GLP-1 receptor dependent and independent effects.

5.4.1 Evidence from in vitro studies and animal studies

It has been shown recently that loss of GLP-1 receptor in C57BL6/Akita mice results in upregulation of NOX-4 expression and increased oxidative stress levels. This results in progressive diabetic nephropathy in this mouse model, which is otherwise resistant to diabetic nephropathy. It was also noted that loss of the GLP-1 receptor increased renal expression of connective tissue growth factor (CTGF) and TGFβ1 in addition to Thrombospondin-1, which is an endogenous activator of TGFβ1. It is proposed that GLP-1 acts via increased intracellular cyclic adenosine mono phosphate (CAMP) and protein kinase A (PKA) to inhibit NADPH oxidase. It has been shown that the GLP-1 analogue exendin-4 inhibited the proliferation of mesangial cells and glucose induced upregulation of TGFβ1 and CTGF expression [167]. Liraglutide, a long acting GLP-1 analogue was noted to suppress the progression of diabetic nephropathy in KK/Ta-Akita mice without major differences in insulin levels, glucose tolerance and metabolic parameters [168]. It has been shown recently by Mima et al that GLP-1 receptor levels were reduced in glomerular endothelial cells in diabetic mice through activation of protein kinase C (PKC) [169]. Exendin-4, a long acting GLP-1 analogue was shown to act through cyclic adenosine mono phosphate (cAMP) induced phosphorylation of c-Raf, which inhibits angiotensin II induced activation of phospho-c-Raf in diabetic mice and prevents progression of diabetic nephropathy.
They showed that exendin-4 did not improve expression of GLP-1 receptor levels in diabetic mice overexpressing protein kinase C in their glomerular endothelial cells (EC-PKCb2Tg). However, exendin-4 was still able to reduce diabetic glomerular changes in these mice in spite of reduced GLP-1 receptor levels suggesting alternative cAMP independent pathways in addition to cAMP dependent pathways [169].

GLP-1 also exerts a protective effect on the kidneys through its anti inflammatory action. It has been shown that exendin-4 ameliorated albuminuria and glomerular changes in a streptozotocin induced diabetic rat model. This was accompanied by reduced macrophage infiltration and release of pro-inflammatory cytokines by macrophages through activation of GLP-1 receptors. It was also shown that intracellular adhesion molecule-1 (ICAM-1) production and expression on glomerular endothelial cells was reduced by activation of GLP-1 receptors on these cells [170]. GLP-1 receptor signaling via AMP activated protein kinase has been shown to increase nitric oxide (NO) production by endothelial cells resulting in vascular relaxation suggesting a direct role in improving hypertension [171]. Furthermore it has been shown that liraglutide increases atrial natriuretic peptide (ANP) release from cardiac atria and there is a meal induced increase in ANP secretion in wild type mice along with increased urinary sodium excretion, which was not seen in GLP1r(-/-) mice [172]. Hence GLP-1 receptor agonists may exert renal benefit by improving blood pressure and endothelial relaxation in small and large blood vessels. However it is to be noted that in human subjects, liraglutide promotes natriuresis without increasing circulating levels of ANP suggesting alternative mechanisms for the natriuresis [173].
5.4.2 Evidence from clinical studies

Exenatide, a GLP-1 agonist has also been shown to reduce urinary albuminuria, collagen IV and TGFβ excretion in patients with type 2 diabetes and microalbuminuria in comparison with glimepride, a sulfonylurea, after attaining similar glycaemic control [174]. GLP-1 infusion over a 2-hour period has been shown to increase urinary sodium excretion and reduce serum angiotensin II concentration in healthy men providing clinical evidence of its effects on the renin angiotensin system [175]. The ELIXA (Evaluation of Lixisenatide in Acute Coronary Syndrome) study comparing the effects of lixisenatide, a GLP-1 agonist, versus placebo in reducing cardiovascular mortality in 6000 patients with acute coronary syndrome over a two year follow up period, did not show any benefit. These data were presented at the American Diabetes Association (ADA) 2015 scientific sessions. More recently the results of the LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results) study were published, which showed cardiovascular mortality benefit of liraglutide in 9340 patients who were randomly assigned to either liraglutide or placebo over a five-year period. In addition, this study also showed significant reduction in incidence of nephropathy compared to placebo [176].

5.5 The enzymatic and non-enzymatic functions of DPP4 in diabetic nephropathy

DPP4 has enzymatic and non-enzymatic attributes, which extend beyond inactivation of GLP-1 and GIP. Animal studies have shown that DPP4i are associated with
improvement in renal function. The study by Mega et al showed improvement in diabetic nephropathy in Zucker diabetic fatty rats (ZDF) although this was associated with improvement in glycaemic parameters [4]. Similarly Liu et al showed improvement in diabetic nephropathy in streptozotocin (STZ) induced diabetic rats treated with vildagliptin. However this was associated with elevated GLP-1 levels making it difficult to estimate the non GLP-1 mediated actions of DPP4 [177]. Linagliptin has been shown to prevent progression of chronic kidney disease in non diabetic rats with 5/6 nephrectomy, hence giving credence to the renoprotective benefit of DPP4i beyond their glucose lowering effect [178].

It has been demonstrated that DPP4 is expressed on the renal tubular brush border. It has also been shown that urinary excretion of an intact peptide substrate by mice lacking tubular brush border DPP4 was much higher than mice possessing DPP4, signifying the enzymatic role of tubular DPP4 over luminal substrates [179]. Mass spectroscopy based global peptide profiling of DPP4 knock out mice has revealed substrates, which may play a role in the pathogenesis of inflammation and more importantly in diabetic nephropathy. One such substrate that has been identified is meprin β [180]. It has been shown that activated forms of pure recombinant meprin β cleave PKC resulting in reduced PKC kinase activity [181]. It is known that PKC is increased in diabetic kidneys and hence meprin cleavage by DPP4 present on the tubular brush border may impact on protective benefits of uncleaved meprin and hence impact diabetic nephropathy. High mobility group box-1 (HMGB-1) is also a substrate of DPP4 [182]. It has also been established that HMGB-1 plays a significant role in inflammation in diabetic nephropathy [183]. It has been shown that tubular expression of HMGB-1 is increased in diabetes and it acts through toll like receptor 2
(TLR2) and toll like receptor 4 (TLR4) to activate downstream inflammatory pathways in diabetic nephropathy [115, 116]. HMGB1 also acts via receptor for advanced glycation end products (RAGE), which in turn activates reactive oxygen species [184]. It is therefore possible that DPP4i may modulate diabetic nephropathy through prevention of HMGB1 cleavage.

With respect to the enzymatic properties of DPP4, several substrates have been found to be important mediators in immune function, inflammation, vascular repair and hypertension [185]. Brain natriuretic peptide (BNP 1-32) is a substrate of DPP4 and is cleaved to BNP 3-32. The uncleaved BNP is shown to be more natriuretic than the cleaved product in dogs and was also shown to significantly reduce blood pressure [186]. Stromal derived factor-1α (SDF-1α) has been shown to be a substrate of DPP4 [187]. SDF-1α has also been shown to be a chemo attractant for progenitor cells and has been suggested to reduce infarct size in Wistar rats with an increased expression of SDF-1α in animals treated with linagliptin [188].

The non-enzymatic actions of DPP4 include interaction with other cellular proteins to exert adhesion molecule like functions and influence extracellular matrix deposition and remodeling [137].

It has been shown that linagliptin, a DPP4 inhibitor is able to ameliorate endothelial to mesenchymal transition and prevent kidney fibrosis in streptozotocin (STZ) induced diabetic mice. This was mediated through microRNA 29 induction and was achieved without alteration of blood glucose levels [189]. It has been shown that DPP4 binds with integrin β1 and this complex activates TGFβ in addition to increased expression
of vascular endothelial growth factor receptor-1 (VEGFR-1) [141]. TGFβ signaling promotes fibrosis and VEGFR1 receptor activation promotes endothelial to mesenchymal transition [141]. Linagliptin has been shown to inhibit the DPP4-integrin β1 interaction blunting TGFβ signaling and endothelial to mesenchymal transition [141].

Our group has shown that DPP4 is expressed in human kidney proximal tubular cells (HK2 cells) and that linagliptin is able to reduce high glucose induced conversion of latent to active TGFβ and downstream reduction in fibronectin [190]. We have shown that linagliptin, a non-peptidomimetic DPP4 inhibitor, potentially reduces activation of TGFβ by inhibiting the high glucose induced interaction of DPP4 with the cation independent mannose-6-phosphate receptor (CIM6PR) in HK2 cells. This results in a demonstrated reduction in downstream Smad2 signaling and consequent tubulointerstitial fibronectin deposition in an STZ induced diabetic eNOS +/- mouse model [8]. We have replicated similar renoprotective benefits using another DPP4 inhibitor, saxagliptin, a peptidomimetic DPP4 inhibitor in a STZ induced diabetic eNOS +/- mouse model, suggesting that this is a class effect and extends across both peptidomimetic and non-peptidomimetic DPP4i [9]. However, it remains possible that the degree of renoprotection varies depending on factors including exposure of the DPP4 inhibitor to the tubular brush border membrane.

It is well known that mortality in CKD is primarily related to cardiovascular disease [191]. Following the unexpected adverse cardiovascular outcomes reported with rosiglitazone, the FDA mandated trials aimed at determining major adverse cardiovascular outcomes with newer anti diabetic agents [102]. In 2009, the
RECORD (Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes) study subsequently demonstrated no increased cardiovascular risk with the use of rosiglitazone. Issues with trial design and data integrity led to an independent readjudication of the data in June 2013 [103]. This process demonstrated the importance of dedicated trials powered to look at major cardiovascular outcomes and hence this is an important focus for manufacturers of newer anti diabetic medications. The SAVOR-TIMI trial looked at cardiovascular outcomes of saxagliptin versus placebo in patients with risk factors for cardiovascular disease or who had a history of cardiovascular events. It did not show any difference in all cause or cardiovascular mortality although the incidence of heart failure was higher in patients treated with saxagliptin [104]. There was an improvement in albuminuria although this was in association with improvement in glycaemic control and potentially a direct result of this rather than due to a specific renoprotective benefit. Similarly the EXAMINE trial looked at cardiovascular outcomes in diabetic patients who were initiated on alogliptin after a myocardial infarction and showed no change in all cause or cardiovascular mortality. This showed non inferiority of DPP4i in relation to cardiovascular outcomes, although it did not reproduce the cardiovascular benefits touted by previous meta analyses. Moreover the changes in eGFR were similar in both placebo and DPP4 inhibitor treated groups [192].

