General introduction and outline of the thesis

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The International Diabetes Federation estimates that one in 11 adults (425 million) is living with diagnosed diabetes, predominantly type 2 diabetes mellitus (T2DM). The prevalence is increasing at such a fast rate that statistical and demographic forecasts have consistently underestimated the trend. Patients with diabetes are at increased risk of developing macrovascular and microvascular complications, which cause physical and psychological distress and result in considerable socioeconomic pressure on affected individuals as well as overwhelming costs to global health-care systems.

Fortunately, management of T2DM and prevention of complications has improved substantially over the past 20 years. Control of modifiable risk factors (such as hyperglycaemia, obesity, hypertension, glomerular hyperfiltration, albuminuria, dyslipidaemia and smoking), and widespread use of renin–angiotensin–aldosterone system (RAAS) inhibitors, statins and platelet inhibitors, have resulted in a more optimistic outlook for patients. The available data from cohort studies (mostly in high-income countries) suggest that the incidences of T2DM-related myocardial infarction, stroke, amputations and mortality have decreased by >50%. The incidence of T2DM-related end-stage renal disease (ESRD) has, however, decreased by only 29% — the lowest rate of decline of all the complications examined. Consequently, a persistently high absolute number of patients with diabetes (20 per 10,000) initiate renal replacement therapy (RRT) every year, and diabetes remains the primary cause of chronic kidney disease (CKD) and ESRD, accounting for ~33% of all patients initiating RRT worldwide. Thus, new therapeutic agents or innovative approaches to prevent the onset and progression of diabetic kidney disease (DKD) are urgently needed. Finding new, safe and effective approaches to halt DKD is challenging and a series of therapies and strategies (bardoxolone-methyl, aldose-reductase inhibitors, sulodexide and maximal RAAS inhibition) have failed over the course of drug development, despite encouraging data from preclinical and small clinical studies.

Over the past decade, glucagon-like peptide 1 receptor (GLP-1R) agonists and dipeptidyl-peptidase 4 (DPP-4) inhibitors, the so called ‘incretin-based drugs, have been introduced as a new class of glucose-lowering drugs for the treatment of T2DM. Shortly after Food and Drug Administration approval of the first GLP-1R agonist (exenatide) in the USA in 2005, sporadic case reports described the occurrence of acute kidney injury after treatment initiation. However, these associations have not been supported by subsequent large database analyses or (ongoing) clinical trials. In contrast, more recent evidence that emerged at the time the studies in this thesis were designed and/or conducted, suggested that incretin-based drugs confer renoprotection beyond glycaemic control. Indeed, in experimental models of diabetes and hypertension, these agents ameliorated histologically verified renal damage and reduced albuminuria. Moreover, in studies in T2DM patients, up to 1 year treatment with a GLP-1R agonist or DPP-4 inhibitor reduced albuminuria, which was independent from changes in HbA1c with the DPP-4 inhibitor linagliptin. Several mechanisms by which incretin-based drugs may affect renal outcome are proposed. First, in clinical trials, GLP-1R agonists and DPP-4 inhibitors reduce blood pressure and improve lipid profiles, while GLP-1R agonists also decrease body weight. Interestingly, preclinical and small-sized studies in humans suggest that these agents have direct renal effects that could lower glomerular hydraulic pressure ($P_{GLO}$) and
Here, we describe the (patho)physiological role of GLP-1 in diabetes and the development of incretin-based drugs. From the role of GLP-1 in the putative gut-renal axis, we next propose direct pathways by which incretin-based drugs agents could favourably affect the kidney.

The entero-endocrine system

In addition to facilitating absorption and maintaining other functions, the gut is increasingly recognized as the largest endocrine organ in the human body. The gut functions as an early warning system that orchestrates a series of complex physiological responses to changes in the external environment. As such, the entero-endocrine system is believed to facilitate the uptake, distribution and disposal of nutrients, while protecting the integrity of the vulnerable intestinal surface and the whole organism through the enteric innate and adaptive immune system.

Sixteen major entero-endocrine cell types have been identified to date; these cells are located throughout the gastrointestinal epithelium from stomach to rectum. In response to specific stimuli from the continuously modified luminal content of the intestine (nutrients, fluid, microorganisms and their products, gastrointestinal secretions and pharmaceuticals), entero-endocrine cells release a cocktail of gut hormones from basolateral secretory granules. Gut-derived hormones can act locally on other cells (including other entero-endocrine cells) or nerve endings or on organs and tissues at remote sites (Figure 1). Locally, gut hormones seem to be involved in barrier function and in food digestion and absorption by regulating intestinal transit, release of digestive enzymes and induction of nutrient transporters. Suggested peripheral effects include actions on the central nervous system (CNS) to regulate food intake (appetite and satiety); as well as on the pancreas (which contains tissues with endocrine and exocrine roles), liver, skeletal muscle, adipose tissue and vascular system to efficiently absorb and dispose of assimilated nutrients; and on the kidney to adjust urinary excretion of fluid and electrolytes according to intake.

Regulation of glucose metabolism

An oral glucose load elicits a much greater increase in insulin secretion than does intravenous glucose administration (Figure 2a), owing to insulinotropic signals from the gastrointestinal tract (hormones and/or glucose-responsive nerves). This phenomenon, which is known as the incretin effect, is responsible for up to ~70% of the overall insulin secretory response after nutrient ingestion in individuals with normal oral glucose tolerance. Two incretin hormones have been identified in the gut: glucose-dependent insulinotropic polypeptide (GIP) is produced by entero-endocrine K cells, which are predominately localized in the upper gastrointestinal tract, whereas GLP-1 is mainly secreted from L cells, which reside throughout the intestine but with increasing abundance towards the distal ileum and colon.

