Summary and General Discussion
The incretin hormone glucagon-like peptide 1 (GLP-1) is secreted by the gut in response to nutrient ingestion. It stimulates insulin and inhibits glucagon secretion by the pancreatic islet cells, thereby lowering postprandial blood glucose levels. Two drug classes have been developed to augment these responses and target hyperglycaemia; GLP-1 receptor agonists and inhibitors of the GLP-1 degrading enzyme, dipeptidyl peptidase (DPP)-4 inhibitors. These GLP-1 (or incretin)-based drugs are widely recommended for treatment of type 2 diabetes (T2DM) [5] and their prescription rate is increasing [511]. Since the GLP-1 receptor is present in many organ systems, including the cardiovascular, renal, gastrointestinal and central nervous system [19,20], finding ‘pleiotropic’ effects (i.e. beyond the endocrine pancreas) of GLP-1-related therapies is to no surprise. Some of these actions are beneficial and contribute to glucose reduction or have other favourable effects, including weight loss, improvement of dyslipidaemia and lowering of blood pressure and systemic inflammation. On the other hand, harmful effects have also been reported, including acute pancreatitis [21–23], heart rate acceleration [24] and renal [26,512] and heart failure [25]. However, a causal relationship between incretin-based therapies and most of these adverse events has not been proven and the underlying mechanisms remain largely unexplored. While large-sized randomized trials and database studies are needed to provide evidence on the clinical risk-/benefit-ratio, mechanistic studies are needed to identify the full spectrum of side effects and their underlying mechanisms. The aim of the studies performed in this thesis was to investigate the acute and prolonged effects of incretin-based therapies on the gastrointestinal and cardiovascular system in patients with T2DM.

Studies Conducted in This Thesis

The data presented in this thesis are based on four clinical trials performed within the framework of the European-funded project ‘Safety Evaluation of Adverse Reactions in Diabetes’ (SAFEGUARD). This project was designed to assess, quantify and understand safety aspects of antihyperglycaemic drugs in T2DM, with a focus on incretin-based therapies [58]. It consisted of eight work packages, including pharmacovigilance database studies, observational database studies, meta-analyses, and mechanistic studies. Within this framework, our task was to provide mechanistic evidence on potential adverse and beneficial effects of incretin-based therapies. The results of these studies are summarized in Table 1.

First, we performed a pilot-study in 10 healthy overweight males, to validate the techniques used in the main study and to assess the effects of the GLP-1 receptor agonist exenatide on cardiovascular endpoints in healthy individuals. Furthermore, by infusion of the nitric oxide (NO) blocker L-NMMA (L-NG-monomethyl arginine), we wanted to understand whether effects of exenatide were NO-dependent. The main study consisted of two separate yet integrated, double-blind, randomized, placebo-controlled trials in 60 patients with T2DM: a short trial to assess the acute effects of exenatide (short-acting GLP-1 receptor agonist) versus placebo, and a 12-week trial to assess the longer-term effects of liraglutide (long-acting GLP-1 receptor agonist) or sitagliptin (DPP4-inhibitor) versus placebo. Finally, in a subset of 12 male T2DM patients of the main study, we performed a randomized, double-blind, cross-over trial to assess the acute effects of exenatide versus placebo on exocrine pancreatic secretion.

In the following sections, the effects of GLP-1 based therapies on specific organ systems are discussed. Results from our own studies are integrated with other studies from the
SAFEGUARD-project and data obtained from the literature, in particular the cardiovascular outcome trials for several incretin-based therapies that were recently published [47,182,183,361,184,362].

Gastro-intestinal

Pancreas

Soon after their introduction, case reports linked GLP-1 based drugs to pancreatitis [21,345]. A subsequent analysis of the United States Food and Drug Administration’s (FDA) adverse events reporting system demonstrated an increased risk of acute pancreatitis and pancreatic cancer [23]; from that point, a plethora of studies with conflicting results have been published, resulting in an on-going discussion on pancreatic safety. As reviewed in chapter 2, several animal studies have demonstrated that both GLP-1 receptor agonists and DPP-4 inhibitors might induce pancreatic inflammation, cellular proliferation and development of neoplasia [27,159–161]. In contrast, other studies found no effect on pancreatic physiology and morphology [162–168]. A similar conflicting pattern applies for observational database studies [182–184,181]. The only consistent finding is a modest increase in pancreatic enzyme levels [47,182,183,361,184,362], of which the cause and consequences are still unclear.

A study in healthy volunteers demonstrated that GLP-1 peptide acutely reduced exocrine pancreatic function [35]. Prior mechanistic studies have shown that both inhibition and stimulation of exocrine secretion may cause pancreatitis [314–316]; thus, a GLP-1 receptor agonist-induced change in exocrine secretion could support an association with pancreatitis. Therefore, in chapter 4, we assessed the acute effects on exocrine pancreatic function. Exenatide or placebo were administered intravenously to 12 T2DM patients, and exocrine pancreatic function was measured using secretin-enhanced magnetic resonance cholangiopancreatography (s-MRCP). Compared with placebo, exenatide had no effect on pancreatic excretion volume, secretion speed or diameter of the pancreatic duct. We then assessed whether GLP-1 receptor agonists acutely increase pancreatic enzyme levels (chapter 5). Patients with T2DM were randomized to intravenous exenatide or placebo, and plasma lipase and amylase were measured repeatedly. Moreover, a high-fat mixed meal was given to stimulate endogenous GLP-1 release. While no changes in lipase and amylase were seen during placebo, indicating that endogenous GLP-1 does not affect pancreatic enzymes, exenatide increased plasma amylase levels within 5 h.

Subsequently, we studied the effects of 12-week treatment with liraglutide or sitagliptin on pancreatic enzymes, exocrine function and morphology in chapter 6. The exocrine function was measured by s-MRCP (bicarbonate secretion), $^{13}$C-mixed triglycerides breath test (lipase function) and faecal elastase-1 and chymotrypsin levels. Changes in pancreatic morphology (pancreas volume, pancreatic duct structure) were assessed by MRI. Sitagliptin marginally increased intra-duodenal pancreatic fluid secretion, while liraglutide did not change exocrine pancreatic secretion. Liraglutide increased plasma lipase levels after 6 weeks, while sitagliptin increased amylase levels after 2 and 6 weeks. After 12 weeks, the effects on lipase and amylase levels had waned, but both agents did increase plasma trypsinogen levels. Neither drug significantly changed pancreatic volume or morphology, although liraglutide tended to increase pancreatic volume. Treatment-induced volume expansion was associated with increased amylase-levels.