There is one clinical trial currently involved in looking at the long-term cardiovascular and renal outcomes in patients with diabetes on linagliptin (CARMELINA, NCT01897532). This trial is being conducted in diabetic patients who are at high risk of cardiovascular events or who have had a prior cardiovascular event. The trial outcomes are major adverse cardiovascular events including
cardiovascular death, non fatal myocardial infarction and non fatal stroke. This trial will also assess renal outcomes including end stage kidney disease and a decline in renal function by greater than 50%. The trial is expected to be completed by 2018 and outcomes of this trial will confirm if linagliptin can offer cardiorenal protection.
**Figure 5.1: The incretin system**

Meal induced stimulation of glucagon like peptide-1 (GLP-1) secretion is short lasting due to deactivation of GLP-1 by dipeptidyl peptidase-4 (DPP-4). However DPP-4 inhibitors prolong action of GLP-1 resulting in prolonged post meal insulin secretion.
Chapter 6: Linagliptin limits high glucose induced conversion of latent to active TGFβ through interaction with CIM6PR and limits renal tubulointerstitial fibronectin

Gangadharan Komala M, Gross S, Zaky A, Pollock C, Panchapakesan U.

Abstract

Background
In addition to lowering blood glucose in patients with type 2 diabetes mellitus, dipeptidyl peptidase 4 (DPP4) inhibitors have been shown to be antifibrotic. We have previously shown that cation independent mannose-6-phosphate receptor (CIM6PR) facilitates the conversion of latent to active transforming growth factor β1 (TGFβ1) in renal proximal tubular cells (PTCs) and linagliptin (a DPP4 inhibitor) reduced this conversion with downstream reduction in fibronectin transcription.

Objective
We wanted to demonstrate that linagliptin reduces high glucose induced interaction between membrane bound DPP4 and CIM6PR in vitro and demonstrate reduction in active TGFβ mediated downstream effects in a rodent model of type 1 diabetic nephropathy independent of high glycaemic levels.

Materials and Methods
We used human kidney 2 (HK2) cells and endothelial nitric oxide synthase knock out mice to explore the mechanism and antifibrotic potential of linagliptin independent of glucose lowering. Using a proximity ligation assay, we show that CIM6PR and DPP4 interaction was increased by high glucose and reduced by linagliptin and excess mannose-6-phosphate (M6P) confirming that linagliptin is operating through an M6P-dependent mechanism. In vivo studies confirmed these TGFβ1 pathway related changes and showed reduced fibronectin, phosphorylated smad2 and phosphorylated smad2/3 (pSmad2/3) with an associated trend towards reduction in tubular atrophy,
which was independent of glucose lowering. No reduction in albuminuria, glomerulosclerotic index or cortical collagen deposition was observed.

Conclusion

Linagliptin inhibits activation of TGFβ1 through a M6P dependent mechanism. However this in isolation is not sufficient to reverse the multifactorial nature of diabetic nephropathy.
6.1 Introduction

The incretin family, including glucagon like peptide 1 (GLP-1), gastrointestinal peptide (GIP) and dipeptidyl peptidase 4 (DPP4), are targets of recent glucose lowering drugs. The DPP4i are now well established as hypoglycaemic agents for use in patients with type 2 diabetes mellitus. The potential for DPP4i to offer beneficial effects beyond glucose lowering lies with the functional ability of DPP4 to cleave a host of peptides apart from GLP-1.

DPP4 is a serine exopeptidase belonging to the S9B protein family, members of which cleave X-proline dipeptides from the N-terminus of polypeptides, such as chemokines, neuropeptides, and peptide hormones [193]. It is a 110-kDa type 11 integral membrane glycoprotein and is expressed ubiquitously in most organs and cell types. Importantly, DPP4 is therefore able to exert pleiotropic effects. DPP4 exists in both a soluble and membrane bound form, both of which are capable of proteolytic activity. The soluble form in the circulation is thought to arise from shedding of the membrane bound DPP4 and is the target for DPP4i as hypoglycaemic agents in clinical use [193]. In contrast, the membrane bound form of DPP4, expressed on the surface of many cell types including kidney tubular cells, endothelial cells and T cells [194], is of major interest with respect to the pleiotropic actions of DPP4. Membrane bound DPP4 also exerts non-enzymatic actions by virtue of colocalising with other membrane proteins and modulating their intrinsic actions [193].

It is widely accepted that transforming growth factor β1 (TGFβ1) is a major driver of fibrosis in diabetic nephropathy. We have recently reported that linagliptin, a DPP4
inhibitor, reduces high glucose induced active TGFβ1 in human kidney proximal tubular cells [195]. This translated to a downstream reduction in phosphorylated Smad2 (pSmad2) and fibronectin transcription and expression. As high glucose induced total secreted TGFβ1 was unchanged by linagliptin, we postulated that the mechanism was related to interference with the conversion of latent to active TGFβ1. TGFβ1 is secreted in a latent form and requires a complex interplay of soluble signaling molecules in the activation process, which releases it from the latency associated peptide (LAP). Once released from the LAP, the unbound TGFβ1 can then bind to its receptor to initiate cell signaling via the Smad pathway. Several other molecules such as plasminogen, thrombospondin-1 (TSP-1) and the cation independent mannose-6-phosphate receptor (CIM6PR) [196] participate in this activation process. Among these candidate molecules, we showed that TSP-1 was not the likely target to explain the inhibition of latent to active TGFβ1 [195].

The CIM6PR is a membrane protein that binds mannose-6-phosphate containing proteins (like DPP4 and LAP). We have shown in our previous studies that CIM6PR is central to the activation process of TGFβ1 in human kidney proximal tubular cells exposed to high glucose [197]. Given the fact that CIM6PR and DPP4 colocalise on the cell membrane [198], we sought to study the interaction between the two in context of high glucose and to delineate the mechanism by which linagliptin reduces active TGFβ1. We also extended our studies to include an in vivo model of diabetic nephropathy, and importantly compared the treatment group to a control group with matched glucose levels, to evaluate whether linagliptin has antifibrotic effects independent of its glucose lowering properties.
6.2 Materials and methods

6.2.1 Cell Culture

HK2 cells, a primary human proximal tubular cell line (American Type Culture Collection), were grown in Keratinocyte Serum Free Media supplemented with bovine pituitary extract 20-30µg/ml and epidermal growth factor 0.1-0.2ng/ml (Gibco, NY, USA) on coverslips and treated with 5mM glucose, 30mM glucose, 30mM glucose plus 1 µM mannose-6 phosphate (M6P) (Santa Cruz) and 30mM glucose plus 30nM linagliptin (generously provided by Boehringer-Ingelheim, Germany) for 48 hours. The IC50 (half maximal inhibitory concentration) of linagliptin is 1nM and the final concentration in our cell culture system was 30nM [195]. Initial experiments were done using increasing concentrations of M6P ranging from 1nM to 1mM. A final concentration of 1µM was chosen. The rationale for adding M6P is to saturate the M6P binding sites on the CIM6PR. If linagliptin reduces the interaction between CIM6PR and DPP4 through a M6P mechanism, then an excess of free M6P in the cell culture system would reduce recognition of the M6P moiety on the DPP4 and hence reduce any CIM6PR: DPP4 interaction.

6.2.2 Proximity ligation assay

Duolink In situ Fluorescence kit (Sigma Aldrich, St. Louis, MO) was used as per manufacturer’s instructions. This is based on the principle that a pair of oligonucleotide labeled secondary antibody probes generate a signal only when the two probes have bound in close proximity to two primary antibodies attached to
proteins that are co-localised [199]. This technique allows direct visualisation of endogenous protein complexes in specific physiological environments. CIM6PR (Novus Biologicals, CO, USA) and DPP4 (Santa Cruz Biotechnology, USA) antibodies were initially optimised for immunofluorescence after cells were fixed using 3.7% paraformaldehyde, blocked with 2% bovine serum albumin and incubated with primary antibodies overnight. Importantly using the same antibodies, we ensured that linagliptin did not alter DPP4 protein expression with immunofluorescence. Cells were then incubated with both primary antibodies overnight, washed, incubated with secondary antibody probes and then subjected to ligation and amplification. Coverslips were mounted on slides using DAPI mounting medium and visualised with a confocal microscope. Images were acquired using Leica TCS SP5 confocal laser scanning microscope with the 63x/1.4NA objective using thick sections and adjusted to ensure that most of the nuclei were in the same Z plane. Resolution (1024x1024 pixels) and parameter settings were standardised for all the images. A minimum of 100 cells per sample was counted. The number of associations (visualised by red dots) was calculated using Image J Analyse Particles function and corrected for the number of cells, which were stained with DAPI. Experiments were done in triplicate and the data was presented as a mean ± standard error. A p value of < 0.05 was considered significant.

6.2.3 Animal model

We used endothelial nitric oxide synthase knockout mice (eNOS -/-) as these have been shown to develop significant changes of diabetic nephropathy [200]. We used linagliptin (provided by Boehringer Ingelheim and at the recommended dose of
3mg/kg per day via oral gavage) as the DPP4 inhibitor in our studies. Current “best practice” for renoprotection rests with administration of an agent that blocks the renin-angiotensin-aldosterone (RAAS) system. Hence we included a comparator with Telmisartan (Sigma Aldrich, St. Louis, MO, at 3 mg/kg /day in drinking water). Animal groups were allocated as shown below for the renal studies, which was conducted for 24 weeks post induction of diabetes. Mice were given intraperitoneal injections of streptozotocin (STZ) at a dose of 55 mg/kg/day (Sigma Aldrich, St. Louis, MO) for 5 consecutive days at 7-8 weeks of age. This is the standard low dose STZ protocol validated by the Animal Models of Diabetic Complications Consortium. Blood glucose was tested using a glucometer (Accuchek Nano, Roche Diagnostics) one week after STZ through tail vein blood collection. Diabetes was defined by blood glucose greater than 16 mmol/L after a six-hour daytime fast. Mice with levels below 16 mmol/L were excluded from the study. Fasting blood glucose levels were measured monthly. Long acting insulin (Insulin Glargine, Sanofi Aventis, Australia) was initiated as required from 10 weeks of age and was administered thrice weekly if the blood sugar exceeded 28 mmol/L or if they had lost weight greater than 25% from baseline. The aim was to match glycaemia and to maintain body weight and avoid ketonuria without achieving euglycaemia. Importantly, in all studies the glycaemic control of the diabetic animals was matched to assess specific renal effects of linagliptin independent of glycaemic control. The study was approved the Royal North Shore Hospital Ethics Committee (Protocol number 1203-009A). The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes were followed in this study. Animals were anaesthetised using short inhalational anaesthesia with 2% Isoflurane for minor procedures. Animals were euthanized under
2% Isoflurane anaesthesia using cardiac puncture terminally. The groups were as below:

(i) Non-Diabetic (control): 12 animals
(ii) Non-Diabetic (control) with linagliptin: 8 animals
(iv) Diabetic: 12 animals
(v) Diabetic with linagliptin: 9 animals
(vii) Diabetic with telmisartan: 9 animals

6.2.4 Measurement of physiological parameters

Body weight was assessed monthly. Blood pressure was measured using a non-invasive tail vein cuff method (CODA BP apparatus, Kent Scientific, USA) preterminally.