GLP-1 is secreted at low tonic rates in the fasting state and the circulating levels of both incretins increase rapidly (within minutes) and transiently upon food intake. K cells and L cells
are directly stimulated by luminal glucose via sodium-glucose cotransporter (SGLT1), but also secrete incretins in response to other carbohydrates, lipids, proteins, amino acids, bile acids and short-chain fatty acids (Figure 1).44 Given the rapid postprandial release of GLP-1 (plasma levels increase from ~5–10 pmol/l when fasting to 15–50 pmol/l within 15–30 mins after eating) and the fairly small number of L cells in the upper gut, an involvement of indirect hormonal (for example, via GIP, cholecystokinin or leptin) or neural loops in relaying the presence of duodenal nutrients to ileal and colonic L cells has been proposed. In addition to acting via neural pathways, incretins exert their insulinotropic activity, at least partly, via interaction with distinct GIP receptors (GIPRs) and GLP-1Rs, which are highly expressed on pancreatic β-cells. In the presence of stimulatory levels of glucose, binding to these G protein-coupled receptors leads to insulin secretion and promotion of insulin transcription and biosynthesis.
Figure 2. Evidence for the incretin effect and the putative gastrointestinal regulation of urinary sodium excretion. a,b) Incremental pancreatic β-cell secretory responses (assessed using C-peptide, which is a marker of endogenous insulin secretion) to an oral glucose load (50 g in 400 ml) or isoglycaemic intravenous (IV) glucose infusion in (part a) healthy individuals (n = 8) and (part b) patients with type 2 diabetes mellitus (T2DM; n = 14). In both groups, the isoglycaemic glucose infusion mimicked plasma glucose concentration profiles after glucose ingestion. The incretin effect — defined as the difference in the area under the curve of the C-peptide response to oral versus IV glucose — is, however, markedly reduced or absent in patients with T2DM. c,d) An equivalent sodium load is more rapidly excreted by the kidneys when given orally than when given by IV infusion. Relative urinary sodium excretion after an oral compared to IV load of 300 mmol sodium in 2 l volume over 60 min in healthy men (n = 8) who were in normal sodium balance with an intake of 150 mmol sodium per day (part c). Cumulative urinary sodium excretion during a balanced 10 mEq constant sodium intake 24 h before and following a 100 mEq oral or IV sodium load in healthy men (n = 8) (part d). Similar to C-peptide, these findings indicate a stimulatory contribution of the gastrointestinal tract to regulation of urinary sodium excretion — the gut–renal axis. *Denotes statistical significance (p<0.05).

A growing body of evidence indicates effects of incretins on pancreatic α-cells and glucoregulatory extrapancreatic tissues (Figure 3). These findings are not surprising given that GIPRs and GLP-1Rs have been putatively localized in multiple organs and cell types. GLP-1 seems to be a glucose-dependent inhibitor of glucagon release, which accounts for ~22% and ~80% of glucagon suppression in the fasting and postprandial states, respectively, mainly through inhibitory factors released from local β-cells (such as insulin) and/ or δ-cells (such as...
The glucagonostatic effect of GLP-1 might be as important to glucose lowering as its incretin effect. By contrast, under certain circumstances, GIP can stimulate glucagon secretion and thereby antagonize GLP-1 actions.\textsuperscript{50,51}

GLP-1 also seems to be involved in the central regulation of homeostatic feeding, a process of modifying the rewarding value of food depending on bodily requirements, as derived from signalling of ingested and stored nutrients.\textsuperscript{52} GLP-1 dose-dependently enhances satiety signals and reduces appetite resulting in abridged food intake, either directly through GLP-1R stimulation in reward-related brain areas or indirectly via vagal afferents.\textsuperscript{52-54} Furthermore, GLP-1 exerts effects on the gastrointestinal tract that facilitate efficient digestion and contribute to energy homeostasis.\textsuperscript{45,55} These actions include inhibition of gastric emptying rate and small intestinal peristalsis; exocrine secretion of bile acids, digestive enzymes and bicarbonate; and suppression of endogenous glucose production.\textsuperscript{45,56} Finally, GLP-1 seems to contribute to the regulation of nutrient distribution and postprandial energy storage by recruiting the microvasculature to peripheral tissues such as skeletal muscle (Figure 3).\textsuperscript{45,56}

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**Figure 3. Putative actions of glucagon-like peptide 1 (GLP-1).** The best elucidated physiological roles of GLP-1 are those related to pancreatic islet cell function. However, GLP-1 and GLP-1 receptor agonists also have pleiotropic effects on various other tissues and organs, with various potential physiological, pathophysiological and pharmacological implications. VLDL, very low density lipoprotein.
**Incretin-based drugs**

The incretin effect is severely reduced or lost in patients with T2DM (Figure 2b)\(^{57-59}\) and a defective incretin system is a key pathophysiological defect that contributes to glucose intolerance.\(^{60}\) Meta-analyses that included small, heterogeneous studies showed no systematic differences in the circulating concentrations of GIP\(^{61}\) and GLP-1\(^{62}\) between individuals with and those without T2DM; however, individual responses to an oral glucose load varied widely, and could be determined by factors such as sex, age and BMI. The most sufficiently powered study to date, which included 1,462 Danish adults, demonstrated that GLP-1 responses to an oral glucose tolerance test are up to 25% lower in individuals with prediabetes or T2DM than in those with normal glucose regulation.\(^{63}\) Whether a defective incretin system in T2DM is caused by decreased responsiveness of β-cells to GLP-1 and GIP\(^{39}\) or by hyposecretion of incretin hormones remains unclear. Importantly, the insulino tropic response to exogenous GIP administration is completely lost in T2DM,\(^{64}\) whereas a partially preserved, substantial dose-dependent response to GLP-1 is observed.\(^{64,65}\) Hence, most pharmacological efforts for treatment of T2DM are directed at amplification of GLP-1-induced glucose lowering in this population.

