THE UNIVERSITY OF
WARWICK

Library Declaration and Deposit Agreement

1. STUDENT DETAILS

Please complete the following:

Full name: ....LOUISE ROBINSON.....

University ID number: .....

2. THESIS DEPOSIT

2.1 I understand that under my registration at the University, I am required to deposit my thesis with the University in BOTH hard copy and in digital format. The digital version should normally be saved as a single pdf file.

2.2 The hard copy will be housed in the University Library. The digital version will be deposited in the University's Institutional Repository (WRAP). Unless otherwise indicated (see 2.3 below) this will be made openly accessible on the Internet and will be supplied to the British Library to be made available online via its Electronic Theses Online Service (EThOS) service.
[At present, theses submitted for a Master's degree by Research (MA, MSc, LLM, MS or MMedSci) are not being deposited in WRAP and not being made available via EthOS. This may change in future.]

2.3 In exceptional circumstances, the Chair of the Board of Graduate Studies may grant permission for an embargo to be placed on public access to the hard copy thesis for a limited period. It is also possible to apply separately for an embargo on the digital version. (Further information is available in the *Guide to Examinations for Higher Degrees by Research.*)

2.4 *If you are depositing a thesis for a Master's degree by Research, please complete section (a) below. For all other research degrees, please complete both sections (a) and (b) below:*

(a) Hard Copy

I hereby deposit a hard copy of my thesis in the University Library to be made publicly available to readers (please delete as appropriate) EITHER immediately OR after an embargo period of ..... months/years as agreed by the Chair of the Board of Graduate Studies.

I agree that my thesis may be photocopied. YES / [redacted] *(Please delete as appropriate)*

(b) Digital Copy

I hereby deposit a digital copy of my thesis to be held in WRAP and made available via EThOS.

Please choose one of the following options:

EITHER My thesis can be made publicly available online. YES / [redacted] *(Please delete as appropriate)*

OR My thesis can be made publicly available only after.....[date] (Please give date)
[redacted] / NO *(Please delete as appropriate)*

OR My full thesis cannot be made publicly available online but I am submitting a separately identified additional, abridged version that can be made available online.
[redacted] / NO *(Please delete as appropriate)*

OR My thesis cannot be made publicly available online. [redacted] / NO *(Please delete as appropriate)*

3. GRANTING OF NON-EXCLUSIVE RIGHTS

Whether I deposit my Work personally or through an assistant or other agent, I agree to the following:

Rights granted to the University of Warwick and the British Library and the user of the thesis through this agreement are non-exclusive. I retain all rights in the thesis in its present version or future versions. I agree that the institutional repository administrators and the British Library or their agents may, without changing content, digitise and migrate the thesis to any medium or format for the purpose of future preservation and accessibility.

4. DECLARATIONS

(a) I DECLARE THAT:

- I am the author and owner of the copyright in the thesis and/or I have the authority of the authors and owners of the copyright in the thesis to make this agreement. Reproduction of any part of this thesis for teaching or in academic or other forms of publication is subject to the normal limitations on the use of copyrighted materials and to the proper and full acknowledgement of its source.
- The digital version of the thesis I am supplying is the same version as the final, hard-bound copy submitted in completion of my degree, once any minor corrections have been completed.
- I have exercised reasonable care to ensure that the thesis is original, and does not to the best of my knowledge break any UK law or other Intellectual Property Right, or contain any confidential material.
- I understand that, through the medium of the Internet, files will be available to automated agents, and may be searched and copied by, for example, text mining and plagiarism detection software.

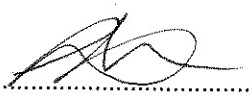
(b) IF I HAVE AGREED (in Section 2 above) TO MAKE MY THESIS PUBLICLY AVAILABLE DIGITALLY, I ALSO DECLARE THAT:

- I grant the University of Warwick and the British Library a licence to make available on the Internet the thesis in digitised format through the Institutional Repository and through the British Library via the EThOS service.
- If my thesis does include any substantial subsidiary material owned by third-party copyright holders, I have sought and obtained permission to include it in any version of my thesis available in digital format and that this permission encompasses the rights that I have granted to the University of Warwick and to the British Library.

5. LEGAL INFRINGEMENTS

I understand that neither the University of Warwick nor the British Library have any obligation to take legal action on behalf of myself, or other rights holders, in the event of infringement of intellectual property rights, breach of contract or of any other right, in the thesis.

Please sign this agreement and return it to the Graduate School Office when you submit your thesis.

Student's signature:  Date: 13-11-13

Obesity and hypertension in Type 2 Diabetes: Exploring the central and peripheral effects of Glucagon-like Peptide-1R signalling

Louise E Robinson, B.Sc. (Dist.), M.Res.

Thesis submitted in full requirement for the degree of Doctor of Philosophy,

University of Warwick

University of Warwick Medical School

July 2012

Contents

Declaration

I am aware of University regulations governing plagiarism and I declare that this PhD document is all my own work except where I have stated otherwise:

Chapter 1: General Introduction. Sections 1.1, 1.2, 1.2.1 and 1.2. were included as part of a review I had written for my previous degree and edited and updated to suit the focus of this degree.

Chapter 4 meta-analysis: The introduction and discussion was co-written with Dr. Paul O'Hare and Dr. Tim Holt.

Louise Emma Robinson

Abstract

Glucagon-like peptide 1 is a hormone involved in the regulation of glucose. Glucose-dependent activation of brain GLP-1R stimulates GLP-1 secretion from the L-cells of the intestine in response to ingested nutrients and it enters the blood stream to direct insulin secretion and to regulate growth and apoptosis of pancreatic cells, regulate the uptake of glucagon secretion as well as other metabolic processes. GLP-1 agonists are a new class of anti-diabetes agent and current research suggests a role in weight and blood pressure reduction. A primary function of GLP-1 is postulated to act as a neuropeptide in the regulation of metabolic and cardiovascular function through gut-brain regulatory feedback. As a secondary effect, GLP-1 may also increase natriuresis by direct action on sodium transport molecules in the kidney and hence play a role in long-term blood pressure homeostasis. The aims of this thesis is to 1) ascertain the effects of GLP-1 agonists on blood pressure, heart rate and body weight in T2DM patients through a systematic review and meta-analysis and 2) to explore the expression of renal GLP-1R during metabolic syndrome (obesity) and diabetes in animals and also hyperglycaemia-like conditions in isolated cells of the human renal proximal tubule and collecting duct. I also investigate for the first time GLP-1R mediated expression of sodium transport molecules in cells of the human collecting duct. I confirm that GLP-1 agonists produce significant weight reducing effects and also a beneficial reduction in blood pressure in T2DM patients. However, there is a tendency for these agents to increase heart rate which may be related to their effects on the autonomic nervous system and direct activation of sino-atrial node GLP-1R. I show evidence that renal

GLP-1R expression is inhibited during obesity and diabetes which might be a consequence of peripheral insulin signalling or regulatory feedback from pancreatic β-cells to the CNS but which needs to be further clarified. GLP-1 was also shown to modestly up-regulate α- ENaC mRNA and protein expression, possibly as a consequence of activation by SGK-1 and PKC-dependent ERK activation in HCD cells. The significance of this paradoxical finding in a clinical scenario is unknown because 1) GLP-1(7-36) should theoretically undergo enzymatic degradation in peripheral tissues and further degradation and elimination in the proximal tubule. However, potential for long-acting GLP-1 agonists to remain in the kidney for longer might mean that these agents reach the collecting duct whereby paradoxical sodium reabsorption may occur. This might also be reflected in the meta-analysis findings that exenatideLAR preparations reduce blood pressure less than shorter acting formulations.