6.2.5 Urine biochemistry

Urine was collected at two different time points (4-6 weeks after initiation of treatment using metabolic cages and terminally using bladder puncture). Urine creatinine was measured using a picric acid method (Creatinine Companion, Exocell Inc., USA). Urine albumin was measured using ELISA (enzyme linked immunosorbent assay) (Albuwell, Exocell Inc., USA).
6.2.6 Kidney tissue harvest

The un-perfused left kidney was harvested and snap frozen, after embedding in OCT compound. The right kidney was perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) and subsequently fixed in 10% neutral buffered formalin for 24-48 hours.

6.2.7 Histology and immunohistochemistry

Formalin fixed paraffin embedded (FFPE) kidney sections were stained with Periodic Acid Schiff and Sirius red stain. Assessment of histological change was done in a blinded manner. The Glomerulosclerotic index (GSI) was calculated based on the formula, 

\[ GSI = \frac{[(1 \times N1) + (2 \times N2) + (3 \times N3) + (4 \times N4)]}{(N0 + N1 + N2 + N3 + N4)} \]

where \( N_x \) is the number of glomeruli with each given score for each section. Atrophic tubules were defined by dilatation, epithelial shedding and thinning of epithelium. Tubular damage was scored by counting the number of atrophic tubules per 400 tubules at 200x magnification. The degree of interstitial collagen content in Sirius red stained slides was assessed in a blinded manner using Image J by identifying the percentage of interstitial collagen positive region at X 200 magnification in 5 randomly selected regions. Immunohistochemistry for nuclear pSmad 2/3, was done on 4 micron paraffin embedded sections using goat anti-mouse pSmad 2/3 (SC11769-G, Santacruz Biotechnology, USA), after an overnight incubation at a concentration of 1:100, followed by donkey anti goat HRP tagged secondary antibody (Santacruz Biotechnology, USA at a concentration of 1:100). With respect to fibronectin, the primary antibody (Sigma, USA) was used at a dilution...
of 1:1000 followed by anti rabbit secondary antibody at a dilution of 1:100 (Dako, Australia). The chromogenic reaction was carried out with 3,3′-diaminobenzidine chromogen (Dako, Australia) solution for 10 minutes.

6.2.8 RNA isolation and RT-PCR analysis

Total RNA was extracted from kidney tissue using Qiagen RNEasy Mini kit on an automated RNA extraction protocol using Qiacube. cDNA was synthesised using Roche Transcriptor First Strand cDNA synthesis kit (Roche, USA). The real time PCR was done using SYBR green (Bioline, Australia) for fibronectin (forward-CACGGAGGCCACCATTACT and reverse-CTTCAGGGCAATGACGTAGAT) using actin (forward-CAGCTGAGGGAAATCGTG and reverse-CGTTGCCAATAGTGATGACC) as the endogenous control. Primers were sourced from Sigma. The RT-PCR was performed using the AB7900 machine (Applied Biosystems, Australia).

6.2.9 Western blot analysis

Frozen tissue was homogenized with Quiagen Tissue Ruptur in 1.5 ml of cold 20mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210mM mannitol, 70mM sucrose and centrifuged at 1,500 x g for 5 min at 4°C. Samples were then analysed by SDS gel electrophoresis (Novex, Life technologies, Australia) and electroblotted to Hybond Nitrocellulose membranes (Amersham Pharmacia Biotech, Bucks, UK). Membranes were then probed with pSmad2 (Ser465/467) antibodies (#3101,Cell Signaling Technology, USA) followed by HRP tagged anti rabbit antibody (Cell
Membranes were stripped and probed for total Smad2 (#5339, Cell Signaling Technology, USA). Proteins were visualized using Luminata Western HRP Substrate (Millipore) in a LAS 4000 image reader (GE Healthcare Life Sciences). Analysis was performed using Image J software (NIH, USA).

### 6.2.10 Statistical analysis

Statistical analysis was done using GraphPad Prism 6. Data are expressed as mean ± standard error of mean. A P value < 0.05 was considered statistically significant. Blood sugar profile during the study was measured using two way repeated measures ANOVA. ANOVA with Bonferroni’s correction was used for all other statistical analysis.
6.3 Results

6.3.1 M6P and linagliptin reduced high glucose induced CIM6PR:DPP4 interaction in HK2 cells

CIM6PR and DPP4 were both present on the cell membrane of HK2 cells. Proximity ligation assay revealed a significant increase in signal when cells were exposed to 30mM glucose suggesting that CIM6PR and DPP4 were interacting under high glucose conditions (P<0.05). Linagliptin reduced this high glucose induced interaction (P<0.05). Excess M6P was also able to reduce this interaction (P<0.05), which suggests linagliptin is competing with M6P for binding at the CIM6PR. This is shown in Figure 6.1A and B.
Figure 6.1

A) Ctrl  HG  HG + 30 nM Lina  HG + 1 μM M6P

B) Treatment Groups
Figure 6.1

(A) Proximity ligation assay demonstrating endogenous protein-protein interaction between membranous DPP4 and CIM6PR in HK2 cells visualised as individual fluorescent dots. This is increased in 30mM high glucose (HG) environment compared to control 5mM glucose (ctrl). M6P at 1µM and linagliptin at 30nM reduced the high glucose induced interaction. A quantitation of this is shown in (B). Data are represented as mean ± standard error, n=3. *=P<0.05 compared to 5mM glucose, #=P<0.05 compared to 30mM glucose.
6.3.2 Blood glucose were elevated and matched in diabetic mice

All diabetic mice had significantly elevated fasting blood glucose compared to control mice (P<0.05). All the diabetic mice had matched fasting blood glucose levels. This is shown in Table 6.1. This was in the absence of urinary ketonuria (data not shown).

6.3.3 Linagliptin did not reduce albuminuria in diabetic mice

The diabetic mice showed significant terminal urinary albumin excretion in keeping with diabetic nephropathy compared to control mice (P<0.01). This was not improved by linagliptin. However telmisartan significantly reduced albuminuria (P< 0.01). This is summarised in Table 6.1.

6.3.4 Physical parameters

The diabetic mice exhibited the expected weight loss during the experiment in comparison to significant weight gain by control mice (P<0.01). The diabetic mice treated with linagliptin also demonstrated weight loss (P<0.01) and were not significantly different to untreated diabetic mice. However diabetic mice treated with telmisartan gained weight, which was significantly better than diabetic mice (P<0.01). The diabetic mice also showed significant renal hypertrophy compared to control mice (P<0.01). Linagliptin did not alter this significantly. Telmisartan reduced diabetes induced renal hypertrophy as signified by the normalised kidney/body weight ratio (P<0.01). The control and diabetic mice had comparable systolic blood pressure. These results are summarised in Table 6.1.
### Table 6.1. Metabolic and physical parameters of mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + linagliptin</th>
<th>Diabetic</th>
<th>Diabetic + linagliptin</th>
<th>Diabetic + telmisartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gain in weight (grams)</td>
<td>5.86 ± 0.70</td>
<td>4.66 ± 0.70</td>
<td>-0.68 ± 0.63**</td>
<td>-0.53 ± 0.60**</td>
<td>2.20 ± 0.64##</td>
</tr>
<tr>
<td>Left kidney/ body weight ratio (%)</td>
<td>0.84 ± 0.05</td>
<td>0.81 ± 0.03</td>
<td>1.07 ± 0.06**</td>
<td>0.97 ± 0.05</td>
<td>0.83 ± 0.02##</td>
</tr>
<tr>
<td>Average blood sugar (mmol/L)</td>
<td>9.9 ± 0.1</td>
<td>10.2 ± 0.1</td>
<td>20.9 ± 0.9*</td>
<td>22.4 ± 0.7*</td>
<td>22.4 ± 1.1*</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>115.6 ± 2.7</td>
<td>115.0 ± 3.2</td>
<td>111.1 ± 4.4</td>
<td>102.9 ± 2.5</td>
<td>101.3 ± 4.9</td>
</tr>
<tr>
<td>24 hour urine albumin excretion (µg/day)</td>
<td>463.4 ± 105.7</td>
<td>360.8 ± 80.6</td>
<td>2319 ± 438.3**</td>
<td>2238 ± 226</td>
<td>766.4 ± 152.3##</td>
</tr>
</tbody>
</table>

Values are shown as mean ± SEM

*=P<0.05 vs control

**=P<0.01 vs control

##=P<0.01 vs diabetic
6.3.5 Linagliptin did not reduce glomerulosclerosis in diabetic mice

The diabetic mice had significant glomerulosclerosis compared to control mice (P<0.01). Linagliptin did not ameliorate the degree of glomerulosclerosis in diabetic mice. Telmisartan showed a significant improvement in the diabetic animals (P<0.01). These results are shown in Figure 6.2.
Figure 6.2

<table>
<thead>
<tr>
<th></th>
<th>ctrl</th>
<th>ctrl+lina</th>
<th>dm</th>
<th>dm+lina</th>
<th>dm+telmi</th>
</tr>
</thead>
</table>

![Bar chart showing glomerulosclerosis score with significance levels](chart.png)
Figure 6.2. Diabetic mice demonstrated increased glomerulosclerosis, which was improved by telmisartan but not by linagliptin as demonstrated by quantification of glomerulosclerosis by glomerulosclerotic index. Representative photographs of PAS stained sections for control (ctrl), control + linagliptin (ctrl + lina), diabetic (dm), diabetic + linagliptin (dm + lina) and diabetic + telmisartan (dm + tel) groups are shown (Magnification=original X400). Data are expressed as mean ± SEM with **=P<0.01 vs ctrl, ###=P<0.01 vs dm.
6.3.6 Linagliptin partially reduced tubular atrophy in diabetic mice

Diabetic mice had significant tubular atrophy (P<0.05). Although both linagliptin and telmisartan showed a reduction in diabetic tubular atrophy, this change did not reach statistical significance. These results are shown in Figure 6.3.