**GLP-1 infusion**

Short-term intravenous infusion of GLP-1 at supraphysiological concentrations normalises or near normalises fasting plasma glucose (FPG) and postprandial glucose levels (PPG) for up to 7 days in patients with varying severities of T2DM.\(^{65-67}\) In 2002, a 6-week proof-of-concept study in 19 obese patients with T2DM showed that subcutaneously infused GLP-1 (to achieve plasma levels of ~60–70 pmol/l) reduced the levels of HbA\(_{1c}\) by ~1.3% and FPG by ~4–6 mmol/l from baseline without inducing hypoglycaemia, improved β-cell function and insulin sensitivity, delayed gastric emptying rate and induced weight loss of 1.9 kg.\(^{68}\)

GLP-1 has low stability *in vivo* and continuous infusion to overcome this problem has limited clinical applicability for long-term treatment of T2DM. Circulating GLP-1 is rapidly inactivated (<2 min), primarily by the ubiquitous proteolytic enzyme DPP-4\(^{69,70}\) and to a lesser extent by various neutral endopeptidases and aminopeptidases.\(^{71-73}\) The contribution of the latter may increase in ESRD.\(^{74}\) DPP-4 is a circulating or membrane-bound serine protease found at numerous sites in the body. This enzyme specifically cleaves dipeptides from the amino terminus of oligopeptides or proteins that contain an alanine (as do incretins) or proline residue at position 2, thereby altering (usually inactivating) their biological activity.\(^{17,75}\) The truncated metabolites of incretin hormones produced by DPP-4 cleavage do not stimulate insulin secretion. These findings prompted two strategies to extend the *in vivo* half-life of GLP-1 for T2DM therapy and to maintain incretin activity: the use of GLP-1R agonists that are resistant to DPP-4 cleavage, and the use of inhibitors of DPP-4, which prevent proteolytic degradation and inactivation of endogenously secreted incretins.
**GLP-1R agonists**

Several GLP-1R agonist formulations have been introduced for glucose lowering in T2DM (Table 1). All of these formulations are administered as a subcutaneous injection and are available for combination therapy with oral glucose-lowering agents and basal insulin. These molecules were developed based on human GLP-1 or exendin 4, which is a 39-amino-acid peptide that has 53% homology with GLP-1 and was originally isolated from the saliva of the Gila monster (*Heloderma suspectum*). In 2005, exenatide (twice-daily) became the first clinically approved GLP-1R agonist for treatment of T2DM. This synthetic version of exendin 4 contains an Ala8Gly substitution that confers resistance to degradation by DPP-4. Exenatide and the structurally similar GLP-1R agonist lixisenatide largely overcome DPP-4 inactivation in vivo, but remain subject to renal elimination and so have a half-life of only ~2–4 h. As such, these compounds are classified as short-acting or prandial GLP-1R agonists with short-lived receptor activation. To improve the pharmacokinetics, modified GLP-1 peptides that bind to large carrier molecules (to limit renal clearance) or are co-administered with other chemicals (to delay subcutaneous tissue absorption) have been developed. These long-acting GLP-1R agonists have half-lives of up to a week. They include the once-daily GLP-1 analogue liraglutide (which has 97% amino acid sequence identity to human GLP-1) and the once-weekly compounds albiglutide, dulaglutide and a long-acting release formulation of exenatide, which is formulated within biodegradable polymeric microspheres. In addition, semaglutide, which is structurally related to liraglutide, albeit with higher affinity for albumin, is filed for regulatory approval as a once-weekly injection.

As pharmacokinetic data and clinical experience with GLP-1R agonists in patients with T2DM and CKD are limited, caution or discontinuation is advised when renal function is severely impaired (Table 1). The results of a trial of liraglutide in patients with T2DM on chronic dialysis, and post hoc safety data from a subgroup of 224 patients with T2DM and estimated glomerular filtration rate (eGFR) <30 ml/min/1.73 m² enrolled in the LEADER trial of liraglutide are eagerly awaited.

**Glucose-lowering mechanisms and efficacy**

In general, GLP-1R agonists reduce HbA₁c levels by ~1.0% compared with placebo, however, the reductions achieved depend on the choice of agent, dose, baseline HbA₁c level and background therapy. Differences between the pharmacokinetic and pharmacodynamic profiles of short-acting and long-acting GLP-1R agonists should be considered when choosing the most appropriate agent for individual patients.