I conclude that GLP-1 signalling is dysregulated in obesity and diabetes and that treatment with GLP-1 agonists confer favourable effects on body weight and blood pressure. However, due to effects on heart rate further safety studies are required to reassure long term cardiovascular safety and autonomic mechanisms behind the cardiovascular and metabolic effects of GLP-1 needs to be explored further.

Acknowledgements

With special thanks to the following people who contributed to the projects contained within this thesis:

Dr. Rosemary Bland and Dr. Claire Hills, for donating HCD and HKC8 cells and for offering valuable technical advice.

Dr. K Lewendowski for supplying samples from pregnant and normal T1DM patients.

Professor Michael Khan and Harpal Randeva for contributing the murine kidneys used in this research.

Dr. Tim Holt, Dr. Karen Rees, Dr. Paul O'Hare and Dr. Harpal Randeva for their kind support and contribution to the meta-analysis and also trial authors for their co-operation in supplying data used for this study

With special thanks to Dr. Paul O'Hare, Dr. Harpal Randeva and Margaret Thorogood for their continued support.

Figures and Tables

Abbreviations

ADA	*American Diabetes Association*
ANOVA	*Analysis of variance*
CENTRAL	*Cochrane Central Register of Controlled Trials*
DBP	*Diastolic Blood Pressure*
EASD	*European Association for the Study of Diabetes*
EMEA	*European Medicines Agency*
FDA	*Federal Drug Administration*
GLP-1	*Glucagon-like peptide 1*
HR	*Heart Rate*
LDL	*Low Density Lipoprotein Cholesterol*
LOCF	*Last Observation Carried Forward*
OAD	*Oral Anti-diabetic Drug*
SBP	*Systolic Blood Pressure*
SD	*Standard Deviation*
SEM	*Standard Error of the Mean*
WMD	*Weighted Mean Difference*
WHO	*World Health Organisation*
PVDF	*Polyvinylidine fluoride*
SGK-1	*Serum/Glucocorticoid regulated kinase 1*
ENaC	*Epithelial sodium channel*
Exendin-4	*Exenatide*
GPCR	*G-protein coupled receptor*
GIP	*Gastric inhibitory peptide*
NHE3	*Sodium hydrogen exchanger 3*
PKA	*Protein kinase A*
PKC	*Protein kinase C*
ERK	*Extracellular- signal regulated kinase 1*
MAPK	*Mitogen activated protein kinase*
HCD	*Human collecting duct cells*
HKC8	*Human proximal tubule cells*

Publications

Robinson LE, Bennett J, Lewendowski KC, Randeva H, O'Hare, PO (2010) Biologically Active metabolite of Glucagon-Like Peptide-1 increases during normal pregnancy and in well controlled type 1 diabetes mellitus patients. *Diabetic Medicine*, **27** (Suppl. 1), 37-188.

Robinson LE, Holt TA, Rees K, Randeva H, O'Hare PO (2012) Effects of Liraglutide and Exenatide on heart rate blood pressure and body weight: Systematic review and meta-analysis: In Press.

1.0 General introduction

1.1 Type 2 diabetes

Recent advances in molecular biology have increased our understanding of type 2 diabetes which is currently considered to be “a complex metabolic disorder with multiple causes” (Leroith, 1999). Impaired insulin action give rise to abnormalities in non-oxidative glucose disposal, as well as defects in glucose uptake by skeletal muscle and phosphorylation (Leroith, 1999; Khan *et al*, 1996). Consequently, release of free fatty acids by adipocytes and enhanced glucose production in liver leads to hyperglycaemia. It is understood that defects in an early post-receptor step in insulin action result in insulin resistance (Abate & Chanalia, 2001; Leroith, 1999). Over a period of years, the ensuing hyperglycaemia results in damage to the heart, blood vessels, kidneys and nerves (WHO, 2008). Global mortality is estimated at 2.9 million deaths attributable to diabetes of which large proportions were the result of concomitant heart disease and stroke. In 2004, 68% and 16% of deaths were attributed to these two disorders, respectively (CDCP, 2011). In 2010, the estimated world-wide prevalence of diabetes was 285 million (6.4%) which is predicted to rise to 439 million (7.7%) by 2030 (Shaw *et al*, 2010). Type 2 diabetes is understood to account for more than 90% of all diabetes cases (Gonzalez *et al*, 2009). The burden to healthcare systems and through loss of earnings is a global concern, especially in low income countries where the disease is most prevalent due to transition to a westernised diet and lifestyle (otherwise coined “nutrition transition”). For example, in 2005 the estimated loss of national income due to heart disease,

stroke and diabetes was $1.6 billion for United Kingdom with China being the country with the highest loss of $18 billion (WHO, 2006).

1.2 Aetiology and pathogenesis of T2DM:

1.2.1 Relationship of diabetes to nutrition and the development of obesity.

Type 2 diabetes mellitus (T2DM) is a progressive disease signified by marked β-cell dysfunction and insulin resistance. Over 90% of those who suffer from T2DM are or have been obese (Nolan *et al*, 2011). Transition from traditional rural environments to urbanisation particularly raises the risk of developing obesity and T2DM. It has been well documented that the important preventative factor seen in the diets of certain ethnic groups is the presence of allelochemicals and complex polysaccharides in unrefined foods that are largely removed from the Western diet to increase palatability and hasten preparation times. These allelochemicals have been shown to regulate the absorption of sugar through the small intestine, for example through inhibition of α-glucosidase and thereby inhibit post-prandial glucose excursions and fasting glucose concentrations (Day & Bailey, 1988; Jenkins *et al*, 1976; Ali *et al*, 1996). Some polysaccharides have been found to slow gastric emptying and act on the endocrine system to increase secretion of gastro-intestinal hormones, as well as the action of short chain fatty acids, coming from fermentation (Dall' Agnoll & Lino von Poser, 2000). The composition of foods is well documented to modulate the release of gastro-intestinal hormones involved in glucose metabolism, hunger and satiety as has been reviewed previously (Karhunan *et al*, 2008). Deregulation of gastro-intestinal hormones such as Glucagon-like peptide-1, leptin and ghrelin

possibly as a consequence of over-nutrition has been implicated in the pathogenesis of T2DM.