Figure 6.3
Figure 6.3. Diabetic mice demonstrated tubular atrophy, which was partially reduced by linagliptin and telmisartan. Representative photographs of PAS stained sections of tubulointerstitium for control (ctrl), control + linagliptin (ctrl + lina), diabetic (dm), diabetic + linagliptin (dm + lina) and diabetic + telmisartan (dm + tel) groups are shown (Magnification=original X 200). Quantification of tubular atrophy in all groups was done by counting the number of atrophic tubules per 400 tubule count (Data are expressed as mean ± SEM with *=P<0.05 vs ctrl).
6.3.7 Linagliptin reduced tubular pSmad 2/3 expression (a marker of transforming growth factor beta activation) in diabetic mice

Diabetic mice showed significant renal nuclear expression of pSmad 2/3 (P<0.01), signifying TGFβ signaling. Both linagliptin and telmisartan treated mice showed a significant reduction in pSmad2/3 expression (both P<0.01). These results are shown in Figure 6.4A. Western blot analysis of pSmad 2 expression showed a trend towards increased expression in diabetic mice, which was ameliorated to some extent by linagliptin (P=0.08), (Figure 6.4B). The results from the Western blot analysis were consistent with the findings from immunohistochemistry.
Figure 6.4

A)  

<table>
<thead>
<tr>
<th>ctrl</th>
<th>ctrl+lina</th>
<th>dm</th>
<th>dm+lina</th>
<th>dm+telmi</th>
</tr>
</thead>
</table>

Bar chart showing the number of psmad 2/3 positive nuclei at X 200 magnification:  
- ctrl  
- ctrl+lina  
- dm  
- dm+lina  
- dm+telmi  

** (p < 0.01)  
## (p < 0.001)
Figure 6.4

B)

pSmad2

tSmad2

Cortical pSmad2 protein over tSmad2 expression
Figure 6.4

A) Diabetic mice demonstrated increased pSmad2/3 nuclear expression with immunohistochemistry, which was reduced by linagliptin and telmisartan. Representative photographs for control (ctrl), control + linagliptin (ctrl + lina), diabetic (dm), diabetic + linagliptin (dm + lina) and diabetic + telmisartan (dm + tel) groups are shown (Magnification=original X 200). Quantification was done by counting the number of positive nuclei at X200 magnification. Data are expressed as mean ± SEM with **=P<0.01 vs ctrl, ##=P<0.01 vs dm

B) Diabetic mice showed a trend towards increase in pSmad 2/total smad2 expression compared to control mice and a reduction with both linagliptin and telmisartan. This trend was consistent with the immunohistochemistry findings but did not reach statistical significance. Quantification was done using Image J. Data are expressed as mean ± SEM, n=6.
6.3.8 Linagliptin reduced fibronectin transcription and expression in diabetic mice

Diabetic mice showed significantly increased renal cortical transcription of fibronectin mRNA in comparison to control mice (P<0.01). This was significantly improved in diabetic mice treated with linagliptin (P<0.05) and telmisartan (P<0.01). These data are shown in Figure 6.5A. In keeping with this, there was also a significant increase in tubulointerstitial fibronectin expression in diabetic mice (P<0.01), which was significantly reduced with linagliptin (P<0.05). These results are shown in Figure 6.5B.
Figure 6.5

A)
Figure 6.5

B) ctrl  ctrl+lina  dm

dm+lina  dm+telmi

Tubulointerstitial fibronectin expression on IHC

ctrl  ctrl+lina  dm  dm+lina  dm+telmi
Figure 6.5

(A) Diabetic mice demonstrated increased cortical fibronectin mRNA transcription by real time PCR. This was significantly reduced by linagliptin and telmisartan.

(B) There was a significant increase in tubulointerstitial FN expression measured by immunohistochemistry in the diabetic animals and this was reduced with linagliptin. Representative photographs for control (ctrl), control + linagliptin (ctrl + lina), diabetic (dm), diabetic + linagliptin (dm + lina) and diabetic + telmisartan (dm + tel) groups are shown (Magnification=original X 200). Data are expressed as mean ± SEM with **=P<0.01 vs ctrl, #=P<0.05 vs dm, ##=P<0.01 vs dm.
6.3.9 Linagliptin did not reduce tubulointerstitial collagen deposition in diabetic mice

Collagen I was increased in diabetic mice compared to control values (P<0.01). Telmisartan reduced collagen I deposition (P<0.05). This is shown in Figure 6.6A. Diabetic mice demonstrated an expected increase in renal picrosirius staining (collagen I and III) in comparison to control mice (P<0.01). Neither linagliptin nor telmisartan reduced picrosirius staining. This is shown in Figure 6.6B.
Figure 6.6

A) ctrl  ctrl+lina  dm  dm+lina  dm+telmi

<table>
<thead>
<tr>
<th>Tubulointerstitial Collagen I expression on IHC</th>
<th>ctrl</th>
<th>ctrl+lina</th>
<th>dm</th>
<th>dm+lina</th>
<th>dm+telmi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>2.0</td>
<td>2.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

** Significant difference

# Significant difference
Figure 6.6

B) ctrl  ctrl+lina  dm  dm+lina  dm+telmi

% of Sirius positive collagen in interstitium

ctrl  ctrl+lina  dm  dm+lina  dm+telmi
**Figure 6.6**

(A) Diabetic mice showed increased collagen I and (B) picrosirius staining. Linagliptin did not change either. Telmisartan reduced collagen I expression.

Representative photographs of collagen I and tubulointerstitial picrosirius red stain for control (ctrl), control + linagliptin (ctrl + lina), diabetic (dm), diabetic + linagliptin (dm + lina) and diabetic + telmisartan (dm + tel) groups are shown (Magnification=original X 200). Quantification was done using Image J. Data are expressed as mean ± SEM with **=P<0.01 vs ctrl, #=P<0.05 vs dm.
6.4 Discussion

These data provide new knowledge on the mechanism by which the DPP4 inhibitor, linagliptin, reduces active TGFβ1 and downstream renal fibrotic markers. An important aspect of this novel finding is that the interaction between CIM6PR and DPP4 is “switched on” by high glucose, and hence is maximally modulated by linagliptin in this environment. In the presence of excess M6P, the CIM6PR binding sites become saturated, resulting in the reduction in CIM6PR/DPP4 interaction, which would suggest that the interaction is occurring through a M6P residue on the DPP4 molecule. The fact that linagliptin also reduced this interaction, suggests a M6P mediated mechanism which is independent of GLP-1 as our in vitro system is lacking in GLP-1. This finding is also confirmed in our in vivo model of diabetic nephropathy. Linagliptin was able to reduce renal cortical fibronectin transcription and tubular pSmad2/3 expression on immunohistochemistry. We also noted a trend towards reduction in pSmad2 expression on western blot analysis of whole kidney tissue. These findings are suggestive of inhibition of active TGFβ1 signaling. There was also a concomitant trend towards a reduction in tubular atrophy. Importantly this anti-fibrotic trend was independent of glucose or blood pressure lowering.

The renal effects of DPP4 inhibition have been previously explored in animal models of Type 1 and Type 2 diabetes. Both studies that have looked at the effect of DPP4 inhibition (using vildagliptin and sitagliptin) show renoprotection. However the HbA1c in the DPP4 inhibitor treated diabetic animals was lower than in the diabetic control animals [4, 177]. So in both of these in vivo studies it is difficult to conclude that the renal effects of DPP4 inhibition are independent of glucose lowering.
Kanasaki et al investigated the antifibrotic effect of linagliptin in a type 1 model of diabetic nephropathy. This study demonstrates that linagliptin after 4 weeks ameliorated diabetic kidney fibrosis independent of glucose lowering and this was in association with the inhibition of endothelial–mesenchymal transition and the restoration of microRNA29a/b/c [189]. Like this study, the advantage of our experimental design is ensuring that the glucose levels are matched so the renal findings are not because of expected changes one would see with glucose lowering itself. In contrast to Kanasaki et al our study has a much longer duration of treatment (20 weeks) with linagliptin in the diabetic mice.

The potential advantage over renin-angiotensin blockade such as telmisartan or other anti-fibrotic therapies targeting TGFβ1, such as monoclonal antibodies, is the finding that CIM6PR and DPP4 interaction seems to be occurring selectively in context of high glucose and hence maximally modulated by linagliptin during hyperglycaemia. Specifically targeting TGFβ1 utilizing antibodies is challenging clinically because of the complexity of its biological role in various organs. Non-selective targeting of TGFβ is unlikely to be a promising therapeutic strategy as TGFβ1 knockout is a lethal phenotype [201] and drugs that non-specifically target TGFβ1 including monoclonal antibodies result in cancer, inflammation and autoimmune disease. Hence more targeted therapies are required. One strategy to overcome this is to exploit the fact that different cells activate latent TGFβ1 using different proteins. For example, unlike the kidney proximal tubular cells, the immune system regulates TGFβ1 through integrins rather than CIM6PR [202], highlighting the potential for DPP4i as antifibrotic in a high glucose environment being limited to CIM6PR dependent TGFβ1 activation. In terms of using M6P analogues directly to achieve the same
effect as linagliptin, their use is currently limited by a short half-life, low bioavailability and poor receptor binding [203, 204].