Short-acting GLP-1R agonists are dosed pre-prandially, strongly suppress postprandial glucagon levels and substantially retard gastric emptying, which prolongs the rate of glucose entry into the duodenum, blunts absorption of meal-derived glucose, and subsequently diminishes PPG and insulin excursions. As plasma drug concentrations decrease rapidly, their effect in the fasting state and on subsequent meals are modest. By contrast, long-acting GLP-1R agonists more strongly reduce FPG owing to uninterrupted glucose-dependent stimula-
tion of insulin secretion (drug concentrations remain elevated throughout the periods between doses).86,87 Unlike short-acting GLP-1R agonists, long-acting compounds do not substantially interrupt gastric motility after prolonged administration, resulting in less effect on PPG, as reported in a head-to-head trial comparing liraglutide with lixisenatide.88 The notable lack of effect on gastric emptying rate is attributed to tachyphylaxis, which is caused by constant GLP-1R activation that induces tolerance to the drug. Further studies should consider which other GLP-1 effects are subject to tachyphylaxis. As such, the ability of liraglutide to inhibit areas in the CNS involved in hedonic feeding after 10 days of treatment are not sustained after 12 weeks,53 and postprandial glucagon suppression was absent at week 12 with liraglutide89 and was sustained after 3 years of exenatide twice-daily.90

Table 1. The pharmacokinetic properties and clinical use of incretin-based drugs76,91-94

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>Approval (year)</th>
<th>EMA</th>
<th>FDA</th>
<th>Half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short acting GLP-1 receptor agonists (subcutaneous injection)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exenatide</td>
<td>5–10 μg BID</td>
<td>2005</td>
<td>2005</td>
<td>2.4</td>
<td></td>
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<tr>
<td>Lixisenatide</td>
<td>10–20 μg QD</td>
<td>2013</td>
<td>2016</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td><strong>Long-acting GLP-1 receptor agonists (subcutaneous injection)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exenatide</td>
<td>2 mg QW</td>
<td>2011</td>
<td>2012</td>
<td>NS†</td>
<td></td>
</tr>
<tr>
<td>Liraglutide</td>
<td>0.6–1.2–1.8 mg QD</td>
<td>2009</td>
<td>2010</td>
<td>11.6–13.0</td>
<td></td>
</tr>
<tr>
<td>Albuglutide</td>
<td>30–50 mg QW</td>
<td>2014</td>
<td>2014</td>
<td>~120.0</td>
<td></td>
</tr>
<tr>
<td>Dulaglutide</td>
<td>0.75–1.5 mg QW</td>
<td>2014</td>
<td>2014</td>
<td>~112.8</td>
<td></td>
</tr>
<tr>
<td>Semaglutide</td>
<td>0.5–1.0 mg QW</td>
<td>Pending</td>
<td>Pending</td>
<td>165.0–184.0</td>
<td></td>
</tr>
<tr>
<td><strong>DPP-4 inhibitors (oral)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>100 mg QD</td>
<td>2007</td>
<td>2006</td>
<td>~12.4</td>
<td></td>
</tr>
<tr>
<td>Vildagliptin</td>
<td>50 mg BID or 50 mg QD plus SU</td>
<td>2007</td>
<td>2007</td>
<td>~2.0</td>
<td></td>
</tr>
<tr>
<td>Saxagliptin</td>
<td>5 mg QD</td>
<td>2009</td>
<td>2009</td>
<td>~2.5 (metabolite ~3.1)</td>
<td></td>
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<tr>
<td>Alogliptin</td>
<td>25 mg QD</td>
<td>2013</td>
<td>2013</td>
<td>~21.0</td>
<td></td>
</tr>
<tr>
<td>Linagliptin</td>
<td>5 mg QD</td>
<td>2011</td>
<td>2011</td>
<td>~12.0</td>
<td></td>
</tr>
</tbody>
</table>

*Dose may vary in some countries. †The pharmacokinetic profile of exenatide QW is similar to that of exenatide BID, except that subcutaneous absorption is prolonged with the QW formulation. No dose adjustment is necessary in patients with severe renal insufficiency, although liraglutide is not recommended in patients with ESRD. BID, twice-daily; CrCl, creatinine clearance;
**DPP-4 inhibitors**

Five selective and competitive inhibitors of DPP-4 for once-daily oral administration have become available globally since 2006 (Table 1). These agents are licensed as monotherapy or as add-on therapy to other glucose-lowering drug classes for T2DM treatment. DPP-4 inhibitors comprise a diverse group of compounds that can be broadly divided into two groups: agents that mimic the dipeptide structure of DPP-4 substrates (sitagliptin, vildagliptin and saxagliptin); and non-peptidomimetic agents (alogliptin and linagliptin). DPP-4 inhibitors vary in their pharmacokinetic properties and elimination pathways, which determine dosing and might influence clinical usage. Although differences in enzyme selectivity between DPP-4 inhibitors clearly exist in *in vitro* studies, no evidence exists for pleiotropic effects related to off-target inhibition when the drugs are used therapeutically.