1.2.2 Maternal and child health

Until 1990, type 2 diabetes was considered to be a disease only affecting people later in life as has been reviewed by Nolan (2009). Nolan points out, that with more and more younger people becoming obese, pregnant women the disorder is now affecting pregnant women (Reinehr *et al*, 2010; McIntyre *et al*, 2009), the majority of whom are from migrant ethnic groups (Mayer-Davis *et al*, 2009). Many young women are not diagnosed as suffering from T2DM until they become pregnant and unfortunately the prognosis is often poor with high rates of congenital malformations and perinatal death (Cundy *et al*, 2000; Clausen *et al*, 2005). Intrauterine growth restriction has been implicated as a causal link for adult diseases such as obesity, hypertension, T2DM and CVD (Chen *et al*, 2009; Plagemann *et al*, 2009). Furthermore there are suggestions that gestational or T2DM in pregnancy can affect diabetes risk in offspring. Interestingly, in his review, Nolan highlights results from a Danish study comparing the offspring of mothers with and without diabetes, the probability for pre-diabetes or T2DM was higher in the offspring of women who had gestational diabetes or type 1 diabetes at age 22 years (Clausen *et al*, 2008, reviewed in Nolan, 2009). The concomitant risk of T2DM in offspring of Type 1 diabetes mothers was found to be greater if maternal hyperglycaemia was present in the third trimester (Clausen *et al*, 2008). Evidence from animal studies indicates that neonatal programming can affect neurohormonal weight control signalling and development of pancreatic islets (Chen *et al*, 2009).

1.2.3 Adiposity and anthropometric determinants

Studies of obesity are highly relevant to the aetiology of T2DM. Genetic factors contribute to the occurrence of T2DM and obesity, respectively. It has been known for many years that obesity is a causative factor of type 2 diabetes and cross-sectional and longitudinal studies have shown that high body mass index (BMI) seems to be associated with increased risk of diabetes in both genders and between all ethnic groups (Nakagami *et al*, 2003). More recently it has been documented that certain ethnic groups convey a greater susceptibility to diabetes than do Caucasians at lower BMI (Misra *et al*, 2005; Nakagami *et al*, 2003).

Another major pathogenic factor seems to be the actual distribution of adipose, namely android (intra-abdominal) distribution. Central abdominal obesity acts as an independent diabetogenic factor due to the fact that it is more closely related to insulin resistance. This has been attributed to its high lipolytic rate that as a consequence elevates portal and peripheral levels of free fatty acids leading to hepatic and muscle resistance, respectively (Abate & Chandalia, 2001).

1.3 Animal models for the study of diabetes and obesity

Many researchers have attempted to construct mouse models of Type 2 diabetes mellitus. Murine models such as *ob/ob* (C57BL/6, BL/6) and *db/db* (C57BL/Ks, BL/Ks) mice and the Zucker fatty rat are primarily models of obesity which has been comprehensively reviewed by Leroith (Leroith, 1999). Obesity is sequelae to diabetes in these animals where massive weight gain is caused by mutations in leptin or its receptor (Zhang *et al*, 1994; Chen *et al*, 1996). Roughly 75-80% of patients with type 2 diabetes is

or has previously been obese. A characteristic of the BL/6 mouse model is that they are massively overweight due to being homozygous for the obese (*ob*) gene. It has also been suggested that obesity in humans involves defects in the brain receptor for the *ob* protein and not a failure to synthesise the protein itself (Blackburn & Kanders, 1994). The finding that the *ob* genes protein product, leptin, is a regulatory protein that, if administered to the obese mice can control the accumulation of adipose, thus aiding weight loss was considered to be a breakthrough in this research area as leptin might alleviate obesity and aid to prevent development of type 2 diabetes. McKeigue and colleagues believe this finding to be especially important in the prevention or maintenance of diabetes due to the fact that it controls maturity- onset obesity (McKeigue *et al*, 1991), and diabetes is largely associated with increasing age. However, suggestions that leptin is important in glucose homeostasis in humans has since been disproved by Froguel (2001) and his team who found evidence to suggest that melanocortin 4 (MC4), which is initiated by leptin, has a more important role in glucoregulation (Froguel & Boutin, 2001). Froguel and Boutin (2001) also state that "although leptin is a prime candidate gene for monogenic obesity, it still may not reside at the core of this metabolic disorder but may instead highlight our ignorance to the intricate biological processes involved" (Froguel & Boutin, 2001). More recently it has been noted that there is considerable inter- individual variability in the relationship of leptin to total adiposity. Therefore, many people who are obese have normal levels of leptin. This finding may suggest that other genes may play a significant role in this disease. Franks and co-workers suggest that disparities between

studies of the role of leptin may be due to differences in experimental design (Franks *et al*, 2003). These researchers carried out a large population based cohort study (The Ely Study). It was demonstrated that a significant inverse association exists between physical activity energy expenditure and plasma leptin concentrations after adjustments for percent body fat or body mass index (Franks *et al*, 2003).

A detailed description of the genetics of the BL/6 and BL/Ks mice is detailed in a review by Clee & Attie (Clee & Attie, 2007) The BL/6 strain shares some *Mus musculus molossinus* genomic regions and is evolutionarily diverged from other transgenic strains except for other C57 and C58 strains (Williams *et al*, 2001). The BL/Ks strain background is composed of BL/6 with contributions from at least 3 mouse strains including C57BL/10, 129 and another unidentified strain that composes approximately 10% of the genome (Clee & Attie, 2007). The severity of diabetes differs in both BL/6 and BL/Ks strains and is dependent on homozygosity for defective genes, Lep^{ob} (encoding the protein product, leptin) or Lep^{db} (encoding for the leptin receptor), respectively. BL/6 mice display decreased insulin secretion and glucose intolerance, although in comparison to BL/Ks these mice are relatively resistant to obesity-induced diabetes (Harris *et al*, 2001). Original experiments by Douglas Coleman over 30 years ago exemplified the differences in strain background to obesity-induced diabetes (Hummel *et al*, 1972; Coleman *et al*, 1973). Coleman showed the obese mice in the BL/Ks strain background to display a high severity of diabetes whereas the obese BL/6 mouse, although displaying severe insulin resistance did not develop overt diabetes (Coleman *et al*, 1992). The BL/6 strain background displays

approximately 220 mg/dl fasting blood glucose, equating to moderate hyperglycaemia but this returns to near normal levels as a result of β-cell expansion and corresponding insulin secretion (Hummel *et al*, 1972). This characteristic is not seen in BL/Ks background and hence these were named *db/db* mice due to ensuing islet atrophy which results in sustained hyperglycaemia (282.9 ± 77.48 mg/dL plasma glucose by 10 weeks of age) and overt diabetes (Sharma *et al*, 2003; Hummel *et al*, 1972). The generally accepted threshold for diagnosis of diabetes in mice is 250 mg/ dL (Clee *et al*, 2007). BL/6 strains are also relatively resistant to diabetic nephropathy.