Although our study suggests that linagliptin has anti-TGFβ1 signaling effects in the kidney, we did not see any significant change in the glomerulosclerotic index or tubulointerstitial collagen deposition and albuminuria, which reflects the multifactorial aetiology of diabetic nephropathy. The polyol pathway is activated in cells such as the proximal tubular cells where glucose entry is independent of insulin. So, this finding is in keeping with our hypothesis and previous findings that DPP4 inhibition is more likely to alter extracellular mediators such as TGFβ1 but not the intracellular regulation induced by hyperglycaemia [195]. Hence we would propose that effective treatments to limit diabetic nephropathy necessarily involve targeting multiple pathophysiological pathways, which include inhibition of membrane bound DPP4.

Our study has a few limitations. We have shown indirectly rather than directly how linagliptin influences the interaction between CIM6PR and DPP4. Linagliptin binds to an internalised site in the enzyme and removed from the M6P moiety on the DPP4 molecule and does not result in significant conformational change (communication from Boehringer-Ingelheim, Germany). So although our data support a M6P related mechanism, this could be through inhibiting either an enzymatic or non-enzymatic property of DPP4. Similar findings were published by Ishibashi et al where blocking the interaction of DPP-4 with M6P/IGF-IIR by the addition of excess amount of free M6P or M6P/IGF-IIR-Ab completely inhibited the DPP-4-induced increase in superoxide generation in HUVECs [205]. It is also important to appreciate that whilst
our *in vitro* data supports a non GLP-1 mediated mechanism, when linagliptin is administered *in vivo* it results in raised GLP-1 levels as expected. Hence our findings do not delineate between a GLP-1 and non GLP-1 mediated mechanism. However, this is mechanistically irrelevant when considering human application.

In summary, we propose novel mechanistic data that add to the existing body of knowledge that DPP4 inhibition with linagliptin can inhibit the TGFβ1 related fibrotic pathway in diabetic nephropathy. Clinical trials are in progress to determine whether linagliptin impacts on renal and cardiovascular (ClinicalTrials.gov Identifier: NCT01897532) outcomes. If this is also borne out in clinical trials, DPP4 inhibition with linagliptin may have an added advantage in those at risk of diabetic nephropathy.
Chapter 7: Saxagliptin reduces renal tubulointerstitial inflammation, hypertrophy and fibrosis in diabetes.

Gangadharan Komala M, Gross S, Zaky A, Pollock C, Panchapakesan U.

Abstract

Aim
In addition to lowering blood glucose in patients with type 2 diabetes mellitus, dipeptidyl peptidase 4 inhibitors (DPP4i) have been shown to be antifibrotic and anti-inflammatory. We have previously shown that DPP4 inhibition in human kidney proximal tubular cells exposed to high glucose reduced fibrotic and inflammatory markers. Hence we wanted to demonstrate renoprotection in an in vivo model.

Methods
We used a type 1 diabetic animal model to explore the renoprotective potential of saxagliptin independent of glucose lowering. We induced diabetes in eNOS -/- mice using streptozotocin and matched glucose levels using insulin. Diabetic mice were treated with saxagliptin and outcomes compared to untreated diabetic mice.

Results
We provide novel data that saxagliptin limits renal hypertrophy, TGFβ related fibrosis and NF-κBp65 mediated macrophage infiltration. Overall there was a reduction in histological markers of tubulointerstitial fibrosis. There was no reduction in albuminuria or glomerulosclerosis.

Conclusion
Our findings highlight the potential of DPP4 inhibition as additional therapy in addressing the multiple pathways to achieve renoprotection in diabetic nephropathy.
7.1 Introduction

Newer glucose lowering agents increasingly used in the treatment of patients with Type 2 diabetes mellitus target the incretin system. These drugs include the glucagon-like peptide 1 (GLP-1) analogues and (DPP4i). The incretin hormone GLP-1 promotes insulin release and inhibits glucagon secretion in a glucose dependent manner, resulting in the regulation of glucose after meals. Although DPP4i were originally designed to lower blood glucose by raising GLP-1 levels, they have pleiotropic actions by virtue of their ability to cleave peptides that have a proline/alanine at the penultimate position in the amino terminal end.

DPP4 is a serine exopeptidase belonging to the S9B protein family, members of which cleave X-proline dipeptides from the N-terminus of polypeptides, such as chemokines, neuropeptides, and peptide hormones [193]. It is a 110-kDa type 11 integral membrane glycoprotein and is expressed ubiquitously in most organs and cell types. Importantly, DPP4 is therefore able to cleave a host of other peptides and exert pleiotropic effects independently of GLP-1. Additionally, DPP4 exists as 2 forms (soluble and membrane bound) both of which are capable of proteolytic activity. The soluble form in the circulation is thought to arise from shedding of the membrane forms and is responsible for the glucose lowering effect of the DPP4i in clinical use. In contrast, the membrane bound form of DPP4 expressed on the surface of many cell types including the apical/brush border surface of the kidney proximal tubular cell (PTC) [206], endothelial cells and T cells [194] is of major interest with respect to the pleiotropic actions of DPP4. We have recently shown in human kidney proximal tubular cells exposed to high glucose that linagliptin reduces activation of TGFβ and
fibronectin production[195], which is likely due to a cation independent mannose 6 phosphate receptor related mechanism. Hence DPP4 also possess non-enzymatic properties and are ideally suited to be used as antifibrotic agents in both type 1 and type 2 diabetes mellitus. This concept is highly attractive given that a third of patients with diabetes suffer kidney complications such as diabetic nephropathy requiring renal replacement therapy.

DPP4i differ significantly in their pharmacokinetic profiles. While the different routes of clearance may not be immediately relevant to glucose lowering effects, it may be directly relevant for renal protection. A DPP4 inhibitor that is not filtered and cleared by the kidneys may not gain access to luminal DPP4 that is bound to the brush border membrane of the proximal tubular cells. For this reason, we chose to use saxagliptin in our experiments, a DPP4 inhibitor which is filtered and excreted by the kidneys.

An important concept in interpreting studies to date evaluating renoprotective benefits with DPP4 inhibition is the confounding effect of concomitant glucose lowering. There are two studies that have looked at the effect of DPP4 inhibition (using vildagliptin and sitagliptin) on the diabetic animal kidney and both show renoprotection. Liu et al reported that vildagliptin was renoprotective with a reduction in albuminuria and improvement in the histological changes in the kidney, which were associated with reduced DPP4 activity and raised GLP-1 levels. The authors concluded that these changes were probably not attributable to the hypoglycaemic effect of vildagliptin. However the HBA1c in the diabetic group was 12.1% (compared to 4.7% in the control group) and the diabetic plus vildagliptin 8mg/kg/day was 10.4%, which was slightly lower than the diabetic group although
this was not significant [177]. We know from the UKPDS study that even small changes in HBA1c levels can lead to significant differences in the development in diabetic microvascular complications [154]. The other study by Mega et al, done in a type 2 animal model of diabetes (Zucker diabetic fatty rat), also showed that DPP4 inhibition (chronic low-dose sitagliptin treatment) was able to ameliorate diabetic nephropathy but once again the changes were associated with a significant reduction of HBA1c levels in the obese diabetic sitagliptin treated group (HBA1c 10.9% vs 9.1% in the diabetic alone group) [4]. So in both these in vivo studies it is difficult to conclude that the renal effects of DPP4 inhibition lie above and beyond glucose lowering. The advantage of our experimental design is ensuring that the glucose levels are matched to remove this confounding variable when interpreting whether an independent renoprotective effect exists.

7.2 Materials and methods

7.2.1 Animal model

All animal use and welfare adhered to the national Institute of Health Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the Institutional Ethics Committee at The Royal North Shore Hospital (Protocol 1203-009A). Animal studies followed the 'Principles of Laboratory animal care' (NIH publication Volume 25, No. 28 revised 1996). We used male endothelial nitric oxide synthase knockout mice (eNOS -/-) as they have been shown to significantly reproduce changes of diabetic nephropathy. We used saxagliptin (provided by BMS and at the recommended dose of 10mg/kg per day via oral gavage) as the DPP4
inhibitor in our studies. Current “best practice” for renoprotection rests with administration of an agent that blocks the renin-angiotensin-aldosterone system (RAAS). Hence we included a comparator with telmisartan (3 mg/kg/day in drinking water), a dose we have previously shown does not induce lowering of BP [7]. Mice were allocated to groups as shown below and sacrificed at 24 weeks post induction of diabetes. Mice were given intraperitoneal injections of streptozotocin (STZ 55 mg/kg/day; Sigma, St Louis, MO) for 5 consecutive days at 7-8 weeks of age. This is the standard low dose STZ protocol validated by the Animal Models of Diabetic Complications Consortium (AMDCC). Blood glucose was tested using a glucometer (Accuchek Nano, Roche) one week after STZ through tail vein blood collection. Diabetes was defined by blood glucose greater than 16 mmol/L after a six-hour daytime fast. Mice with blood glucose levels below 16 mmol/L were excluded from the study. Treatment with saxagliptin and telmisartan commenced at week 12 of age once diabetes was established. Fasting blood glucose levels were measured monthly. Long acting insulin (Insulin Glargine, Sanofi Aventis, Australia) was initiated as required from 10 weeks of age and was administered thrice weekly if the blood sugar exceeded 28 mmol/L or if they had lost weight greater than 25% from baseline. HBA1c was measured in mouse whole blood terminally using calorimetric method (Crystal Chem, USA). There was no difference in overall insulin requirements among the three diabetic groups. The aim was to match glycaemia, maintain body weight and avoid ketonuria without achieving euglycaemia. In all studies the glycaemic control of the diabetic animals was matched to assess specific renal effects of saxagliptin independent of glycaemic control. The groups were as below:

(i) Non - Diabetic (control): 12

(ii) Non - Diabetic (control) with saxagliptin: 8
(iii) Diabetic (control): 12
(iv) Diabetic with saxagliptin: 8
(v) Diabetic with telmisartan: 9

7.2.2 Measurement of physiological parameters

Body weight was assessed monthly. Blood pressure was measured using a noninvasive tail vein cuff method (CODA BP apparatus, Kent Scientific, USA) preterminally.

7.2.3 Urine biochemistry

Urine was collected at two different time points (4-6 weeks after initiation of treatment using metabolic cages and terminally using bladder puncture). Urine creatinine was measured using a picric acid method (Creatinine Companion, Exocell Inc., USA). Urinary glucose was measured using Abbot Architect C16000 analyser. Urine albumin was measured using Elisa (Albuwel, Exocell Inc., USA).