<table>
<thead>
<tr>
<th>DPP-4 inhibition (24 h post-dose*)</th>
<th>Elimination</th>
<th>Use in patients with renal insufficiency</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Use in patients with renal insufficiency</strong></td>
<td>Mild (CrCl 50/60–89 ml/min)</td>
<td>Moderate (CrCl 30–50/60 ml/min)</td>
<td>Severe or ESRD (CrCl &lt;30 ml/min)</td>
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<tr>
<td>NA</td>
<td>Mainly renal</td>
<td>No adjustment</td>
<td>Conservative dose escalation</td>
</tr>
<tr>
<td>NA</td>
<td>Mainly renal</td>
<td>No adjustment</td>
<td>No adjustment</td>
</tr>
<tr>
<td>NA</td>
<td>Mainly renal (~10 weeks to fully clear)</td>
<td>No adjustment</td>
<td>Not recommended</td>
</tr>
<tr>
<td>NA</td>
<td>Peptidases and renal 6%, faeces 5%</td>
<td>No adjustment</td>
<td>No adjustment</td>
</tr>
<tr>
<td>NA</td>
<td>Peptidases and renal</td>
<td>No adjustment</td>
<td>No adjustment</td>
</tr>
<tr>
<td>NA</td>
<td>Peptidases and renal</td>
<td>No adjustment</td>
<td>No adjustment</td>
</tr>
<tr>
<td>NA</td>
<td>Peptidases and renal</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>&gt;80%</td>
<td>Renal ~87%; faeces ~13%</td>
<td>No adjustment</td>
<td>Dose reduction (50 mg QD)</td>
</tr>
<tr>
<td>&lt;40%</td>
<td>Renal ~85%; faeces ~15%</td>
<td>No adjustment</td>
<td>Dose reduction (50 mg QD)</td>
</tr>
<tr>
<td>(~80% after 12 h)</td>
<td>Renal 12–29% (renal metabolite 21–52%); faeces 22%</td>
<td>No adjustment</td>
<td>Dose reduction (2.5 mg QD)</td>
</tr>
<tr>
<td>~70%</td>
<td>Renal ~76%; faeces ~13%</td>
<td>No adjustment</td>
<td>Dose reduction (12.5 mg QD)</td>
</tr>
<tr>
<td>~75%</td>
<td>Renal ~5%; faeces ~80%</td>
<td>No adjustment</td>
<td>No adjustment</td>
</tr>
</tbody>
</table>

*Dose may vary in some countries. *-Not recommended is not specified for patients with renal insufficiency.*

DPP-4, dipeptidyl peptidase 4; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; FAPα, fibroblast activation protein-α; GLP-1, glucagon-like peptide 1; NA, not applicable; NS, not specified; SU, sulfonylurea. QD, once-daily; QW, once-weekly.
The kidneys are important for the final elimination of most DPP-4 inhibitors, which involves glomerular filtration and active tubular secretion through unknown mechanisms. Sitagliptin and alogliptin rely almost exclusively on renal clearance, whereas hepatic metabolism (which generates an active metabolite with ~50% potency) contributes to the elimination of saxagliptin, and hydrolysis adds to the elimination of vildagliptin (~60% of which is achieved by DPP-4 itself). By contrast, the main elimination route for linagliptin is biliary excretion. At a therapeutic dose, linagliptin is mostly protein-bound, which minimizes its renal clearance to <6%; this compound does not, therefore, require dose-adjustment for renal impairment. For the other DPP-4 inhibitors, total exposure to the drug increases proportionally to the degree of GFR decline. Although most of these agents are well tolerated in advanced CKD and even in patients with ESRD (in whom the fraction of the dose removed by haemodialysis is small), specific recommendations for appropriate dose reductions according to CKD severity are in place (Table 1).

**Glucose-lowering mechanisms and efficacy**

The glucose-lowering effect of DPP-4 inhibitors is most notably mediated by preventing the postprandial fall of endogenous GLP-1, thereby enhancing and prolonging insulin secretion and glucagon suppression. As such, DPP-4 inhibitors induce a 1.5–3.0-fold increase in circulating levels of biologically active GLP-1. This increase is low in comparison to the therapeutic concentrations of GLP-1R agonists, which are equivalent to an ~10-fold increase in endogenous GLP-1.

In addition to increasing circulating GLP-1 levels, experimental studies in rodents and humans indicate the existence of non-classical glucose-lowering mechanisms of DPP-4 inhibitors. These include inhibition of gut DPP-4 activity, which augments GLP-1-induced activation of autonomic nerves as well as high portal GLP-1 levels that suppress endogenous glucose production; inhibition of pancreatic islet DPP-4 activity, which augments islet cell-produced GLP-1 that directly stimulates insulin and inhibits glucagon secretion; and reduced inactivation of DPP-4 substrates other than GLP-1, which may increase islet cell function and induce additional actions (see below). In contrast to GLP-1R agonists, DPP-4 inhibitors do not delay gastric emptying or increase satiety owing to the relatively low incretin levels that are achieved and/or the antagonizing influences of other DPP-4 substrates. Once DPP-4 is inhibited, glucose lowering plateaus and, consequently, reductions in glucose levels are similar across this drug class without an obvious basis for differentiation regarding efficacy. DPP-4 inhibitors reduce PPG excursions by ~3.0 mmol/l, and FPG levels by 1.0–1.5 mmol/l. In a 2015 meta-analysis of 98 trials of ≥12 weeks duration, DPP-4 inhibitors as monotherapy or as add-on therapy to other oral agents reduced HbA1c levels by 0.77% (95% CI 0.82–0.72%) from a mean baseline of 8.05%.