a)

b)

Figure 1.3.0.1 - Typical glycaemic profiles of a) *ob/ob* (C57BL/6, BL/6) and b) *db/db* (C57BL/Ks, BL/Ks) strains. Figures obtained from Jackson laboratory website, 2011: BL/Ks mice show marked hyperglycaemia by 8 weeks of age as compared to BL/6 mice which display moderate hyperglycaemia. Compensation of high circulating glucose by increased β-cell proliferation and hyperplasia protects BL/6 strains from the development of overt diabetes.

The tamoxifen-induced hyperglycaemic mouse model is a model of diabetes cleverly designed to allow a direct comparison to be made between the effects of high circulating glucose levels and normoglycaemia in the same animal. During chronic hyperglycaemia there are alterations in β-cell gene expression, survival and function. Therefore in models of hyperglycaemia, β-cells display decreased expression of genes important for glucose stimulation of insulin secretion such as *glucose transporter 2* (*GLUT2*), *glucokinase* and *pre-proinsulin* (*Ppi*) and some transcription factors that control their expression (Pascal *et al*, 2008). The c-MYC proto-oncogene is understood to be a transcription factor involved in modulating a large number of genes involved in cell proliferation, differentiation and apoptosis (Evan & Littlewood, 1993; Harrington *et al*, 1994). Up-regulation of c-MYC during chronic hyperglycaemia has been shown to trigger apoptosis in β-cells and reduce the expression of *Ppi* and other genes important for β-cell function, leading to diabetes. Furthermore, forced activation of c-MYC has also been shown to reduce expression of *Ppi*, induce β-cell apoptosis and thus result in the induction of hyperglycaemia (Pascal *et al,* 2008).

In the tamoxifen model, fusion of the hormone binding domain of the oestrogen receptor to the *Ppi* promoter initiates c-MYC activation upon administration of oestrogen, and thus makes c-MYC dependent on exposure to oestrogen. The major drawback to this method is the potential for endogenous oestrogens to bind to the hormone binding domain *in vivo* which therefore hinders the interpretation of consequent transcriptional activation of β-cell regulatory genes (Littlewood *et al*, 1995). Therefore, Littlewood and co-workers modified this method to use a transcriptionally inactive mutant of

the murine oestrogen receptor which binds to the synthetic ligand, 4-hydroxytamoxifen. This method was further developed to specifically alter c-MYC in the β-cell by Professor Khan and co-workers to enable researchers to directly study the effects of hyperglycaemia *in vivo*. Fusion of the mutant oestrogen receptor to the C-terminus of the c-MYC protein results in β-cell apoptosis (more so than proliferation) which is dependent on 4-hydroxytamoxifen, but is not influenced by 17- β-oestradiol (Littlewood *et al*, 1995).

1.3.1 Hypertension in obesity and diabetes: Focus on sodium transport by epithelial sodium channel (ENaC)

The kidneys play a pivotal role in regulation of blood volume (BV), and hence blood pressure by adjusting sodium and water loss into urine. Renal control of BV is achieved in various ways. An increase in BV raises arterial pressure, renal perfusion and glomerular filtration rate (GFR) through an increase in renal water and sodium concentration and is termed “pressure natriuresis”. Key hormones, angiotensin and aldosterone stimulate distal tubular sodium reabsorption by activating intracellular sodium transporters and thus decrease sodium and water loss by the kidney. Activation of the renin angiotensin aldosterone system (RAAS) is complimented by another important hormone, vasopressin (antidiuretic hormone, ADH) which is secreted from the posterior pituitary gland. One of the actions of vasopressin is to stimulate water reabsorption by the collecting duct thereby decreasing water loss and increasing BV. The principle role of counter-regulatory hormones such as atrial natriuretic peptide is to increase water and sodium loss by the kidney and thus a concert of mechanisms ultimately maintains blood volume (and blood pressure) within set limits.

An increase in BV results in a corresponding increase in arterial pressure by raising central venous pressure, right atrial pressure and right ventricular end diastolic pressure and volume. An increase in left ventricular preload and stroke volume results from the augmented right ventricular stroke volume and hence pulmonary venous blood flow to the left ventricle. The resultant increase in stroke volume raises cardiac output and arterial blood pressure, a phenomenon coined, the Frank Starling mechanism.

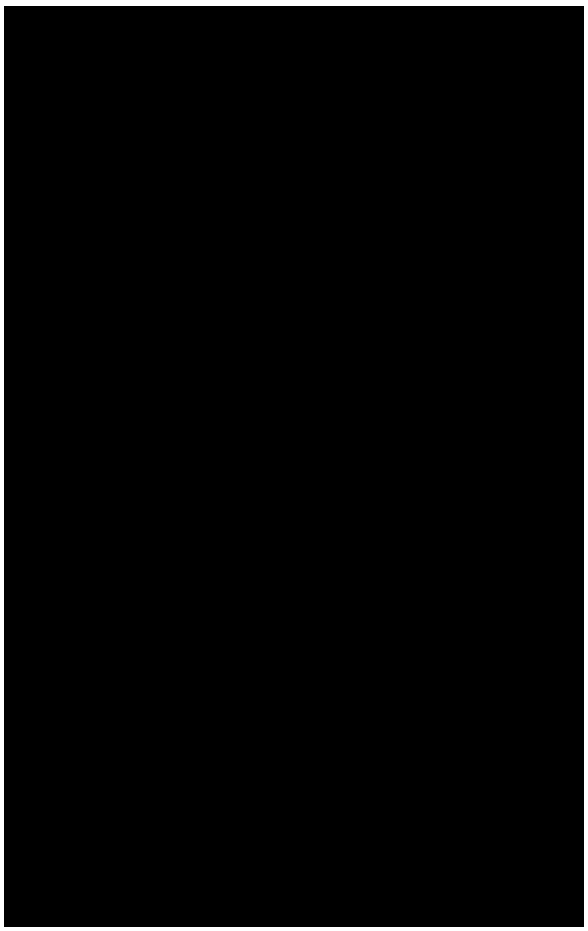
Obesity alone and as sequelae to T2DM results in abnormal renal function and treatment resistant hypertension. In human obesity, excess renal sodium reabsorption in the Loop of Henle results in a shift of pressure natriuresis which initiates raised blood pressure as a result of weight gain. Mechanisms behind the increased sodium reabsorption and altered pressure natriuresis in obesity include activation of renin, aldosterone-angiotensin system (RAAS) and sympathetic nervous systems, physical compression of the kidneys due to excess intra-renal adipose tissue and increased extra-cellular matrix (Hall, 1999). In human obesity, sympathetic activation is mediated in part by elevated leptin and interactions with neuropeptides in the hypothalamus. In the early phases of obesity, glomerular hyperfiltration and increased arterial pressure help to compensate for increased tubular reabsorption and homeostasis is maintained. However, a subsequent increase in glomerular capillary wall stress, activation of neurohormonal systems, increased lipids and glucose intolerance, eventually lead to loss of nephron function (Hall, 1999). Over time the compensatory increases in arterial pressure to maintain sodium balance results in hypertension which is difficult to resolve with anti-hypertensive therapy.