The most apparent finding of these studies is that GLP-1 based therapies (transiently) increase plasma pancreatic enzyme levels. Although not novel, we expand current literature by demonstrating that this enzyme elevation occurs immediately and also includes
### Table 1: Major effects found in the performed studies.

The ‘↑’ sign indicates a treatment-induced increase, the ‘↓’ a decrease, and the ‘=’ indicates no effect. **Abbreviations = CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; MTG, mixed triglycerides; SNS, sympathetic nervous system; UDCA, ursodeoxycholic acid**

<table>
<thead>
<tr>
<th>Effect</th>
<th>Healthy Volunteers (acute)</th>
<th>Type 2 Diabetes (acute)</th>
<th>Type 2 Diabetes (12-week)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exenatide</td>
<td>Exenatide</td>
<td>Liraglutide</td>
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<tr>
<td>s-MRCP pancreatic secretion</td>
<td>=</td>
<td>=</td>
<td>= / ↑</td>
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<tr>
<td>13C-MTG breath test</td>
<td>=</td>
<td>=</td>
<td>=</td>
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<tr>
<td>Faeces elastase-1 / chymotrypsin</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Lipase / Amylase / Trypsinogen</td>
<td>↑</td>
<td>= / ↑</td>
<td>= / ↑</td>
</tr>
<tr>
<td>Pancreas volume</td>
<td>=</td>
<td>(trend ↑)</td>
<td>=</td>
</tr>
<tr>
<td>Gallbladder emptying</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Bile acids in serum</td>
<td>DCA ↑</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Bile acids in faeces</td>
<td>DCA ↑</td>
<td>CDCA ↑</td>
<td>CA ↑ UDCA ↑</td>
</tr>
<tr>
<td>Gastric emptying</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Hepatic fat content</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Resting heart rate</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Diastolic blood pressure</td>
<td>=</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Stroke volume</td>
<td>=</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Cardiac output</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Total peripheral resistance</td>
<td>=</td>
<td>↑</td>
<td>=</td>
</tr>
<tr>
<td>Cardiovascular SNS-activity</td>
<td>↑</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Arterial stiffness</td>
<td>↓</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Baseline capillary density</td>
<td>↑</td>
<td>=</td>
<td>=</td>
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<tr>
<td>Post-occlusive capillary density</td>
<td>↑</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Skin SNS-activity</td>
<td>= / ↓</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Glucose levels (fasting)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Insulin levels (fasting)</td>
<td>=</td>
<td>↑</td>
<td>=</td>
</tr>
</tbody>
</table>

**Summary and General Discussion**
trypsinogen. The acute increase in amylase but not lipase suggests active secretion instead of acinar damage, as the latter would have caused congruous enzyme changes [354, 513]. This hypothesis is supported by a recent in vitro study, where GLP-1 increased amylase secretion through a GLP-1 receptor dependent cyclic-AMP pathway [339].

An important question is whether this increase in enzyme levels reflects pancreatic pathology. We have demonstrated that the acute rise in enzyme levels was not accompanied by inhibition of the exocrine pancreatic function. Neither did the 12-week intervention have a profound effect on pancreatic function or morphology, although liraglutide tended to increase pancreatic volume and sitagliptin marginally increased bicarbonate secretion. This tendency towards increased volume is in line with animal studies, where GLP-1 receptor agonists augment enzyme production, thereby increasing acinar cell size and, consequently, pancreas weight [357,514]. The marginal increase in bicarbonate secretion with sitagliptin is a novel finding. Interestingly, other aspects of the exocrine excretion were unchanged. While test variability and sensitivity could explain this discrepancy, it may also be that DPP-4 inhibition only affects bicarbonate secretion. This hypothesis is strengthened by the observation that the DPP-4 enzyme is present on pancreatic duct cells [355]. Whether these modest changes are detrimental in the long term is hard to predict. This may be the case, as for example continuous pancreatic stimulation with low-dose caerulin was shown to have negative effects after years of treatment [360].

In 2015 and 2016, several large studies were published regarding pancreatic safety. For example, within the SAFEGUARD framework, we performed a case-control study (n=3,990 pancreatitis-cases matched with n=19,543 controls), and observed no increased risk of acute pancreatitis in patients treated with GLP-1 based therapy [515]. In another study, a cohort of over 1 million patients with T2DM initiating antihyperglycaemic agents were studied, finding no association between GLP-1 based therapies and pancreatitis [516]. However, even the largest observational studies might be hampered by potential confounders, including diabetes duration, co-morbidities and co-medication.

![Figure 1: Pancreatic effects of GLP-1. GLP-1 affects both the endocrine and exocrine aspects of pancreatic physiology. Effects with the asterisk (*) are only described in rodent studies, not in humans.](image)
Unfortunately, dedicated RCTs on pancreatic risk are lacking. Nevertheless, some data can be subtracted from the cardiovascular safety trials, where pancreatic safety was also evaluated [47,182–184,361,362]. For GLP-1 receptor agonists, no statistical significant increase in acute pancreatitis risk was observed in ELIXA (lixisenatide), LEADER (liraglutide) or SUSTAIN-6 (semaglutide) [184,361,362]. A recent analysis of the LEADER trial demonstrated elevations in both amylase and lipase levels, which were not predictive of an event of acute pancreatitis [517]. In contrast, for DPP-4 inhibitors, a recent meta-analysis, which combined the results of SAVOR-TIMI 53 ( saxagliptin), EXAMINE ( alogliptin) and TECOS ( sitagliptin), demonstrated a significant increased risk of acute pancreatitis, although the risk may differ between the compounds [346]. The authors calculated that, with an odds ratio of 1.79 and a low background prevalence, the number needed to harm is 1940 per year. Thus, although DPP-4 inhibitors may increase the risk of acute pancreatitis, the absolute risk is very low.