**Other incretin-independent effects**

DPP-4 is a widely and abundantly expressed multifunctional enzyme that transduces numerous actions as a transmembrane and soluble circulating molecule. The challenge of understanding
DPP-4 action is amplified by the fact that, in addition to GLP-1 and GIP, DPP-4 cleaves many bioactive peptides, including chemokines, neuropeptides and regulatory peptides.\textsuperscript{17,75} Cleavage by DPP-4 can inactivate peptides and/or generate new bioactive moieties, which might impact numerous cell types and organs, including the kidney, theoretically resulting in numerous pleiotropic benefits and risks.\textsuperscript{17,75} Despite a host of preclinical studies that have identified and characterized putative DPP-4 substrates \textit{ex vivo} and in experimental models,\textsuperscript{75} the understanding of key non-incretin substrates with clinically relevant actions remains limited.

### The gut–renal axis

Harnessing the pharmacological properties of gut hormones has sparked interest in the properties and therapeutic potential of entero-endocrine cells in various medical disciplines, including nephrology, as well as in defining other regulatory actions of GLP-1 (and consequently incretin-based drugs) beyond glycaemia.

Entero-endocrine cells seem to contribute to the physiological control of water and electrolyte balance upon meal ingestion by affecting the CNS to adjust thirst and, to a lesser extent mechanisms that affect solute intake; intestinal transport to control fluid and electrolyte absorption and secretion; intracellular and extracellular compartments to dispose the absorbed content; and the kidney to stimulate excretion or reabsorption of fluid and electrolytes. This system is very flexible. For example, consumption of a potassium-rich meal, which often contains more potassium than the total extracellular potassium content, would be potentially lethal if absorbed potassium were to remain in the extracellular fluid.\textsuperscript{100,101} Intracellular redistribution and urinary excretion rapidly removes excessive potassium, particularly in the replete state. As renal electrolyte homeostasis is slowly regulated by circadian rhythms and numerous circulating hormones, a putative rapid-acting gut–renal axis might assist renal solute excretion in response to acute solute ingestion,\textsuperscript{17,101-103} forming a crucial feed-forward loop.

### Effects on tubular transport

Seminal studies that support the concept of the gut–renal axis used similar methodologies to experiments that led to discovery of the incretin effect. For example, depending on sodium balance, an equivalent sodium load is more rapidly excreted by the kidneys when given orally than when administered intravenously in many, but not all, animal and human studies (Figure 2c and 2d).\textsuperscript{37,102,104-106} This result seems to be independent of changes in the levels of circulating atrial natriuretic peptide (ANP) and aldosterone.\textsuperscript{102} In addition to gut-assisted sodium homeostasis, several lines of evidence suggest similar feed-forward loops for potassium\textsuperscript{101-103} and phosphate balance,\textsuperscript{102,103} and perhaps other electrolytes.\textsuperscript{102} The gut has been suggested to directly detect changes in the levels of ingested electrolytes (Figure 1) and couple these changes to release of hormones and/or activation of neural pathways that regulate renal tubular and gastrointestinal transport. Several gut hormones and peptides have been proposed to be effectors of, or have a role in, the gut–renal (natriuretic) axis, including gastrin (via interaction with renal dopamine\textsuperscript{107}), ghrelin, uroguanylin, guanylin, secretin, vasoactive intestinal polypeptide, peptide YY (PYY) and GLP-1.\textsuperscript{102,106}
From a pathophysiological perspective, an impaired gut–renal axis in urinary sodium excretion might contribute to salt-sensitive hypertension. Whether this impairment would involve hyposcretion or reduced receptor signalling of entero-endocrine-cell-derived hormones, or conversely, inability of these signals to suppress antinatriuretic systems (such as the RAAS) in response to a salt load, is unclear. Unravelling the gut–renal axis concept, the mediators and their potential pathophysiological contribution to salt-sensitive hypertension might be important for the development of novel targeted therapies.

**Effects on renal haemodynamics**

Although gut hormones can vary tubular electrolyte handling without any substantial fluctuation in the filtered load, direct or indirect signals from the gastrointestinal tract on postprandial renal haemodynamics can also facilitate renal solute excretion. For example, after ingestion of a high-protein meal, a physiological increase in renal blood flow increases GFR independent of changes in arterial pressure, which enhances the filtered load of circulating solutes.\(^{108,109}\)

This postprandial GFR increase might have an important function, as protein catabolism produces nitrogen waste products (urea, uric acid, ammonia and creatinine) and other metabolites (including phosphates, sulphates and protons) that require renal excretion. Postprandial hyperfiltration might thus be a useful mechanism to rapidly excrete excess or potentially injurious gut-absorbed solutes and catabolic wastes. Numerous gut-derived hormones have been reported to influence renal haemodynamics.\(^{102}\) If total nephron capacity is already maximally used in the fasting state (that is, glomerular hyperfiltration), as regularly seen in patients with T2DM and/or advanced CKD to compensate for loss of renal function,\(^{109}\) the contribution of gut-mediated renal haemodynamic changes to acute solute excretion in the postprandial state may be minimal.

**Role of GLP-1**

An acute oral NaCl load in rats\(^{110,111}\) and an acute water load in humans\(^{112}\) have both been reported to increase total circulating GLP-1 levels within 5 min. However, these experiments had important limitations (such as limited GLP-1 measurements and inadequate control experiments) and other studies in rats\(^{113}\) and humans\(^{114}\) have as yet not confirmed these observations. How L cells (or other GLP-1-producing cells, for example in the hindbrain) could be stimulated by an oral sodium or water load is unknown. Although sodium arising at the luminal side of the L cell may directly trigger GLP-1 secretion (for example, through glucose-coupling via SGLT1 or Na\(^-\)dependent amino acid transporters\(^{44}\)), indirect neural or hormonal stimuli might be more likely given the rapidity of the response (Figure 1).