Sodium intake has long been understood to be positively correlated with high blood pressure upon increasing age. To exemplify this, epidemiological research has shown that certain indigenous communities who survive on a low sodium diet, such as the Yanamamo Indians of the Brazilian Amazon, as a consequence do not suffer increasing blood pressure with age compared to people from developed countries who in contrast, consume on average twenty times more sodium per day (Takahashi *et al*, 2011). Furthermore, the obese and diabetic state exacerbates sodium retention by increasing sodium reabsorption at the kidney tubules (Defronzo, *et al,* 1975). For example, during metabolic syndrome (obesity) and early stages of T2DM, hyperinsulinemia leads to metabolic insulin resistance. Contrary to this, insulin resistance does not to occur in the kidney where insulin has been shown to enhance renal sodium reabsorption (Defronzo *et al,* 1975). Of the sodium transporters involved the main regulators of sodium reabsorption are epithelial sodium channel (ENaC) in the distal portion of the nephron and sodium-proton exchanger type 3 (sodium-hydrogen exchanger 3, NHE3) in the proximal tubules (Horita *et al,* 2011). Similarly, hyperglycaemia and hyperaldosteronism which also occur during obesity and diabetes increase sodium reabsorption through similar mechanisms. This effect is compounded by the “obesogenic” or modern day diet which is also low in potassium which under normal conditions enhances excretion of sodium by the kidneys (Takahashi *et al,* 2011). Therefore, obesity and diabetes results in dysregulation of key sodium transport molecules exacerbating the impact of the western diet on blood pressure regulation.

Discovery of a severe monogenic form of hypertension, Liddle's syndrome was an important breakthrough in our understanding of the critical role of ENaC in the pathogenesis of hypertension. Liddle's syndrome is characterised by severe hypertension and hyperkalaemia with negligible secretion of aldosterone. Dysregulation of the sodium channel ENaC is able to independently produce severe and sustained hypertension (Bubein *et al,* 2010). Furthermore, obesity-related hypertension and Liddle's syndrome, although physiologically distinct with regards to circulating insulin, glucose and aldosterone levels, display hyperactive ENaC and decreased plasma potassium levels. Furthermore, both disorders are resistant to anti-hypertensive agents, emphasizing the importance of increasing our understanding of the physiological role of ENaC in the pathogenesis of hypertension (Horita *et al*, 2011; Bubein *et al*, 2010).

As already mentioned, extra-cellular fluid volume is maintained by regulation of Na^+ by the kidney. This process is essential for maintaining fluid and electrolyte balance and regulation of blood pressure. The majority of sodium is reabsorbed in the proximal tubule (**Figure, 1.3.1.1 The Renal Commission of the International Union of Physiological Sciences (IUPS) schema of the nephron**). However, the distal portion of the nephron (**Figure, 1.3.1.1**) is ultimately responsible for blood pressure regulation as a result of changes in urine flow and Na^+ excretion (Petri-Pederdi *et al*, 2002). In the cortical collecting duct, sodium is transported across the apical membranes of salt- reabsorbing epithelia. ENaC moves sodium ions into the cell by electrodiffusion and is sensitive to amiloride (and its analogues), a K^+-sparing natriuretic and mild diuretic. In the late distal convoluted tubule,

connecting tubule and collecting duct, sodium reabsorption is mainly controlled by aldosterone. During this process, salt enters the epithelial cell via ENaC at the apical plasma membrane and sodium is expelled into the interstitial fluid via the basolateral Na^+-K^+-ATPase in exchange for potassium (Christensen *et al*, 2010). It is well understood that aldosterone stimulates P13-K which promotes the activity of ENaC through upregulation of serum/ glucocorticoid regulated kinase-1 (SGK-1) (Thomas *et al*, 2007; 2008). ERK activation has been shown to suppress ENaC activity and is dependent on activation of PKC (Thomas *et al*, 2008) and therefore, aldosterone is responsible for dual control of ENaC by both activating and suppressing its activity. Interestingly, recent evidence also suggests that another GPCR and component of RAAS, angiotensin II regulates ENaC and this effect has been found to be due to activation of PKC, independent of Ca^{2+} and which acts to activate NOX and consequent superoxide generation. This effect is understood to be through activation of the angiotensin receptor AT_1 (Petri-Pederdi *et al*, 2002; Sun *et al*, 2011).



1. Renal corpuscle including Bowman's capsule and the glomerulus (glomerular tuft).
2. Proximal convoluted tubule
3. Proximal straight tubule
4. Descending thin limb
5. Ascending thin limb
6. Distal straight tubule (thick ascending limb)
7. Macular densa located within the final portion of the thick ascending limb
8. Distal convoluted tubule
9. Connecting tubule
10. Cortical collecting duct
11. Outer medullary collecting duct
12. Inner medullary collecting duct

Figure, 1.3.1.1- The Renal Commission of the International Union of Physiological Sciences (IUPS) schema of the nephron: Depicts the short-looped and long-looped nephron as well as the collecting duct system. The dashed line within the cortex represents a medullary ray (from Kriz & Bankir, 1988).

Figure, 1.3.1.2 Aldosterone signalling in the kidney. Binding of aldosterone to MR and a distinct non-genomic receptor is understood to initiate rapid signalling events including activation of PKC family isoforms which is central to rapid stimulation of membrane ion transport in the distal nephron. ENaC activity is the rate-limiting step in Na^+ reabsorption. Trafficking and stability of pre-expressed ENaC is subject to delayed regulation by SGK-1 expression. ERK1/2 activation and increasing intracellular pH promotes basolateral recycling through activation of Ca^{2+} and ATP-dependent K^+ channels which is also dependent on PKC (From, Thomas *et al,* 2007).