Identifying a potential risk of pancreatic cancer is even more important. The larger observational database studies found no increase in pancreatic cancer risk [348,518]. In the cardiovascular safety trials no statistically significant risk increment was observed [47,182–184,361,362]. However, in LEADER, 13 out of 4668 patients receiving liraglutide developed acute pancreatitis [184,361,362]. A recent analysis of the LEADER trial demonstrated an increase in acute pancreatitis risk was observed in ELIXA (lixisenatide), LEADER (liraglutide) or SUSTAIN-6 (semaglutide) [184,361,362]. A recent analysis of the LEADER trial demonstrated.

**Biliary System**

Two recent RCTs with liraglutide, the SCALE-trial (liraglutide 3.0 mg daily; effects on body weight) and LEADER (liraglutide 1.8 mg daily), demonstrated a 1.6- to 2.2-times elevated risk of cholelithiasis and cholecystitis [112,361]. Moreover, we recently reviewed the adverse event reporting system (EudraVigilance) of the European Medicines Agency, and observed that gallbladder-related events occur more frequently in patients treated with GLP-1 receptor agonists or DPP-4 inhibitors [378]. While weight loss with GLP-1 receptor agonists could be involved in gall stone development, no weight effects are seen during DPP-4 inhibitor treatment [519]. Therefore, it is of interest to understand the effects of these agents on the biliary system.

As reviewed in chapter 2, few studies have assessed the effects of GLP-1 and GLP-1 based therapies on the biliary system. In DPP-4 knockout-mice, a reduction in bile acid production and an increase in canalicular excretion was observed [189]. The same was found in wild type animals during GLP-1 infusion [189]. In vitro, GLP-1 and the GLP-1 receptor agonist exendin-4 increased cholangiocyte proliferation and reduced apoptosis [190]. Moreover, a single dose of exenatide decreased CCK-induced gallbladder contractions in healthy humans [38]. A combination of reduced gallbladder emptying (inducing bile stasis) and reduced bile acid secretion (leading to cholesterol supersaturation) could well explain an increase in gall stones. We therefore assessed the effects of GLP-1 based therapies on gallbladder emptying (using ultrasonography) and bile acid levels in faecal and (postprandial) serum samples (chapter 7).

Against expectations, neither liraglutide nor sitagliptin affected gallbladder fasting volume
or emptying. Liraglutide increased serum and faecal levels of deoxycholic acid (DCA), a secondary bile acid which is formed by 7-dehydroxylation of the primary bile acid cholic acid (CA) by the intestinal microbiome. In the absence of changes in other bile acids, these findings point towards changes in the intestinal microbiome. Indeed, a recent study in mice demonstrated that GLP-1 receptor agonists, but not DPP-4 inhibitors, have the potential to alter gut microbiota [520]. Studies that investigate whether the human intestinal microbiome is altered by GLP-1 based medication are currently ongoing using faecal samples that were obtained in the present study.

Sitagliptin had no effect on serum bile acids, but increased faecal levels of CA, chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA). This can only be explained by an increase in hepatic bile acid production, since faecal bile acids reflect hepatic synthesis. However, in a recent study by Nunez et al [388], sitagliptin had no effect on 7-alpha-hydroxy-4-cholesten-3-one (C4), a marker frequently used as a proxy of bile acid synthesis. Since C4 is an intermediary metabolite in the bile acid synthetic pathway, this marker is only reliable when external factors do not influence activity of enzymes more downstream in the bile acid synthesis pathway nor transport or removal from the plasma. As such, since it is still unclear how sitagliptin could increase bile acid production, C4-measurements might not adequately reflect the effects of sitagliptin.

If the liraglutide-induced increase in DCA also occurs in bile, this could induce gall stone formation, since DCA stimulates cholesterol excretion [389]. CA is a strong stimulator of the farnesoid X receptor (FXR) and G protein-coupled bile acid receptor 1 (TGR5). Stimulation of these receptors reduces hepatic gluconeogenesis and improves insulin sensitivity [391]. Combined, these suggestions highlight the need for future studies to assess the effects on bile composition. The sitagliptin-induced increase in faecal bile acids and lack of effect on biliary motility argue against DPP-4 inhibitors increasing the risk of bile stones. Indeed, in a recent cohort study, Faillie et al demonstrated that DPP-4 inhibitors were not associated with cholelithiasis (hazard ratio 0.99; 95%-CI 0.75 – 1.32) [521]. GLP-1 receptor agonists on the other hand were found to increase this risk by 3.7 (95%-CI 3.5 – 4.0). Thus, future studies should focus on how GLP-1 receptor agonists cause biliary side effects, while for DPP-4 inhibitors, they should be directed at unravelling the cause and consequences of the increased faecal bile acids.

Liver
Although the presence of a hepatic GLP-1 receptor remains uncertain, several studies have demonstrated that GLP-1 and GLP-1 based therapies have hepatic effects. As discussed in chapter 3, GLP-1 increases glycogen synthesis and reduces glycolysis and gluconeogenesis, independent of insulin and glucagon, leading to reduced glucose production and increased (postprandial) glucose uptake and storage [39,186]. This effect is particularly interesting for diabetic patients with little to no residual β-cell function. In a pilot study in obese but otherwise healthy men (chapter 9 and chapter 13), glucose levels decreased during exenatide-infusion, despite stable insulin levels, which could suggest a decrease in hepatic glucose output.