In line with the role of GLP-1 in the central regulation of feeding,\(^{52}\) peripheral and central GLP-1R agonist administration reduces water and saline intake,\(^{113,115-119}\) which seems to be independent of food intake.\(^{118}\) Conversely, rats injected with the GLP-1R blocker exendin 9 drank more fluid than vehicle-treated rats in dipsogenic and nondipsogenic conditions, suggesting that endogenous GLP-1 tone suppresses drinking behaviour. This effect might
involve GLP-1-producing cells in the nucleus solitarius. In healthy males, GLP-1 infusion decreased *ad libitum* water intake after a salty breakfast by 36%, without affecting serum sodium concentration. In addition to directly affecting drinking behaviour, GLP-1 has been suggested to reduce the need for water consumption by decreasing sodium uptake by the gut, possibly by inhibiting intestinal sodium–hydrogen exchanger isoform 3 (NHE3) activity. In line with this hypothesis, three of the nine volunteers in the study dropped out during GLP-1 infusion owing to osmotic diarrhoea.

GLP-1R is expressed in the kidney, with studies of various quality reporting the presence of a functional receptor in proximal tubular cells and in the renal vasculature (Box 1). Although a study that used the most extensively validated monoclonal antibody for GLP-1R to date showed that GLP-1R was exclusively expressed in the preglomerular vascular smooth muscle cells and juxtaglomerular cells of primate kidneys, uncertainties remain regarding receptor localization. DPP-4 is highly active as a transmembrane molecule in several renal cell types, particularly podocytes and proximal tubular cells.

**Incretin-based drugs and the kidney**

Involvement of GLP-1 in the gut–kidney axis was first suggested in 1996 when exogenous GLP-1 was shown to dose-dependently increase natriuresis and diuresis in rats. GLP-1-induced renal sodium excretion and increased urine flow was confirmed in short-term infusion studies involving nondiabetic and diabetic animals, healthy volunteers and insulin-resistant obese

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**GLP-1**

The majority of glucagon-like peptide 1 (GLP-1) actions in vivo are transduced by the GLP-1 receptor (GLP-1R). This G-protein-coupled receptor was originally identified in islet β-cells, but its expression has now been reported in numerous extrapancreatic tissues, including the lung, brain, enteric and peripheral nervous systems, lymphocytes, heart and blood vessels and various locations in the kidney. Most of the commercially available antisera that are used to detect GLP-1R expression (by immunohistochemistry or western blot analysis) are neither sensitive nor specific, and important control experiments are frequently absent. Furthermore, interspecies differences might hamper exact mapping of the distribution of a functional GLP-1R. A study that used the most extensively validated monoclonal antibody to date showed that in primate kidneys, GLP-1R is exclusively expressed in preglomerular vascular smooth muscle cells and juxtaglomerular cells.

**Membrane-bound DPP-4**

Dipeptidyl peptidase 4 (DPP-4) is highly active and abundantly expressed in the kidney. The highest level of expression of DPP-4 is at the brush border of the S1–S3 segment of the proximal tubule (where it is functionally coupled to intestinal sodium–hydrogen exchanger isoform 3). Lower expression levels are present at other sites in the nephron and tubulointerstitium. The results of studies that analysed the renal distribution of GLP-1R and/or DPP-4 in the kidneys of various organisms are summarised below (Box Table 1).

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*Box 1. Putative renal distribution of the GLP-1 receptor and membrane-bound DPP-4*
## Box Table 1.

<table>
<thead>
<tr>
<th>Location</th>
<th>Species</th>
<th>mRNA or protein</th>
<th>Detection method</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1R</td>
<td>Preglomerular* vascular smooth muscle cells</td>
<td>Monkey; human Rat</td>
<td>Protein</td>
<td>Immunohistochemistry</td>
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<td></td>
<td></td>
<td>Rat</td>
<td>Protein</td>
<td>Autoradiography of $^{125}$I-labelled GLP-1, exendin 4 (GLP-1 agonist) and exendin 9–39 (GLP-1R antagonist)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mRNA, protein</td>
<td>In situ hybridization, Immunohistochemistry</td>
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<td></td>
<td></td>
<td></td>
<td>Protein</td>
<td>RT-PCR, Western blotting</td>
</tr>
<tr>
<td>Hilar and intralobular arteries</td>
<td>Human</td>
<td>Protein</td>
<td>Autoradiography of $^{125}$I-labelled GLP-1</td>
<td>124</td>
</tr>
<tr>
<td>Glomerular capillary and vascular walls</td>
<td>Mouse</td>
<td>mRNA</td>
<td>In situ hybridisation, RT-PCR</td>
<td>121</td>
</tr>
<tr>
<td>Glomerular endothelial cells and macrophages</td>
<td>Rat</td>
<td>Protein</td>
<td>Immunofluorescence</td>
<td>123</td>
</tr>
<tr>
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<td>mRNA</td>
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</tr>
<tr>
<td>Juxtaglomerular cells</td>
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<td>Protein</td>
<td>Immunohistochemistry</td>
<td>125</td>
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<td>Proximal tubule</td>
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<tr>
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<td>Protein</td>
<td>Autoradiography of $^{125}$I-labelled GLP-1, exendin 4 (GLP-1 agonist) and exendin 9–39 (GLP-1R antagonist)</td>
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<tr>
<td>Membrane-bound DPP-4</td>
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<td>Podocytes</td>
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<tr>
<td>Proximal tubule</td>
<td>Pig; human Rat</td>
<td>RNA, protein</td>
<td>RT-PCR, immunocytochemistry</td>
<td>126</td>
</tr>
<tr>
<td>Loop of Henle, distal convoluted tubule, connecting tubule, cortical collecting duct</td>
<td>Rat</td>
<td>mRNAs</td>
<td>Deep sequencing of RNA species</td>
<td>131</td>
</tr>
</tbody>
</table>