ENaC is a member of the ENaC/ degenerin gene family (Kellenberger & Schild, 2002) and cloning has identified it to be composed of five ENaC subunits: α, β, γ, δ, and ε-ENaC (Ji *et al*, 2006). It is understood that β and γ subunits act as regulators of channel activity, whereas α, δ, ε- ENaC subunits are conductive and form heteromers in epithelial cells (**Figure, 1.3.1.3 Structure of ENaC and membrane topology)**(Ji *et al*, 2006). ENaC channels are understood to be selective for cations over anions and display a higher affinity for Na^+ over other cations (Kucher *et al*, 2011). Ji and co-workers describe ENaC channels to vary in Na^+ conductance, P_{Na}/P_k and amiloride affinities depending on the tissues and species where the channels are located. This is believed to be the consequence of differing heteromers of ENaC being present in the various types of epithelial cells (Ji *et al*, 2006). ENaC subunits share a common secondary and tertiary structure that consists of a large extracellular domain separated from shorter cytosolic NH_2- and COOH-tails by two transmembrane domains (Kucher *et al*, 2011). Complex regulatory mechanisms exist for ENaC resulting in rapid responses by signalling cascades which occur within minutes and later transcriptional changes occurring within days (Thomas *et al*, 2007, 2008). Serum glucocorticoid kinase-1 (SGK-1) is known to be associated with the regulation of a wide variety of channels including ENaC, Na^+ K^+ ATPase and NaCl co-transporters (Lang *et al*, 2009). SGK-1 is also understood to be activated by a variety of mechanisms including insulin, and growth factors and its expression is stimulated by many mechanisms such as glucocorticoids, mineralocorticoids and hyperglycaemia (Lang *et al*, 2009).

The critical role of ENaC in sodium homeostasis has been exemplified by our understanding of Liddle's syndrome which is caused by sodium retention resulting from mutations in the C-terminus of the β- or γ- subunit (Hansson *et al*, 1995). Reports in the literature suggest that α- ENaC is vital for the functionality of the ENaC channel and that it is the availability of the α-subunit that influences the level of ENaC activity in the distal portion of the nephron (Thomas *et al*, 2008; Canessa *et al*, 1994; Snyder *et al*, 2000). It has been demonstrated that during co-expression of β- and/ or γ- subunits with α- ENaC there is a resultant increase in Na^+ current (Canessa *et al*, 1994; Snyder *et al*, 2000). The functional significance of α- ENaC required for Na^+ transport can be further exemplified by Hummler and co-workers intricate studies in mice genetically lacking α- ENaC (Hummler *et al*, 1996). Hummler and co-workers describe mice lacking α- ENaC were born with morphologically normal lungs but were unable to clear fluid from the airways and died within the first two days after birth (Hummler *et al*, 1996). However, this lethal effect appears to highlight the importance of functional α-ENaC in the lung. Depletion of renal α-subunit results in metabolic acidosis but the renal pathology is not as severe as in β (-/-) or γ (-/-) mice (Bonny *et al*, 2000). In contrast mice lacking β- and γ- ENaC subunits show a delay in lung water clearance and sodium reabsorption and subsequently die from an electrolyte imbalance as a consequence of abnormal renal function (Giallard *et al*, 2000).

Figure, 1.3.1.3 Structure of ENaC and membrane topology. ENaC is composed of three partly homologous subunits inserted into the membrane with a suggested topology of 2α: 1β: 1γ. Subunit α-ENaC is able to produce small amiloride sensitive Na^+ currents which are greatly enhanced by β and γ-ENaC subunits (From Gormley *et al,* 2003).

1.4 Diagnosis of T2DM

A diagnosis of diabetes is made by evidence of either recurrent or persistent hyperglycaemia, confirmed by one of the following blood plasma concentrations of glucose (WHO, 2006):

1) Fasting plasma glucose (FPG) level of ≥7.0 mmol/l (whole blood, ≥6.0 mmol/l).

2) Two hour plasma glucose concentration of ≥11.1mM following ingestion of 75g anhydrous glucose in an oral glucose tolerance test (OGTT)

3) A random venous plasma glucose concentration of ≥11.1 mmol/l

Pre-diabetes is confirmed by:

1) Impaired fasting glucose (IFG) levels between 6.1 and 6.9mM and/ or 2h plasma glucose **<**7.8mM.

2) Impaired glucose tolerance (IGT) of FPG <7.0mM and 2h plasma glucose >7.8mM (but <11.1mM).

In addition to this the latest WHO guidelines state a cut-off value HbA1c of 48 mmol/mol (6.5%) and that in asymptomatic patients a repeat laboratory test and if deemed to be high risk (<48 mmol/mol, 6.5%) repeat at 6 monthly intervals. Patients with HbA1c less than 6.5% may still meet criteria for T2DM (high risk, 42-47 mmol/mol with the possibility that patients with HbA1c under this value may still be high risk) WHO, 2011.

1.4.1 Treatment approaches and algorithms

There are a number of algorithms which recommend haemoglobin A1C targets and clinical treatment of T2DM. These are developed by the regulatory authorities: American Diabetes Association, European Association for the Study of Diabetes, ADA/EASD (**Figure, 1.4.1.1 ADA/ EASD**

algorithm for the pharmacological management of type 2 diabetes), American Association of Clinical Endocrinologists (AACE), US Department of Veteran Affairs (VA/DoD), International Drug Federation (IDF) Canadian Diabetes Association (CDA) and National Institute for Health and Clinical Excellence (NICE). All of the proposed algorithms vary slightly in the approach to diabetes care and haemoglobin A1C targets. One of the important recent changes to the ADA/EASD algorithm is the initiation of drug therapy as well as lifestyle changes at start of treatment. Previously it was recommended that patients begin with lifestyle changes and after review be put on first line therapy such as metformin (**Figure, 1.4.1.1**). The AACE algorithm is more complex and features drugs such as colesevolam which is a bile acid sequestrant used to reduce low-density lipoprotein cholesterol, rather than an OAD. The main strength of this algorithm is the recommendation that the approach to therapy is tailored to the individual depending on AIC levels at diagnosis (Rodbard *et al,* 2009). Most algorithms are metformin based. The NICE guideline is unique in that its A1C targets are based on more achievable goals depending on success of initial treatment (from 6.5% in monotherapy to <7.5% in combination therapy). It may be argued that this is a logical approach for patients who are not able to reach glycaemic targets and to minimise the number of OADs prescribed in such circumstances (NICE, 2012). Currently, newer agents such as the glucagon-like peptide agonists (GLP-1 agonists) are incorporated into combination therapy in patients who fail to maintain target values or who are not tolerant to other agents.