Another important hepatic effect of GLP-1 is its ability to improve lipid metabolism. GLP-1 stimulates mitochondrial degradation of fatty acids and inhibits triglyceride production [187,188]. Combined with the beneficial effects on hepatic insulin sensitivity, body weight and inflammation, this may be beneficial for patients with non-alcoholic fatty liver disease (NAFLD) [202–208], as reviewed in chapter 2. Although the prevalence of NAFLD exceeds
30% of the Western population, treatment strategies are virtually non-existing. Several studies have demonstrated a steatosis-reducing potential for GLP-1 based therapies, though these trials lacked adequate control groups or used inferior techniques [192–196,522,523]. We therefore tested this hypothesis in a double-blind RCT (chapter 8). Using hydrogen-magnetic resonance spectroscopy (1H-MRS), 12-week treatment with liraglutide or sitagliptin had no effect on hepatic steatosis or fibrosis. These results were unexpected, especially since most other studies did confirm that GLP-1 based therapies improve hepatic steatosis [197–199,401,402]. Moreover, a recent meta-analysis demonstrated efficacy of GLP-1 receptor agonists for the treatment of NAFLD [524]. Our 12-week intervention may have been too short to induce hepatic changes, but a recent well-designed 24-week study with sitagliptin also failed to show an effect [401]. Maybe differences in measurement techniques (MRI, biopsy) or study population (NAFLD, non-alcoholic steatohepatitis, T2DM) are responsible for these discordant findings. Nevertheless, we feel that current evidence is strong enough to support treatment of patients with T2DM and NAFLD with these agents, especially since adverse effects appear to be limited.

**Stomach**

The inhibitory effect of GLP-1 on gastric emptying is likely its most recognized gastrointestinal effect. As reviewed in chapter 2 and 3, GLP-1 prolongs the lag time, inhibits propulsion waves, stimulates pyloric tone, doubles time to empty 50% of gastric contents, without affecting maximal secretion speed [32,86–90]. All GLP-1 receptor agonists mimic these effects after single-dose administration, yet after prolonged intervention, important differences occur [96,97,266,267]. Short-acting agents retain their effect on gastric emptying [93,94], while with long-acting agents, it tends to wane, presumably due to receptor tachyphylaxis [93,94]. Interestingly, although DPP-4 inhibitors raise plasma active GLP-1 levels, they have limited to no effect on gastric emptying [32,79,98,99]. It has been suggested that other peptides also degraded by DPP-4, such as peptide YY (PYY), counteract the inhibiting effect of GLP-1. In our main study, we assessed the effects of 12-week treatment with liraglutide (long-acting GLP-1 receptor agonist) or sitagliptin (DPP-4 inhibitor) on gastric emptying, by performing an acetaminophen absorption test (chapter 6). In line with previous studies, neither agent affected gastric emptying compared with placebo.

Gastric inhibition is likely accountable for several side- and therapeutic effects of GLP-1 based therapies. First, reduced gastric emptying causes less duodenal glucose loading, thereby reducing postprandial glucose excursion [261]. In chapter 11 and 13, the postprandial glucose levels were studied. Since GLP-1 based therapies did not alter gastric emptying in the 12-week trial, this explains why postprandial glucose levels rose to the same extent as during placebo. In contrast, acute exenatide reduced postprandial glucose excursions. Although not measured in this setting, reduced gastric emptying probably explains this difference.

Second, as indicated in chapter 10 and others, participants treated with exenatide (acute study) or liraglutide (12-week study) frequently experienced nausea and/or vomiting. This well-known adverse effect of GLP-1 receptor agonists occurs in at least 25% of patients [123]. While it has been suggested that the gastric inhibitory effect is responsible, recent studies implicate involvement of neurological nausea centres. This is in concordance with our observations that nausea occurred in the fasting state, as well as in absence of changes in gastric emptying. Nausea does not occur with DPP-4 inhibitors. Although this could be explained by their lack of gastric inhibition, that does not account for the central effects of
GLP-1. Potentially, GLP-1 levels are not high enough to induce central effects during DPP-4 inhibition. Alternatively, as discussed, other peptides normally degraded by DPP-4 could be involved. An important unanswered question is whether GLP-1 based therapies can be used in patients with gastroparesis, or strongly reduced gastric emptying.

**Cardiovascular**

One of the main triggers of the SAFEGUARD-project is the so-called “rosiglitazone-affair.” In 2007, a meta-analysis found a significant increased risk of acute myocardial infarction in patients treated with the thiazolidinedione rosiglitazone [525], which lead to marketing suspension in the EU and restrictions in the USA. As a result, the regulatory agencies now require demonstration of cardiovascular safety for all new antihyperglycaemic agents, either by showing cardiovascular safety in their phase-III programme, or by conducting a large cardiovascular outcome trial [526]. Based on their registration trials, all currently available GLP-1 receptor agonists and DPP-4 inhibitors were granted marketing authorization, yet so-called cardiovascular safety trials needed to be performed for final FDA approval. Meanwhile, several mechanistic studies have been performed, demonstrating both beneficial and adverse effects of GLP-1 receptor agonists and DPP-4 inhibitors on systemic haemodynamics and the microcirculation.

**Resting Heart Rate**

One cause of concern regarding treatment with GLP-1 receptor agonists is the increase in resting heart rate (RHR). While physiological GLP-1 levels do not affect RHR [427], infusion of supraphysiological levels or administration of a GLP-1 receptor agonist increases RHR [426]. This occurs immediately [408] and sustains throughout intervention [429]. On average, an acceleration of ~2 beats/minute is reported, yet RHR increases by ~10 beats/minute [429,430] have been observed. This may be worrisome, since elevated RHR has been associated with all-cause mortality [431]. In one analysis, an increase of 5 beats/minute was associated with a 17% increase in mortality [415]. While some suggest that RHR simply reflects general fitness, and should by itself not be considered a risk factor for mortality, others believe that RHR-elevation independently causes or aggravates atherosclerosis and increases the risk of myocardial ischaemia [432].