7.2.4 Kidney tissue harvest

The unperfused left kidney was harvested and snap frozen after embedding in OCT compound. The right kidney was perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) and subsequently fixed in 10% neutral buffered formalin for 24-48 hours.
7.2.5 Histology

Formalin fixed paraffin embedded (FFPE) kidney sections were stained with Masson’s Trichrome, Picrosirius red and Periodic Acid Schiff. Assessment of histological change was done in a blinded manner. The Glomerulosclerotic index (GSI) was calculated based on GSI = [(1 x N1) + (2 x N2) + (3 x N3) + (4 x N4)]/(N0 + N1 + N2 + N3 + N4), where N is the number of glomeruli with each given score for a given section. Immunohistochemistry for nuclear pSmad 2/3 was done on 4 micron paraffin embedded sections using goat anti mouse pSmad 2/3 (SC11769-G, Santacruz Biotechnology, USA) after an overnight incubation at a concentration of 1:100 followed by donkey anti goat HRP tagged secondary antibody (Santacruz, USA at a concentration of 1:100). The chromogenic reaction was carried out with 3, 3'-diaminobenzidine chromogen (Dako, Australia) solution for 10 minutes. F4/80 assessment was done on frozen sections and anti-F4/80 was obtained from AbD Serotec (Oxford, U.K.) The degree of interstitial collagen content in Masson and Picrosirius stains were assessed in a blinded manner using Image J by identifying the percentage of interstitial collagen positive regions at X 200 magnification in 20 randomly selected regions. The number of cells positive for F4/80 were counted in 20 fields at X400 magnification. Cells positive for nuclear pSmad 2/3 and nuclear NF-κBp65 were counted in 20 random fields at X200 magnification.

7.2.6 Western blot analysis

Frozen tissue was homogenized with Quiagen Tissue Ruptur in 1.5 ml of cold 20mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210mM mannitol, 70mM sucrose
and centrifuged at 1,500 x g for 5 min at 4°C. Samples were then analysed by SDS gel electrophoresis (Novex, Life technologies, Australia) and electroblotted to Hybond Nitrocellulose membranes (Amersham Pharmacia Biotech, Bucks, UK). Membranes were then probed with pSmad2 (Ser465/467) antibodies (#3101, Cell Signaling Technology, USA) followed by HRP tagged anti rabbit antibody (Cell Signaling Technology, USA). Membranes were stripped and probed for actin (Goat anti mouse, Santa Cruz). Proteins were visualized using Luminata Western HRP Substrate (Millipore) in a LAS 4000 image reader (GE Healthcare Life Sciences). Analysis was performed using Image J software (NIH, USA).

7.2.7 Statistical analysis

Statistical analysis was done using GraphPad Prism 6. Data are expressed as mean ± standard error of mean. A P value < 0.05 was considered statistically significant. Blood sugar profile during the study was measured using two way repeated measures ANOVA. ANOVA with Bonferroni’s correction was used for all other statistical analysis.
7.3 Results

7.3.1 Blood glucose levels in diabetic mice

All diabetic mice had significantly elevated fasting blood glucose compared to control mice (P<0.01). All the diabetic mice, had matched fasting blood glucose levels independent of treatment. Similarly the average HbA1c levels in diabetic mice in all groups were matched and significantly elevated compared to control mice (P<0.01). This is shown in Table 7.1. This was in the absence of urinary ketonuria (data not shown).

7.3.2 Effect of saxagliptin on albuminuria in diabetic mice

The diabetic mice showed significant elevations in terminal urinary albumin excretion in keeping with diabetic nephropathy compared to control mice (P<0.01). This was not improved by saxagliptin. However telmisartan significantly reduced albuminuria (P< 0.01). These data are summarised in Table 7.1.

7.3.3 Physical parameters

The diabetic mice showed weight loss during the experiment in comparison to significant weight gain by control mice (P<0.01). The diabetic mice treated with saxagliptin gained only minimal weight and were not significantly different to untreated diabetic mice. However diabetic mice treated with telmisartan gained
weight, which was significantly better than diabetic mice (P<0.01). The diabetic mice groups had comparable systolic blood pressure independent of treatment (Table 7.1).

7.3.4 Effect of saxagliptin on diabetic kidney hypertrophy

The diabetic mice showed significant renal hypertrophy as measured by left kidney/body weight ratio compared to control mice (P<0.01). Both saxagliptin (P<0.05) and telmisartan (P<0.01) were able to reduce diabetic renal hypertrophy as signified by the improved kidney/body weight ratio. This is summarised in Table 7.1.
### Table 7.1. Metabolic and physical parameters of mice.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control +saxagliptin</th>
<th>Diabetic</th>
<th>Diabetic +saxagliptin</th>
<th>Diabetic +telmisartan</th>
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<tr>
<td><strong>Gain in weight (gram)</strong></td>
<td>5.83 ± .70</td>
<td>4.95 ± 0.67</td>
<td>-0.68 ± .63**</td>
<td>0.52 ± .60**</td>
<td>2.20 ± .64**</td>
</tr>
<tr>
<td><strong>Left kidney/ body weight ratio (%)</strong></td>
<td>0.84± .05</td>
<td>0.69 ± .03</td>
<td>1.07 ± .06**</td>
<td>0.89 ± .03#</td>
<td>0.83 ± .02##</td>
</tr>
<tr>
<td><strong>Average blood sugar (mmol/L)</strong></td>
<td>9.9 ± 0.1</td>
<td>9.9 ± 0.2</td>
<td>20.9 ± 0.9**</td>
<td>21.7 ± 1.1**</td>
<td>22.4± 1.1**</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>3.8 ± 0.56</td>
<td>4.1 ± 0.43</td>
<td>6.4 ± 0.46**</td>
<td>6.9 ± 0.55**</td>
<td>7.6 ± 0.64**</td>
</tr>
<tr>
<td><strong>Systolic BP</strong></td>
<td>115.6 ± 2.7</td>
<td>117.1 ± 3.4</td>
<td>111.1 ± 4.4</td>
<td>110.4 ± 4.4</td>
<td>101.3 ± 4.9</td>
</tr>
<tr>
<td><strong>24 hour urine albumin excretion (µg/day)</strong></td>
<td>463± 105</td>
<td>497 ± 90</td>
<td>2319 ± 438**</td>
<td>2463 ± 574</td>
<td>766 ± 152##</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM

**= P<0.01 vs control

#=P<0.05 vs diabetic

###=P<0.01 vs diabetic
7.3.5 Effect of saxagliptin in diabetic glomerulosclerosis

The diabetic mice had significant glomerulosclerosis compared to control mice (P<0.01). Saxagliptin did not ameliorate the degree of glomerulosclerosis in diabetic mice. Telmisartan showed a significant improvement in the diabetic animals (P<0.01). These results are shown in Figure 7.1.

Figure 7.1
Figure 7.1. Saxagliptin did not reduce glomerulosclerosis in diabetic mice.

Diabetic mice demonstrated increased glomerulosclerosis, which was improved by telmisartan but not by saxagliptin as demonstrated by quantification of glomerulosclerosis by glomerulosclerotic index. Representative photographs of PAS stained sections for a) ctrl, b) ctrl + saxa, c) dm, d) dm + saxa and e) dm + tel groups are shown (Magnification=original X400). Data are expressed as mean ± SEM with **=P<0.01 vs ctrl, ##=P<0.01 vs dm.
7.3.6 Effect of saxagliptin on diabetic renal tubulointerstitial fibrosis

Diabetic mice had significant tubulointerstitial fibrosis with Masson’s positive collagen staining (P<0.01) and Picrosirius red staining (P<0.01). Treatment with saxagliptin and telmisartan resulted in a significant reduction in collagen deposition. This is shown in Figure 7.2A (Masson’s staining) and 7.2B (Picrosirius red).
Figure 7.2A
Figure 7.2B
Figure 7.2. Saxagliptin improved tubulointerstitial fibrosis in diabetic mice.

(A) Diabetic mice demonstrated increased Masson’s staining which was significantly improved by telmisartan and saxagliptin.

(B) Diabetic mice demonstrated increased sirius positive collagen staining, with telmisartan and saxagliptin showing significant improvement. Representative photographs for a) ctrl, b) ctrl + saxa, c) dm, d) dm + saxa and e) dm + tel groups are shown (Magnification=original X200). Quantification was done using Image J software. Data are expressed as mean ± SEM with **=P<0.01 vs ctrl, #=P<0.05 vs dm and ###=P<0.01 vs dm.
7.3.7 Effect of saxagliptin on tubular pSmad 2/3 expression in diabetic mice (a marker of transforming growth factor beta activation)

Diabetic mice showed significant nuclear expression of pSmad 2/3 (P<0.01 vs control), signifying activation of the downstream fibrotic pathways of TGFβ. Both saxagliptin (P<0.01) and telmisartan (P<0.01) showed a statistically significant reduction in pSmad2/3 expression (Figure 7.3A). Diabetic mice showed significant increase in pSmad2 expression on western blot (P<0.05). Saxagliptin showed a trend towards improvement in diabetic mice (P=0.08). This is shown in Figure 7.3B.
Figure 7.3B

![Graph showing pSmad2 protein expression with actin as control. The graph compares different samples: ctrl, ctrl+saxa, ladder, ctrl+saxa, dm, dm, dm+saxa, dm+tel, and dm+tel. The y-axis represents pSmad2 protein expression, while the x-axis lists the different sample groups. A bar graph illustrates the protein expression levels with error bars indicating variability. The dm sample group shows significantly higher expression compared to the ctrl group, marked with an asterisk (*).]
Figure 7.3. Saxagliptin reduced tubular nuclear pSmad 2/3 expression (a marker of transforming growth factor beta activation) to control levels in diabetic mice. Western blot showed a trend towards improvement in pSmad2 expression in diabetic mice with saxagliptin.

A) Diabetic mice demonstrated significantly increased pSmad2/3 nuclear expression with immunohistochemistry, which was significantly reduced to control levels by saxagliptin and telmisartan. Representative photographs in a) ctrl, b) ctrl + saxa, c) dm, d) dm + saxa, e) dm + tel groups are shown (magnification X200). Quantification was done by counting the number of positive nuclei in 20 random fields at X200 magnification.