Figure, 1.4.1.1- ADA/ EASD algorithm for the pharmacological management of type 2 diabetes. ADA/ EASD instruct to reinforce lifestyle regimen during each clinical evaluation. “A) Consider beginning at this stage in patients with very high HbA1c (e.g., ≥9%). B) Consider rapid-acting, non-sulfonylurea secretagogues (meglitinides) in patients with irregular meal schedules or who develop late postprandial hypoglycaemia on sulfonylureas... D) Usually a basal insulin (NPH, glargine, detemir) in combination with noninsulin agents. E) Certain noninsulin agents may be continued with insulin”. (From, Inzucchi *et al,* 2012).

2.0 From the identification of GLP-1 to the development of GLP-1 drugs

Hormones secreted from the gastrointestinal tract after food intake act to stimulate insulin secretion from islet beta cells in response to glucose. This phenomena is coined the “incretin effect” and was first noticed during experiments whereby plasma insulin responses were compared in patients given either intravenous or oral glucose load. It could be seen that insulin secretion is far less (≈40%) after intravenous administration than when given orally. These findings indicate that mechanisms in addition to arterial blood glucose levels regulate insulin secretion (Perly & Kipnis, 1967). The first incretin hormone was identified in the 1970s and named glucose-dependent insulinotropic polypeptide (GIP). GIP was shown to be a potent stimulator of glucose-dependent insulin secretion but removal of GIP from gut extracts did not abolish the incretin effect, indicating the existence of additional peptides with incretin-like activity. Many such hormones have been identified and include a superfamily of hormones such as, Glucagon-like peptides (GLP-1 and GLP-2), secretin, glucagon, vasoactive intestinal peptide (VIP), glucose-dependent insulinotropic peptide (GIP), glicentin and oxyntomodulin (Nancy *et al*, 2000; Manucci & Rotella, 2008).

The novel Glucagon-Like Peptide- 1 (GLP-1) derivatives are a new class of type 2 diabetes drugs which are based on the incretin hormone. The primary action of these pharmacotherapies is significant improvement in postprandial glucose excursions and reduced incidence of hypoglycaemia (Grieve *et al,* 2009). Ceriello and colleagues propose management of postprandial glucose levels to be vital in reducing incidence of macrovascular and

microvascular complications as postprandial hyperglycaemia (plasma glucose > 7.8 mmol/l) is a common feature, despite sufficient overall metabolic control (Ceriello *et al*, 2008). Endogenous GLP-1 secretion has been shown to be diminished in T2DM and obese patients (Muscilli *et al*, 2007; Manucci *et al*, 2000; Holst & Gromada 2004; Vilsbøll *et al*, 2003). Perhaps, as mentioned in the introduction, a diet rich in high calorific foods but lacking the essential complex polysaccharides found in traditional ethnic diets, could be loosely speculated to reduce liberation of this gastro-intestinal hormone. In particular, GLP-1 has not been found to be diminished in type 1 diabetes the pathogenesis of which is not directly related to a westernised lifestyle (Huml *et al*, 2011; Visbøll *et al*, 2003).

Exenatide (termed Exendin-4 at its developmental stage) which shares 53 per cent homology with GLP-1 and Liraglutide, a GLP-1 analogue which shares 99 per cent homology are the primary GLP-1 drugs to enter the market, although other preparations including long-acting once-weekly formulations are now becoming available. The main actions of this class of drug are to stimulate insulin secretion, increase β-cell neogenesis, inhibit β-cell apoptosis, inhibit glucagon secretion, and slow gastric emptying and to increase satiety (Ceriello *et al*, 2008) (**Figure 2.0.1- Biological functions of GLP-1**). An exciting discovery is that GLP-1 derivatives have been reported to display beneficial effects on blood pressure and body weight, making these drugs extremely promising therapeutic agents (Nauck *et al*, 2009; Garber *et al*, 2008). Importantly, it is suggested that the blood pressure effects reported in clinical trials might be independent of concurrent weight loss (Varanasi, *et al*, 2011), although this is yet to be confirmed.

Figure 2.0.1- Biological functions of GLP-1. GLP-1 acts directly on endocrine pancreas, GI- tract, heart and brain. (From: Drucker *et al,* (2006) *Cell Metab*. **3**: 154).

2.1 GLP-1 and the distribution and characterization of its receptor

The polypeptide precursor of the glucagon gene, proglucagon encodes two glucagon-like peptides (GLP-1 and GLP-2) that shares roughly 50% amino acid homology to pancreatic glucagon (**Figure 2.1.1 The proglucagon gene**, **localized on human chromosome 2q36eq37**.). GLP-1 is a peptide which is synthesized in intestinal endocrine cells (namely, L- cells), in two chief molecular forms, GLP-1 (7-36) amide and GLP-1 (7-37). In 1985, cloning of preproglucagon showed the full length N-terminal extended forms (GLP-1 (1-36 amide) to display an insulinotropic effect but the truncated form GLP-1 (7-36) produces a stronger effect (Schmidt *et al*, 1985). By 1987 this had been demonstrated by several groups (Orskov *et al*, 1987; Kreymann *et al*, 1987).

Further to this, it was reported that GLP-1 (7-37) increases the intracellular messenger, cyclic AMP (cAMP) which is involved in a vast number of processes such as glycogen metabolism and regulation of Ca^{2+} through ion channels. This is supported by studies using cultured rat insulinoma cells and rat gastric glands where it was identified that GLP-1 receptors are coupled to a cAMP- dependent adenylate cyclase system (Goke *et al*, 1989; Uttenhal and Blazquez, 1990). Characterization of the GLP-1 (7-37) receptor in various cells including rat lung membranes confirmed this. It was also demonstrated that specific binding of radio-labelled GLP-1(7-37) is dependent on pH, time and temperature and is proportional to membrane protein concentration. In these experiments the addition of unlabelled GLP-1 (7-36), inhibited binding in a linear fashion consistent with a single class of binding site (plots will be curvilinear in the presence of multiple binding sites on the protein or if it binds to multiple protein sites) (Richter *et al*, 1990). Cloning and functional experiments of the rat and human receptor revealed that it belongs to Class B of the seven transmembrane, G-protein coupled receptor superfamily (Van Eyll *et al*, 1994; Thorens *et al,* 1992; Dillon *et al*, 1993; Grazanio, *et al* 1993; Richter *et al*, 1990; Goke *et al*, 1988).

Figure 2.1.1- The proglucagon gene, **localized on human chromosome 2q36eq37**. Proteolytic processing occurs in the intestine to yield GLP-1, over 70% of which is secreted from intestinal L-cells in its amidated form, and GLP-2. Glucagon is secreted from the pancreas (From: Yabe & Seino, 2011).