Mechanisms underlying the RHR-accelerating effect of GLP-1 receptor agonists remain incompletely understood. Therefore, we performed three trials to provide insight. First, in chapter 9, healthy overweight volunteers received, in a non-blinded cross-over setting, placebo, exenatide, the nitric oxide (NO) blocker L-NMMA, and a combination of exenatide and L-NMMA. Automated oscillometric blood pressure measurements and finger photoplethysmography were performed to measure systemic haemodynamics, while sympathetic nervous system (SNS) activity was measured by heart rate variability and rate-pressure product. Exenatide increased RHR, systolic blood pressure (SBP) and SNS-activity, without affecting total peripheral resistance. During concomitant L-NMMA-infusion, exenatide had the same effect. These data argue against exenatide-induced reflex tachycardia as response to vasodilation, and rather suggest involvement of SNS activation. In the main study in patients with T2DM (chapter 10), acute exenatide-infusion increased RHR, systolic and diastolic blood pressure and vascular resistance, while stroke volume and arterial stiffness decreased. SNS-activity and cardiac output were unaffected. Twelve-week treatment with liraglutide increased RHR, while reducing SBP and stroke volume, without affecting the other parameters. The combination of the acute and 12-week data
suggest that RHR-acceleration with GLP-1 receptor agonist treatment in T2DM patients is not explained by changes in systemic haemodynamics, vascular resistance/stiffness or SNS-activity, implicating direct sino-atrial stimulation. The recent observation that sino-atrial cells express GLP-1 receptors supports our data [19]. Moreover, stimulation of the GLP-1 receptor in other myocardial tissue increased cyclic-AMP signalling [26], a pathway known to stimulate sino-atrial-mediated RHR-acceleration [38]. It would be of great interest for future studies to assess in vitro whether exposure of sino-atrial cells to GLP-1 or GLP-1 receptor agonists leads to increased pacing, and thus RHR-acceleration. The differences between the effects on RHR of GLP-1 based therapies in healthy controls and T2DM are not understood. One explanation could be the presence of insulin resistance. Since insulin is a potent activator of SNS activity [43], this may have attributed to SNS stimulation in healthy volunteers, but not in T2DM patients. Another explanation may lie in a slight difference in the study protocols for the healthy volunteers and T2DM patient, since a time-dependent effect could have occurred in the pilot-study due to its cross-over design. Future studies should include T2DM patients and matched controls with identical protocols to further assess any differences between these groups.

Chronic SNS-activation is associated with atherosclerosis, arrhythmia, heart failure, kidney failure [27], and as such, it can be considered beneficial that the RHR-acceleration appears not to be caused by SNS-activation. Whether drug-induced RHR-acceleration on itself is harmful is unknown, since all current evidence linking increased RHR to mortality is derived from observational studies, not distinguishing the cause of the increase in RHR. Of interest are two drugs that also affect heart rate. Doxazosin, an α-blocking agent that increases RHR by ~25% [28], has been shown to increase heart failure incidence compared with the diuretic agent chlorthalidone [29]. Second, ivabradine, a selective inhibitor of the cardiac “funny-channel” did not affect mortality in patients with stable coronary artery disease, despite a reduction in RHR of ~10 beats/minute [30]. These data are confusing, suggesting an increase in RHR is harmful, yet a reduction is not beneficial. All in all, it can be concluded that drug-induced increases in RHR are at least worrying and require further study.

Blood Pressure
In healthy volunteers (chapter 9), SBP increased with exenatide-infusion, while DBP was not affected. The increase in SBP was likely explained by an increase in cardiac output. In T2DM patients (chapter 10), exenatide infusion increased both SBP and DBP; likely due to an increase in vascular resistance, as this parameter also increased. Similar to differences in RHR changes, these discrepancies between healthy volunteers and T2DM patients remain unexplained. As stated, potential explanations include a differential effect of exenatide on SNS-activity and differences in study design. However, in patients with T2DM, endothelial dysfunction and different endothelial responsiveness to GLP-1 receptor agonists may have contributed, as was recently demonstrated in miniature Ossabaw swine [499]. During prolonged intervention in patients with T2DM (chapter 10), liraglutide reduced SBP, but had no effect on DBP. The conflicting effect of acute and prolonged treatment with GLP-1 receptor agonists on blood pressure is not understood. Sitagliptin-treatment did not affect blood pressure. These findings are in line with most of the published literature. In recent meta-analyses, an average SBP-lowering between 1.84 and 4.6 mmHg was calculated for GLP-1 receptor agonists [29], and ~3.04 mmHg for DPP-4 inhibitors [31]. The current trial was not designed to assess mechanisms underlying this antihypertensive effect, yet some aspects can be highlighted. First, we observed no reduction in SNS activity. Second,
blood pressure lowering was not associated with a reduction in vascular resistance. Third, liraglutide had no significant effect on body weight, excluding this as likely explanation. Fourth, liraglutide did not increase renal sodium excretion after 12 weeks, nor did it affect the renin-angiotensin-aldosterone system (RAAS)[532]. We did not measure serum levels of atrial natriuretic peptide (ANP), which has been suggested to increase with GLP-1 therapy [476]. However, several recent trials showed conflicting evidence regarding the effect of GLP-1 based therapies on ANP concentrations [533]. Moreover, ANP would lower blood pressure by promoting natriuresis and/or reducing vascular resistance [533], both of which have not been observed in the current study. A large trial, integrating all of the above aspects, is needed to understand why blood pressure reduces with GLP-1 based therapies. The clinical relevance of these modest changes in blood pressure needs further study. The acute, temporary GLP-1 induced increment in blood pressure is unlikely to have clinical consequences. On the other hand, even a modest decrease in blood pressure over a prolonged period of time is likely beneficial, as was shown in the ADVANCE trial, where a reduction of 5.6 mmHg in SBP reduced major cardiovascular events by 9% [534].