B) Western blotting showed a significant increase in pSmad2 expression in diabetic mice with saxagliptin showing a trend towards improvement (P=0.08 vs dm). Quantification of western blot was done using Image J software. Data are expressed as mean ± SEM with *=P<0.05 vs ctrl, **=P<0.01 vs ctrl, ##=P<0.01 vs dm.
7.3.8 Effect of saxagliptin on fibronectin expression in diabetic mice

Diabetic mice showed significant tubulointerstitial fibronectin expression signifying extracellular matrix deposition (P<0.01 vs control). Both saxagliptin and telmisartan showed significant improvement in fibronectin expression in diabetic mice (P<0.01) (Figure 7.4).

Figure 7.4
Figure 7.4. Saxagliptin reduced fibronectin expression in diabetic mice. Diabetic mice demonstrated a significant increase in tubulointerstitial FN expression with immunohistochemistry and there was significant improvement with saxagliptin and telmisartan. Data are expressed as mean ± SEM. Representative photographs in a) ctrl, b) ctrl + saxa, c) dm, d) dm + saxa, e) dm + tel groups are shown (magnification X200). Quantification was done using Image J software. Data are expressed as mean ± SEM with **=P<0.01 vs ctrl, ##=P<0.01 vs dm.
7.3.9 Effect of saxagliptin on nuclear NF-κBp65 subunit translocation in diabetic mice

Diabetic mice had a significant increase (P<0.01) in NF-κBp65 subunit translocation into the nucleus of proximal tubular cells indicating activation of tubular inflammation. Both saxagliptin and telmisartan reduced this significantly (P<0.01; Figure 7.5).

Figure 7.5

![Image of nuclear NF-κBp65 subunit translocation in diabetic mice](image_url)
Figure 7.5. Saxagliptin reduced nuclear NF-κBp65 subunit translocation to control levels in diabetic mice. Diabetic mice showed significantly increased tubulointerstitial NF-κBp65 nuclear expression and this was significantly reduced with saxagliptin and telmisartan. Representative photographs in a) ctrl, b) ctrl + saxa, c) dm, d) dm + saxa, e) dm + tel groups are shown with positive nuclei shown using arrows (magnification X400). Quantification was done by counting the number of positive nuclei in 20 random fields at X200 magnification. Positive nuclei are shown with arrows. Data are expressed as mean ± SEM with **=P<0.01 vs ctrl, ##=P<0.01 vs dm.
7.3.10 Effect of saxagliptin on expression of F4/80 positive macrophages in tubulointerstitium of diabetic mice

Diabetic mice had a significant increase (P<0.05) in F4/80 expression, which is a macrophage marker in diabetic mice. Saxagliptin reduced this significantly and this result is in keeping with the NF-κBp65 reduction (P<0.05; Figure 7.6).

Figure 7.6
Figure 7.6. Saxagliptin reduced the number of F4/80 cell to control levels in diabetic mice. Diabetic mice showed significantly increased tubulointerstitial F4/80 positive macrophages and this was significantly reduced with saxagliptin. Representative photographs in a) ctrl, b) ctrl + saxa, c) dm, d) dm + saxa, e) dm + tel groups are shown (magnification X400). Quantification was done by counting the number of positive cells in 20 random fields at X400 magnification. Data are expressed as mean ± SEM with *=P<0.05 vs ctrl, #=P<0.05 vs dm.
7.4 Discussion

We provide novel data that saxagliptin limits renal hypertrophy, TGFβ related fibrosis and NF-κBp65 mediated macrophage infiltration in an animal model of diabetic nephropathy. There was a reduction in histological markers of tubulointerstitial fibrosis. It has been well described that glomerular and tubulointerstitial fibrosis occur sequentially and although there was no improvement in glomerulosclerosis in our model, the improvement in the tubulointerstitial markers may reflect the effect of DPP4 inhibition on filtered luminal profibrotic and proinflammatory mediators. In contrast to previously published literature, we have specifically designed our studies to show that these renal effects are independent of glucose lowering. As DPP4i are used clinically as oral glucose lowering agents in patients with type 2 diabetes mellitus the potential for these agents to have additional renoprotective effects makes them attractive therapeutic agents applicable to all patients with type 2 diabetes mellitus.

There are several DPP4i available clinically and in context of the potential to prevent or reduce the burden of diabetic nephropathy it is important to appreciate the differences in terms of pharmacokinetics. Linagliptin has the advantage of being useful in patients with unstable renal impairment, as it is not predominantly cleared by the kidneys. In contrast, saxagliptin is freely filtered at the level of the kidney glomeruli and hence is able to access luminal membrane DPP4 which is abundantly expressed at the proximal tubular cell.
In our animal model of diabetic nephropathy, we demonstrate that saxagliptin reversed several typical changes of diabetic nephropathy such as renal hypertrophy, fibrosis and inflammation, an effect that was also seen with telmisartan (current best practice therapy in diabetic nephropathy). Given the nature of the DPP4 enzyme, it is likely that the mechanism involves several putative peptides rather than one well defined pathway. In addition our model does not distinguish whether the renal benefits are mediated by or independent of GLP-1 acting at receptors in the kidney cells or vasculature. Apart from cleaving putative substrates, membrane bound DPP4 can interact with other cell membrane proteins to signal intracellularly or extracellularly. We have previously published that DPP4 interacts with membrane cation independent mannose 6 phosphate receptor which is involved in the TGFβ activation process which is GLP-1 independent [195, 207]. This mechanism is in keeping with our in vivo data showing a significant reduction in pSmad2/3 expression resulting in a reduction of fibronectin expression.

High mobility group box protein 1 (HMGB1) was identified as a DPP4 substrate by Marchetti et al several years ago with cleavage altering its biological function [182]. HMGB1 is a well described proinflammatory cytokine activating NF-κB in diabetes and has been shown to positively correlate with macro and microalbuminuria in patients with Type 1 diabetes [208]. We have previously shown in human kidney proximal tubular cells that DPP4 inhibition significantly reduced HMGB1 induced NF-κB activation. The mechanism of this is unclear as although HMGB1 is a substrate of DPP4, we showed no alteration in the cleaved product in our in vitro experiments [195]. It has been reported that saxagliptin enhances endothelial nitric oxide release resulting in lowering of blood pressure and inflammation [209].
However this effect is unlikely in our model given that the animals are eNOS knockout mice. This reduction in NF-κB activation has been reproduced in our in vivo studies, which supports existing literature [210].

From a mechanistic perspective it is likely that the pleiotropic effects of DPP4 inhibition are a combination of its enzymatic and non-enzymatic properties on several putative substrates and signaling pathways. It is also likely that the net effect of DPP4 inhibition is dependent on the disease process and the extracellular substrate milieu and may explain our discordant findings, as we did not see a reduction in the glomerulosclerotic index or albuminuria.

In summary we provide novel data that saxagliptin reduces renal hypertrophy, TGFβ related fibrosis and NF-κBp65 mediated macrophage infiltration in a model of diabetic nephropathy independent of glucose lowering. Although there was no reduction in overall glomerulosclerosis or albuminuria, the improvement in tubulointerstitial profibrotic and inflammatory markers highlights the potential of DPP4 inhibition as additional therapy in addressing the multiple pathways to achieve renoprotection in diabetic nephropathy.
Chapter 8: Summary and future directions
8.1 Summary

Diabetic nephropathy is the commonest cause of progressive kidney disease and end stage kidney disease requiring dialysis in the world. The role of angiotensin converting enzyme inhibitors (ACE inhibitors) and angiotensin receptor blockers (ARBs) in attenuating diabetic nephropathy independent of their anti-hypertensive effects, have been proven through multiple trials over the last two decades [211-213]. Current treatment is modest at best and more effective therapeutic measures are critical to prevent kidney failure and dialysis in this population. The focus has shifted to interventions, which go beyond blood glucose control, anti hypertensive management and blockade of the renin angiotensin pathway in preventing diabetic nephropathy.

The most important therapeutic developments in diabetes over the last decade have been the evolution of DPP4i and SGLT2i. Both these drugs are oral hypoglycaemic agents that are safe to use in mild to moderate kidney disease. Indeed DPP4i retain significant efficacy in patients with ESKD, whereas as renal function declines the glucose lowering potential for SGLT2i is reduced. There have been significant advances and consequent evolution of knowledge in this area since the commencement of this PhD, which will be discussed later on in this chapter. The roles of both SGLT1 and SGLT2 in glucose metabolism in the kidney were reviewed in chapter 2. This Chapter has been peer reviewed and published in Current Opinion in Nephrology and Hypertension (Sodium glucose cotransporter 2 and the diabetic kidney) [5]. This review examined the predominant role of SGLT2 in reabsorbing most of the filtered glucose from the luminal filtrate and also looked at the role of the
proximal tubule (PT) in the pathogenesis of diabetic nephropathy. The early and enhanced glucose reabsorption by the PT in states of hyperglycaemia and its contribution to glomerular hyperfiltration and tubular glycotoxicity was discussed. This chapter explored the role of SGLT2 inhibitors in preventing the initial physiological changes of diabetic nephropathy that was available at the time of commencement of this PhD. There were concerns regarding increased incidence of bladder and breast cancer with these agents although these fears are currently unfounded after clinical use of these drugs began over the last three years.

The content of Chapter 3 has been peer reviewed and published in Expert Review in Clinical Pharmacology (Empagliflozin for the treatment of type 2 diabetes) [6]. Essentially, the clinical and basic scientific data available for empagliflozin, the SGLT2 inhibitor that we used in our studies, was reviewed. Empagliflozin was established as an effective agent to improve glycaemic control with robust evidence from animal studies and phase III human trials. There were no studies examining the renoprotective potential of SGLT2 inhibitors independent of the glucose lowering effect.

The content of Chapter 4 has been peer reviewed and published in PLoS One (Inhibition of kidney proximal tubular glucose reabsorption does not prevent against diabetic nephropathy in type 1 diabetic eNOS knock out mice) [7]. The aim was to determine the role of SGLT2i in renoprotection over and above glycaemic control. This in vivo study was conducted in an endothelial nitric oxide deficient (eNOS -/-), streptozotocin induced type 1 diabetic mouse model. This model is endorsed by the
Animal Models of Diabetic Complications Consortium (AMDCC) and reproducibly develops diabetic kidney nephropathy.