*Afferent arterioles, interlobular arteries and arcuate arteries.*
GLP-1R-mediated natriuresis and diuresis seem to involve inhibition of NHE3, which is located at the brush border of the renal proximal tubule bound to a complex that also contains DPP-4. In animals and humans, acute GLP-1 infusion increases renal lithium clearance (a marker of proximal tubular sodium reabsorption) and urinary pH in rats and humans supporting the notion that NHE3 is indeed involved. The natriuretic actions of incretin-based drugs may also involve the RAAS. Indeed, GLP-1R agonists inhibit post-receptor actions of angiotensin-II, while acute GLP-1 infusion lowers angiotensin-II levels in healthy males and plasma renin activity in obese insulin-resistant subjects.

Notably, patient with diabetes have increased proximal tubular sodium (and glucose) reabsorption, resulting in reduced sodium delivery at the distally located macula densa and subsequent increases in P_GLO and single-nephron GFR via tubuloglomerular feedback. Incretin-based therapy-related natriuresis could restore this disrupted response. Indeed, in early studies in diabetic rodents, 4 to 8 weeks of administration of exendin-4, liraglutide or linagliptin reduced glomerular hyperfiltration. In line with these findings, acute GLP-1 infusion lowered creatinine clearance from 151 ml/min to 142 ml/min in 16 obese insulin-resistant males (four of whom had T2DM).

At the time that the trials in this thesis were designed, no studies examined the actions of clinically used GLP-1R agonists or DPP-4 inhibitors on renal haemodynamics and tubular functions in humans.

**Aim of the thesis**

In the present thesis, of which the SAFEGUARD (Safety Evaluation of Adverse Reactions in Diabetes) and ELIXIRS (Effect of LIXIsenatide on the Renal System) studies form the backbone, we aimed to determine the effects of incretin-based drugs on ‘gold standard’ measured renal haemodynamics, as well as intrarenal haemodynamics, tubular functions and renal damage markers, in healthy overweight males and T2DM patients. In particular, we sought to assess whether these agents confer renoprotection by improving glomerular hyperfiltration and related P_GLO.

**Outline of the thesis**

In **Chapter 2**, we give an overview of the risk factors and treatment of DKD, and discuss the pleiotropic effects of widely used drugs in T2DM management on renal outcomes, with a special emphasis on glucose-lowering drugs.

In **Chapter 3** we focus on glomerular hyperfiltration as renal risk factor in diabetes. We discuss its mechanisms and clinical significance, and summarise available and emerging interventions that could attenuate the renal haemodynamic abnormality.

In **Chapter 4**, we discuss the hitherto reported interactions between the incretin- and RAAS pathways, thereby focusing on potential clinical consequences when pharmacological compounds interfering with these pathways are prescribed simultaneously.
Figure 4. Trial designs
In Chapter 5, we report an open-label, non-randomised, placebo-controlled study that determined the acute renal effects of exenatide compared to placebo infusion in 10 healthy overweight males (Figure 4A and Table 2). We assessed the potential involvement of nitric oxide by performing examinations with and without concurrent infusion with the nitric oxide-synthase inhibitor L-N\textsuperscript{G}-monomethyl arginine.

In Chapter 6, we describe a double-blind, placebo-controlled, randomised clinical trial (RCT), which scrutinized the acute renal effects of exenatide infusion in insulin-naïve 60 T2DM patients (Figure 4B and Table 2).

In Chapter 7 we report an additional double-blind RCT performed in the same study population, to assessed the renal effects of 12 week treatment with the long-acting GLP-1R agonist liraglutide or sitagliptin compared to placebo (Figure 4C and Table 2).

In Chapter 8, we describe an open-label RCT in 40 insulin glargine-treated T2DM patients, which determined the postprandial renal effects of 8 week treatment with the short-acting GLP-1R agonist lixisenatide versus once-daily titrated insulin glulisine (Figure 4C and Table 2).

In Chapters 9 and 10 we report predefined secondary analyses of that RCT to further detail the magnitude and mechanisms by which lixisenatide affects electrolyte homeostasis (Chapter 9) and postprandial systemic haemodynamics (Chapter 10).
In Chapter 11, we report a post-hoc analysis of the previous four clinical trials, in which we determined the effects of acute and prolonged GLP-1R agonists administration on uric acid levels (an emerging cardiovascular and renal risk factor) and its renal clearance.

In Chapter 12, we report a response to a secondary analysis of a landmark RCT, which addresses the glucose-, bodyweight- and blood pressure-independent renoprotective effects of liraglutide.

In Chapter 13, we provide a summary of the results and a general discussion of the direct pathways by which incretin-based drugs may affect the kidney. In addition, we summarise other (indirect) pathways through which these drugs are suggested to reduce DKD-burden. Finally, based on evidence that came available after our studies were initiated, we review the effects of GLP-1R agonists and DPP-4 inhibitors on renal outcomes in T2DM patients.
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Chapter 1


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Chapter 1