Cardiovascular Safety (Trials)
Within the SAFEGUARD-project, a case-control study was performed to assess the safety of non-insulin blood glucose lowering drugs, including cardiovascular safety. We matched 25,979 cases of acute myocardial infarction to 127,570 controls [535]. In this analysis, the use of metformin/exenatide (odds ratio 0.44 [95%-CI 0.22 – 0.88]) and the use of metformin/sitagliptin (OR 0.79 [95%-CI 0.66 – 0.94]) were associated with a significantly lower risk of acute myocardial infarction compared with metformin/sulphonylurea users, while metformin/liraglutide was neutral (OR 0.72 [95%-CI 0.39 – 1.32]). This observational study suggests that GLP-1 based therapies have no adverse cardiovascular consequence after long-term intervention, and might even be beneficial. However, residual confounding always remains a risk with database studies. Therefore, the results of the recent cardiovascular safety trials are of great interest. To date, three trials with GLP-1 receptor agonists (lixisenatide, liraglutide, semaglutide) and three with DPP-4 inhibitors (saxagliptin, alogliptin and sitagliptin) have been conducted [47,182–184,361,362]. The trials regarding GLP-1 receptor agonists (ELIXA, LEADER and SUSTAIN-6) are of particular interest in light of their accelerating effect on RHR. Lixisenatide (ELIXA-trial) only marginally increased RHR [239,430], without an increased mortality risk (HR 1.02 [95%-CI 0.89 – 1.17]). In LEADER, liraglutide increased RHR by 3 beats/minute [361], yet reduced systolic blood pressure (-1.2 mmHg), body weight (-2.3 kg), and HbA1c (-0.4%). This resulted in a decrease in the primary composite outcome (cardiovascular death, nonfatal myocardial infarction and nonfatal stroke), with an HR of 0.87 (95%-CI 0.78 – 0.97). While designed to only show non-inferiority of liraglutide compared with placebo (to exclude harm of liraglutide), this study actually showed a superior effect (p=0.01). This reduction in primary outcome was mainly driven by a decrease in death by cardiovascular causes, while nonfatal myocardial infarction and stroke were lowered without reaching statistical significance. All-cause mortality was also significantly reduced by liraglutide. The SUSTAIN-6 trial with semaglutide, demonstrated to some extent similar results. Semaglutide increased RHR by 2-2.5 beats/minute, yet reduced SBP (1.3-2.6 mmHg), body weight (3-4.5 kg), and HbA1c (-0.7%)[362]. Semaglutide significantly reduced the primary composite outcome (same as in LEADER), with an HR of 0.74 (95%-CI 0.58 – 0.95). Again, while aiming for non-inferiority, SUSTAIN-6 showed superiority compared with placebo (p=0.02). In contrast to LEADER, in
SUSTAIN-6 the reduction in primary outcome was mainly caused by reduction in non-fatal stroke, while a non-significant reduction in nonfatal myocardial infarction was seen. Death from cardiovascular causes was not affected. Preliminary results from the EXSCEL-study (long-acting exenatide) demonstrate no increase in cardiovascular events compared with placebo [536]. While exenatide numerically reduced cardiovascular events, this did not reach statistical superiority compared to placebo. Similarly, the FREEDOM-CVO trial showed that continuous subcutaneous delivery of exenatide (ITCA 650) does not increase the risk of cardiovascular events [537]. It is currently debated whether the (marginal) improvements in glycaemic control, indicating a lack of glycaemic equipoise, with liraglutide and semaglutide are fully responsible of the beneficial effects on cardiovascular events, since the large ACCORD, ADVANCE and VADT trials demonstrated no effect of intensive glucose control versus normal glucose control on cardiovascular endpoints [538–540]. Extraglycaemic effects of GLP-1 receptor agonists, such as improvements in blood pressure, body weight and lipid profile are likely involved.

It remains uncertain whether the results of these cardiovascular safety trials can be extrapolated to all T2DM patients who receive GLP-1-based therapies. For example, these trials only included patients with a high cardiovascular risk profile. Moreover, one can wonder whether the relatively short follow-up time (2-4 years) was long enough to allow for adverse effects due to an increase in RHR to become apparent. As such, it is unclear whether a drug-naïve T2DM patient without cardiovascular disease who will use an incretin-based drug for decades will have the same beneficial effects. Nevertheless, two of the three cardiovascular safety trials demonstrate safety and cardiovascular benefit.

The cardiovascular safety trials with DPP-4 inhibitors (EXAMINE, SAVOR TIMI-53 and TECOS) did not show difference between treatment and placebo on the primary endpoints: death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke. This may seem surprising, since a glucose-lowering agent is tested against placebo. It appears that patients at high cardiovascular risk are not helped by short term glucose reduction alone [541]. In contrast to GLP-1 receptor agonists, DPP-4 inhibitors have no effect on blood pressure and body weight. However, these studies were powered to show non-inferiority, and not superiority. As such, they do not rule out that DPP-4 inhibitors prevent cardiovascular events in low-risk patients in the long term. Nevertheless, with hazard ratios close to one, this is not likely. In contrast, one sign of concern is the increased risk of hospitalization for heart failure with saxagliptin in the SAVOR-TIMI 53 study [47]. A recent meta-analysis of all RCTs examining the effects of DPP-4 inhibitors on heart failure demonstrated that as a group, DPP-4 inhibitors were not associated with heart failure, but when analysed individually, saxagliptin increased heart failure risk by 21% [542]. An explanation for this difference could be that saxagliptin has the least specificity toward DPP-4 [543], and thus has more effect on other enzymes. However, no signal for increased risk exists for vildagliptin, which is also only moderately selective for DPP-4. Unfortunately, no cardiovascular safety trial is performed for vildagliptin and data only exist from smaller and shorter RCTs. Another explanation could be the different populations in which the effects of the different DPP-4 inhibitors were studied, with more high-risk patients receiving saxagliptin [542].

We hypothesized that an interaction between DPP-4 inhibitors and angiotensin converting enzyme (ACE)-inhibitors might induce adverse effects [421,544]. Both DPP-4 and ACE degrade substance P and neuropeptide Y (NPY), and by double inhibition, levels of these vasoactive peptides may become very high, leading to activation of the sympathetic nervous system, vasoconstriction and heart rate acceleration. In a post-hoc analysis of SAVOR TIMI-
53, no interaction between the use of saxagliptin and ACE-inhibitors was demonstrated with regards to cardiovascular outcomes [545]. However, this analysis was not adequately powered, and it would be interesting to take such an interaction into account in future studies.

Based on our mechanistic data, combined with the observational studies and RCTs, it appears that liraglutide and sitagliptin do not have an adverse cardiovascular safety profile. However, this conclusion cannot be transferred to other GLP-1 receptor agonists and DPP-4 inhibitors, given the within-class drug differences. Therefore, data of the other cardiovascular outcome trials – e.g. HARMONY outcomes (albiglutide), PIONEER 6 (oral semaglutide), CAROLINA and CARMELINA (linagliptin) and REWIND (dulaglutide) – are eagerly awaited.