This mouse model developed all clinical characteristics of diabetic nephropathy, namely albuminuria, glomerulosclerosis, tubulointerstitial inflammation and fibrosis. In this study matched comparable high glucose levels were maintained in diabetic mice with the use of insulin. This was the first study designed to ensure that any renal effects that were observed were not confounded by concomitant glucose lowering. Moreover the renoprotective benefits that are demonstrated in the setting of matched glucose levels could also be applicable in chronic kidney disease of non diabetic etiology as the benefits are independent of glucose lowering and reflect alternative protective mechanisms. We demonstrated that while empagliflozin was effective in reducing insulin requirements in diabetic mice for the same matched high glucose levels, it did not ameliorate diabetic nephropathy in our model. In contrast diabetic mice treated with telmisartan, which is a current standard treatment for patients with diabetic nephropathy, demonstrated renoprotection. This study demonstrated that inhibition of proximal tubular glucose reabsorption was not associated with improvement in renal outcomes in the setting of persistently high blood glucose. A study by Vallon et al published in 2013 showed that empagliflozin improved diabetic nephropathy in a mouse model in accordance with improved glycaemic parameters reinforcing the well known fact that glycaemic control is essential to limit diabetic nephropathy [131]. Our study uniquely demonstrated that empagliflozin did not have renoprotective benefits independent of glucose lowering. However, it has to be noted that the eNOS −/- diabetic mouse model does not develop hyperfiltration [214]. Hence this study was not designed to evaluate the renal effects of empagliflozin on
glomerular hyperfiltration and hence the results obtained may have been limited by the model studied.

Since publication of these findings, Cherney et al showed that by limiting proximal tubular sodium chloride (NaCl) reabsorption and hence increased distal delivery of NaCl, SGLT2i activate tubuloglomerular feedback with a consequent reduction in hyperfiltration [215]. It has also been shown that the sodium hydrogen exchanger 3 (NHE3) in the proximal tubule is closely associated with SGLT2 and SGLT2 mediated glucose uptake regulates NHE3 activity [216]. Hence reducing tubular Na reabsorption is a mechanism by which SGLT2i can have a positive impact on systemic hypertension in addition to reducing glomerular hyperfiltration by activation of tubuloglomerular feedback. Most importantly, in the recently published EMPA-REG study, it has been shown that empagliflozin improved cardiovascular mortality in patients with diabetes and established cardiovascular disease compared to placebo [217]. This was observed in with the context of better glycaemic control. Whilst the absolute renoprotective benefits of SGLT2i may not be apparent in the setting of poorly controlled diabetes in an animal model, the overall long term cardiovascular and potentially renal benefits have thus been shown in humans. The EMPA-REG study was not powered to demonstrate renoprotective benefits, although a reduction in hard renal endpoints and a slowing in the progression of renal deterioration compared to the control group has been reported recently [218]. The CREDENCE study is designed to specifically determine whether the SGLT2i canagliflozin is renoprotective. It is currently recruiting patients with macroalbuminuria and various degrees of renal dysfunction (clinicaltrials.gov identifier: NCT02065791). This trial aims to recruit nearly 4200 patients who will be assigned to either canagliflozin 100
mg or placebo. It is important to appreciate that although SGLT2i block glucose reuptake and glycotoxicity at the level of the PTC, our study and the rest of the literature demonstrate that the inhibition of sodium reabsorption is more likely to account for the cardiovascular risk reduction and renoprotective effects [219].

As alluded to above, DPP4i, which act via the gut based incretin pathway to sustain postprandial insulin secretion are increasingly used as second line therapy after metformin in patients with type 2 diabetes mellitus to improve glycaemic control. Chapter 5 reviews the available evidence regarding DPP4i in preventing diabetic nephropathy. DPP4 inhibition is potentially able to exert renoprotective effects through GLP-1 mediated and GLP-1 independent pathways. In addition, the enzymatic and non enzymatic role of DPP4i in reducing diabetic nephropathy in animal models was summarised. Our group has previously established that DPP4i reduce high glucose induced conversion of latent to active TGFß and therefore limit downstream pSmad2 expression in human kidney proximal tubular cells (HK2 cells) [190]. Our group has also previously demonstrated that cation independent mannose-6-phosphate receptor (CIM6PR) is integral to the activation of TGFß in HK2 cells exposed to high glucose [197]. Given that CIM6PR is a membrane protein that binds mannose-6-phosphate containing proteins such as DPP4, the aim was to study the interaction between membrane bound DPP4 and CIM6PR and the role of DPP4i in preventing this interaction and thereby possibly preventing activation of TGFß, a prominent profibrotic cytokine in diabetic nephropathy. This mechanistic study was conducted in vitro and then validated in vivo. Once again, the animal model was designed to investigate renoprotective effects of DPP4i independent of its effect on glycaemic control. DPP4i differ in the way they bind to DPP4 as well as in their mode
of excretion. DPP4i can be divided into two groups based on whether they establish a covalent (vildagliptin, saxagliptin) or non covalent (sitagliptin, linagliptin and alogliptin) bond with the enzyme [148]. Unlike other DPP4i, linagliptin is not eliminated through the kidneys and so does not require a dose reduction in CKD. However, this also limits the exposure of linagliptin to the proximal tubular brush border membrane, which contains considerable membrane bound DPP4. Given pharmacological differences between DPP4i, 2 different clinically available DPP4i were employed in the in vivo model.

The results presented in Chapter 6 have been peer reviewed and published in PLoS One (Linagliptin limits high glucose induced conversion of latent to active TGFß through interaction with CIM6PR and limits renal tubulointerstitial fibronectin) [8]. It was shown, using a proximity ligation assay, that there was increased interaction between membrane bound DPP4 and CIM6PR when HK2 cells were exposed to high glucose. DPP4 inhibition using linagliptin reduced this interaction, conceptually through a M6P mediated mechanism. This provided mechanistic data for the reduction in high glucose induced TGFß signaling observed in the presence of linagliptin. The biological relevance of these in vitro findings were then determined in a mouse model of diabetic nephropathy. High blood glucose levels were comparable and matched using long acting glargine insulin in linagliptin treated and untreated diabetic mice to demonstrate its benefits beyond its blood glucose lowering effect. Diabetic mice treated with linagliptin demonstrated a reduction in tubulointerstitial pSmad 2/3 expression and fibronectin transcription and protein expression, which are downstream markers of TGFß activation. However there was no improvement in diabetic glomerulosclerosis.
In Chapter 7, a similar in vivo study was undertaken using saxagliptin, a DPP4 inhibitor predominantly eliminated by the kidneys. All data contained in Chapter 7 has been peer reviewed and published (Saxagliptin reduces renal tubulointerstitial inflammation, hypertrophy and fibrosis in diabetes) [9]. There was reduced renal tubulointerstitial inflammation and fibrosis with reduced tubular pSmad 2/3 expression and tubulointerstitial collagen deposition, which are downstream markers of TGFβ activation. Saxagliptin also reduced nuclear NF-κB expression in tubular epithelial cells and tubulointerstitial macrophage infiltration, demonstrating improvement in inflammation associated with diabetic nephropathy. Similar to the study with linagliptin, there was no improvement in diabetic glomerulosclerosis.

Collectively from these two studies on linagliptin and saxagliptin we demonstrated that DPP4i have renoprotective benefits at least in reducing tubulointerstitial fibrosis. The lack of improvement in glomerulosclerosis could be because DPP4 inhibition is more likely to alter extracellular mediators such as TGFβ1 but not the intracellular regulation induced by hyperglycaemia highlighting the need to target multiple pathways concurrently to prevent nephropathy. Our results suggest that the renoprotective benefits of saxagliptin may be greater for linagliptin. We hypothesise that this may be due to greater exposure of the DPP4 inhibitor to the proximal tubular brush border membrane due to the renal excretion of saxagliptin. However, this hypothesis has not been specifically tested in the studies to date.

A reduction in all cause mortality, cardiovascular and renal end points remain the key indicators of the long term benefits of any diabetic medication designed to lower
glucose. The large CARMELINA trial is in progress to determine whether linagliptin impacts on renal and cardiovascular outcomes (clinicaltrials.gov identifier: NCT01897532), and is due to be completed in 2018. This trial aims to recruit 8000 patients with preexisting macrovascular disease and/or impaired renal function looking at major adverse cardiovascular effects (a composite of non-fatal myocardial infarction, non-fatal stroke, and cardiovascular death) as well as renal endpoints including 50% drop in eGFR or end stage kidney disease. Similarly the cardiovascular outcome of linagliptin is being compared to that of glimepride, a sulfonylurea drug in the CAROLINA trial (clinicaltrials.gov identifier: NCT01243424). This is currently the largest head to head trial comparing a DPP4 inhibitor to a sulfonylurea and is aimed at comparing the cardiovascular outcomes of these two classes of anti diabetic medications [220]. The MARLINA trial aims to identify the renal benefit of linagliptin in diabetic patients with suboptimal diabetic control, micro or macroalbuminuria and eGFR greater than 30 ml/min and who are on an angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB). The trial is currently complete and results will inform us of the potential renal benefit in patients with established diabetic nephropathy.

8.2 Future directions

A fixed dose combination of empagliflozin and linagliptin has been shown to have additional glycaemic benefits with improved weight loss and a low risk of hypoglycaemia [221]. Both these drugs have a complementary mode of action as the increased endogenous glucose production (EGP) due to SGLT2 inhibitors is offset by the DPP4i, which reduce glucagon secretion and reduce EGP [222].
In this postgenomic era, therapy is shifting towards gene therapy. ISIS 388626 is an anti sense oligonucleotide (ASO) that targets human SGLT2 mRNA. Clinical trials are currently looking at the outcome of this ASO in the management of diabetes. In preclinical trials it has shown to reduce SGLT2 expression in a dose dependent manner in animal models without any toxic effects [223]. ASOs targeting glucagon receptors are in preclinical trials as they reduce the hepatic glucose production and can reduce insulin requirement (information from Ionis Pharmaceutical website).

The treatment of diabetes and diabetic complications is entering an interesting era with newer therapeutic approaches such as gene therapy and significant clinical trial outcomes expected in the foreseeable future. Concurrent evaluation of short-term efficacy in lowering glucose as well as the longer term impacts on cardiovascular and renal endpoints are essential to improve the short-term and longer terms co-morbidity associated with diabetes mellitus.
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