Other Aspects of Incretin-Based Therapies Addressed in This Thesis

Postprandial Hypotension

While blood pressure lowering in general is beneficial, a drop in SBP of ≥20 mmHg within 2 h after meal ingestion, termed postprandial hypotension (PPH), causes dizziness, light-headedness, syncope, angina, and, in severe cases, myocardial infarction and stroke [445]. In the elderly, PPH has even been associated with increased mortality [446]. We previously demonstrated that PPH occurs in over 25% of T2DM patients [447], yet numbers may be as high as 40% [445]. In chapter 11, we looked more closely at the effects of GLP-1 based therapies on postprandial BP. In the placebo-group of the acute trial, we observed a PPH-prevalence of 28.6%. While acute exenatide reduced the postprandial drop in DBP and vascular resistance, no effect on SBP was seen. The 12-week treatment with liraglutide or sitagliptin did not alleviate the postprandial SBP and DBP drop. In contrast, sitagliptin even further reduced postprandial SBP and vascular resistance, and consequently, markers of myocardial perfusion. Combined, these data suggest that prolonged intervention with GLP-1 based therapies has no beneficial effect on postprandial haemodynamics, while sitagliptin might even tend to worsen myocardial blood flow. Although very speculative, such a reduction in myocardial perfusion could link the use of saxagliptin to the increase in heart failure (discussed above), if these postprandial effects are also induced by this DPP-4 inhibitor [47].

Microcirculation

Changes in microcirculation play an important role in the development of an increased cardiovascular risk in T2DM. Reduced skin capillary density, or capillary ‘rarefaction,’ has been associated with microvascular complications, such as proliferative diabetic retinopathy, cerebral microbleeds and albuminuria [546,547]. Moreover, it is linked to hypertension, likely being a cause and consequence [469,548]. Finally, the microcirculation might be an important regulator of glycaemic control, since impaired microvascular perfusion reduces glucose delivery to (muscle) cells, thereby impeding (insulin-stimulated) skeletal muscle glucose uptake [494].

Several studies showed effects of GLP-1-based therapies on the microvascular system, which may be beneficial. However, to date, only studies with GLP-1 peptide have been performed, or, when GLP-1 based therapies were studied, the effects on macrovascular endothelial function were assessed. Since the effects of GLP-1 based therapies on microvascular function remained unknown, we studied these in healthy volunteers (chapter 12) and
T2DM patients (chapter 13). Microvascular function was measured by nailfold skin capillary videomicroscopy and laser Doppler fluxmetry. In healthy volunteers, exenatide increased baseline and post-occlusive capillary density, compared with placebo, which was not affected by concomitant L-NMMA infusion. A trend towards decreased skin SNS-activity was observed from the laser Doppler fluxmetry data. In patients with T2DM, acute exenatide did not affect capillary density compared with placebo. However, exenatide increased skin SNS-activity. Twelve-week intervention with liraglutide or sitagliptin had no effect on capillary density or skin SNS-activity.

Thus, exenatide acutely increases capillary perfusion in healthy volunteers, but not in T2DM patients; while in T2DM patients, effects on skin SNS-activity appear to be different for acute and prolonged intervention. This difference may be explained by differences in study design. In the pilot study, placebo was given before exenatide in a crossover setting. In the main

**Figure 2: Primary outcomes for the LEADER and SUSTAIN-6 trials.** Kaplan-Meier plots of the primary outcome (composite of cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke) for A) liraglutide versus placebo, up to 54 months of treatment; and B) semaglutide versus placebo, up to 109 weeks of treatment. Adapted from [361] and [362].
study, both were given at the same time, in a parallel setting. Thus, a circadian rhythm may have influenced results. Alternatively, endothelial resistance to GLP-1 might be involved. This phenomenon is known to occur in animals with metabolic syndrome, but not in healthy lean animals [499]. A similar difference could be present in healthy volunteers and T2DM patients.

An increase in microvascular perfusion would be beneficial, not only because of the link with microvascular complications, but also since microvascular perfusion is associated with increased muscle glucose uptake and myocardial function. However, our lack of effect on perfusion does not rule out beneficial effects on muscle glucose uptake or myocardial function. In several studies, GLP-1 based therapies were associated with an increase in muscle glucose uptake [460,473,549] and improvements in myocardial function [325].

Metabolic and Anthropometric Effects

GLP-1 induced glucose lowering is thought to be mainly caused by increased insulin secretion. Indeed, in both trials, insulin levels increased in T2DM patients. However, in the healthy volunteers, exenatide did not affect insulin levels, while it did reduce glucose. This might be explained by other glucose-lowering effects of GLP-1 based therapies, including a reduction in hepatic gluconeogenesis, intestinal glucose uptake and gastric emptying. Also, in a different study, we confirmed that exenatide reduces glucagon levels [550]. As discussed in chapter 3, these non-insulin glucose-lowering effects of GLP-1 based therapies are most relevant, as they create opportunities to employ these drugs also in patients with little to no residual β-cell function (i.e. type 1 diabetes), or they can provide beta-cell rest in patients with stressed beta cells.

While GLP-1 receptor agonists are hailed for their weight-loss promoting effect, we did not observe a statistically significant effect on body weight after 12-week treatment with liraglutide. From a mean baseline of 105.9 kg, patients receiving liraglutide dropped 2.1 kg, or 1.8%, of their body weight (corrected for placebo-effects: -1.7 kg or -1.8%). In a recent meta-analysis including data from 51 RCTs, the average reduction with liraglutide was 1.43 kg (with a range between 1.24 and 2.61 kg)[111]. Thus, the observed weight loss in our study is within the expected effect of liraglutide. Statistical significance was likely not reached due to the relatively small sample size and large variation.

Methodological Considerations

Our four studies comprised many different techniques for endpoint measurements. Unfortunately, we were not always able to use gold-standard measurements, since some techniques were too invasive (duodenal aspiration for pancreatic exocrine function) or logistically not available (scintigraphy for gallbladder and gastric emptying). Instead, we chose a combination of alternative techniques, which correlate well with the gold-standard. Several of these tests needed to be operationalized, such as gallbladder emptying ultrasonography, s-MRCP and the $^{13}$C-mixed triglycerides breath test. Their feasibility was assessed in the pilot study. Using multiple tests is one of the strengths of this thesis, since it allowed us to deeply phenotype participants.

For the main study, we included 60 participants. Although this number may seem low, a priori sample size calculations were performed to ensure sufficient power for the primary endpoints. Subsequent analyses have demonstrated that also for the secondary endpoints, a satisfactory number of participants were included. Nevertheless, it should be acknowledged
that the sample size was too small to detect minor changes and did not allow extensive multivariable analyses.

Generally, acute drug interventions lead to the most pronounced physiological changes. However, during prolonged intervention, compensatory mechanisms and/or tachyphylaxis may occur, leading to reduced effects. This is the reason why we assessed responses in the acute and prolonged setting. However, one can question whether 12-week treatment is truly ‘prolonged’ and whether a longer intervention may have yielded different results. For example, a reduction in body weight and hepatic fat content takes time to develop. Moreover, if GLP-1 based therapies increase pancreas volume, this is likely a slow process. A placebo arm was used in all the performed trials, to allow optimal analysis of drug-induced changes. However, an important drawback of comparing with placebo is the inability to exclude effects of glucose-lowering. Nonetheless, since we were simply interested in the effects of GLP-1 based therapies, whether or not caused by effects on glucose, we do not consider this to be a limitation of the thesis.

For the acute intervention studies, exenatide was used; while for the 12-week study, we used liraglutide. As exenatide placebo-pens were not available, we chose intravenous infusion of exenatide for the acute study, since this allowed us to perform a blinded study by comparing IV exenatide with IV saline (‘placebo’). Liraglutide was chosen for the 12-week study because of its once daily administration and possibility to use placebo pens. Both are GLP-1 receptor agonists, yet they differ in their pharmacokinetic properties, which could lead to differential results.

**Future Perspectives**

With the positive results of the currently available large cardiovascular safety trials, GLP-1 based therapies will be increasingly prescribed. However, results of ongoing trials are still eagerly awaited to identify possible differences in safety profiles. Moreover, the results of the GRADE trial (“The Glycemia Reduction Approaches in Diabetes”) are needed to determine how GLP-1 receptor agonists and DPP-4 inhibitors weigh up against sulphonylurea and basal insulin [247]. An interesting future evolution is the current development of respiratory and oral GLP-1 receptor agonists. Since these administration routes bypass the need for subcutaneous injection, they enhance patient comfort, which likely results in better drug adherence.

**Conclusions**

The aim of this thesis was to assess the acute and prolonged effects of incretin-based therapies on the gastrointestinal and cardiovascular systems, in order to better understand their risks and benefits.

First, regarding pancreatic safety, our data are in agreement with recent large-scale observational database studies and RCTs, suggesting that treatment with sitagliptin may slightly increase the risk of development of acute pancreatitis, while no increased risk appears present for liraglutide. Given the low background incidence of acute pancreatitis, the absolute risk of this adverse effect is very small. At this point, there are no data to suggest that GLP-1 based therapies increase the risk of pancreatic cancer, although we feel that the long-term consequences of a tendency to increase pancreatic enzymes, exocrine secretion (sitagliptin) and volume (liraglutide) require longer-term follow up. Nevertheless, the
pancreatic adverse effects of GLP-1 do not seem a major concern. However, we recommend to have reservations with DPP-4 treatment in patients with increased risk for pancreatitis or pancreatic cancer, such as those with hypertriglyceridemia, alcohol abuse or a family history of pancreatic cancer.

Second, GLP-1 based therapies affect biliary physiology; liraglutide increased the levels of deoxycholic acid, likely by altering the intestinal microbiome, while sitagliptin increased hepatic bile acid production. An increase in bile acids could explain the favourable metabolic effects of GLP-1 based therapies, including improved insulin sensitivity and reduced hepatic gluconeogenesis. However, liraglutide also increases the risk for gallstones.

Third, against expectations, we observed no effect of liraglutide or sitagliptin on hepatic fat content. However, a plethora of other studies with more patients and longer follow-up found reductions in hepatic fat and inflammation, demonstrating the potency of these agents for the treatment of NAFLD.

Fourth, GLP-1 receptor agonists increased resting heart rate, likely through direct stimulation of sino-atrial cells in patients with T2DM, although activation of the sympathetic nervous system appears to play a (supplementary) role in healthy volunteers. Large-scale cardiovascular safety trials did not show that the increase in resting heart rate translates to increased mortality, although these trials may not have been of sufficient duration to detect any adverse effects of a small increase in RHR. In contrast, liraglutide and semaglutide treatment improves cardiovascular outcomes compared with placebo.

Figure 3: Weighing the risks and benefits of GLP-1 based therapies. Abbreviations = DPP-4I, dipeptidyl peptidase 4 inhibitor; GLP-1RA, glucagon-like peptide 1 receptor agonist
Fifth, exenatide acutely increased blood pressure, due to effects on cardiac output and/or vascular resistance, yet after prolonged intervention with liraglutide, a reduction in blood pressure was observed. For the latter, no explanation was found, although we excluded vascular effects, sustained natriuresis and weight loss as potential underlying mechanisms. This reduction in blood pressure is a likely contributor to the beneficial effects on cardiovascular outcomes. Importantly, while sitagliptin had no effect on blood pressure in the fasting state, it significantly reduced systolic blood pressure after food intake. This led to a tendency to reduce myocardial blood flow, and future studies should assess the clinical relevance of this.

Sixth, although GLP-1 receptor agonists increased microvascular perfusion in healthy volunteers, this was not observed in our cohort of patients with T2DM.

In conclusion, combining our mechanistic data with those of larger recently published trials, the gastrointestinal and cardiovascular effects of GLP-1 based therapies are predominantly beneficial. We feel that the beneficial effects on glucose, body weight and blood pressure outweigh an increase in resting heart rate with GLP-1 receptor agonists, and a slight increased risk of pancreatitis with DPP-4 inhibitors. The rising popularity of these agents seems justified, especially after the results of the large cardiovascular safety trials demonstrated their cardiovascular safety (and superiority in LEADER and SUSTAIN-6), but also their neutrality when it comes to pancreatic adverse effects. Thus, the extra-glycaemic effects of GLP-1 based therapies, which mediate the benefit in LEADER and SUSTAIN-6, should be considered rather as a friend than a foe.