Genetic susceptibility to mutations at the GLP-1 receptor has also been considered. Transfection experiments whereby a single point mutation within a non-conserved motif of the N-terminal, extracellular domain of GLP-1 receptor, results in impaired receptor function (Van eyll *et al*, 1996). Previous analyses of GLP-1 receptor gene polymorphisms could not show linkage to T2DM (Van Eyll *et al*, 1996; Tanizawa *et al*, 1994; Zhang *et al*, 1994). However, Van Eyll and co-workers state that linkage analysis is not sensitive enough to locate single point mutations making this an important finding. More recently a GLP-1R missense polymorphism at position 149 threonine has been identified with reduced binding affinities (60-fold for GLP-1 and 5-fold for exendin-4 (exenatide) (Beinborn *et al*, 2005). It may be postulated that due to GLP-1's insulinotropic action, through glucose sensing pathways, receptor mutations might lead to impaired glucose tolerance, a

feature of T2DM. Going beyond glucose regulation, this may have implications with regards to blood pressure regulation as GLP-1R have been located in the heart, vascular smooth muscle, endocardium and coronary endothelium, suggesting GLP-1 may be involved in important cardiovascular actions (Wei & Mosjov, 1995; Grieve *et al*, 2009). Further to this, recent personal communication with Novo Nordisk researchers confirmed presence of GLP-1R in sinoatrial node tissue which suggests GLP-1 might directly modulate heart rhythm.

It has been speculated that other receptors exist which bind exenatide as it has been shown to initiate effects not strictly related to GLP-1 such as activation of vagal afferent nerves (Grieve *et al,* 2009). Some studies also demonstrate that, despite the absence of the classical GLP-1R, exenatide GLP-1 and exendin (9-39) were able to initiate signalling components and transcription (Ban *et al,* 2010; Ban *et al,* 2008). Receptors for GLP-1 are expressed in a vast number of tissues including the GI tract, pancreatic islet cells, the lung, breast, heart, central nervous system and the kidney (Thorens *et al,* 1992; Wei and Mosjov, 1995; Yu *et al*, 2003; Körner *et al*, 2007).

2.2 GLP-1 and glucose homeostasis

The most widely studied area concerning the action of GLP-1 is in the β-cell. In brief, this is where GLP-1 acts to induce insulin secretion in response to glucose stimulation. Activation of the GLP-1 receptor stimulates adenylyl cyclase and the generation of cyclic adenosine monophosphate (cAMP), leading to activation of second messenger pathways such as protein kinase A (PKA) and exchange proteins activated by cAMP (Epac). Continuous GLP-1 receptor activation increases insulin synthesis and beta cell proliferation and neogenesis (Doyle and Egan, 2007). Various factors contribute to the pathogenesis of hyperglycaemia in humans. It is widely understood that GLP-1R contributes to the regulation of glucose in a variety of tissues and therefore the potential for dysregulation of GLP-1R signalling in each system highlighted in **Table 2.2.1 GLP-1R- mediated effects on glucose homeostasis** may contribute to metabolic abnormalities and hence T2DM. Research is making great progress into piecing together GLP-1s actions in individual tissues.

Table 2.2.1 GLP-1R- mediated effects on glucose homeostasis.

Tissue	Defect	Pathophysiological effect	GLP-1R present?	Role in glucose homeostasis
Pancreas- β cells	Decreased Insulin secretion	Fasting and postprandial hyperglycaemia	Y	Increase insulin secretion
Pancreas- α cells	Increased glucagon secretion	Excessive stimulation of hepatic glucose production	N	
Liver	Insulin resistance Increased hepatic glucose output	Increased fasting and postprandial glucose	Y	Fatty acid oxidation, insulin sensitivity
Muscle	Insulin resistance Decreased glucose uptake	Increased fasting and postprandial glucose	Smooth muscle, cardiac	Release GLP-1 intestine in response to food; ANS; No affect on cardiac glucose uptake.
Gut	Decreased GLP-1/ GIP secretion B- cell glucose resistance to GLP-1/ GIP	Reduced postprandial insulin secretion	Y	Post-prandial glucose homeostasis Satiety
Adipose	Increased lipolysis	Increased plasma free fatty acids, exacerbating insulin resistance in muscle and liver and impairing β-cells	Y	Lipolysis/ lipogenesis Beneficial effects on body weight
Brain	Neurotransmitter dysfunction and insulin resistance	Impaired satiety signals and impaired neurohormonal signalling	Y	Increase satiety Haemodynamics, CNS effects
Kidney	Increased glucose reabsorption	Increased plasma glucose	Y	Poorly understood

Adapted from Defronzo *et al*, 2011

2.3 Renal effects

At the physiological level, there is a wealth of evidence to suggest that GLP-1 derivatives are able to produce a dose dependent effect on sodium handling in renal tubules in animals and man and that this confers significant long term blood pressure reduction in patients prescribed GLP-1 agonists (Gutzwiller *et al,* 2004, 2006; Moreno *et al,* 2002; Bojanowska & Stempniak, 2003; Hirata *et al,* 2009; Yu *et al,* 2003). For instance, Moreno and co-workers demonstrated that recombinant GLP-1 increased urine Na^+ excretion 13-fold in kidneys of Sprague- Dawley rats (Moreno *et al,* 2002). Kidneys of the rats were denervated and compared to that of the innervated animals showing a greater increase in glomerular filtration in the innervated animals. This might be explained if there was GLP-1 activation of the sympathetic outflow and consequent renin release. Interestingly, increased Na^+ excretion was not postulated to be the result of hemodynamic changes as no significant elevations of renal hydrostatic pressure were recorded between innervated and denervated rats despite a 39% increase in glomerular filtration rate in the innervated animals. Moreover, the elevation of lithium clearance in these experiments was a strong indicator that sodium reabsorption was being inhibited in the tubules, mechanisms for this being inhibition of cellular sodium transport machinery (Moreno *et al,* 2002; Thomsen *et al,* 1981). Furthermore, GLP-1 receptors have been located in the kidney (Körner *et al,* 2007) and renal GLP-1 receptors have been identified to be primarily located in the proximal tubular cells of the cortex (Moreno *et al,* 2002; Schlatter *et al,* 2007). Proximal tubule cells are concerned with regulating sodium-reabsorption as well other factors such as

excretion of drugs, recycling of proteins and other nutrients, and as discussed previously the kidney is the main controller of long-term blood pressure via regulation of BV. Therefore, a reduction in sodium reabsorption results in higher urinary output of sodium (natriuresis) and leads to decreased blood volume and hence decreased blood pressure. Surprisingly, there is paucity in available literature to investigate whether GLP-1 is able to directly inhibit sodium reabsorption. In studies using porcine and opossum kidney cells, Schlatter and colleagues were unable to show GLP-1, exendin 4 and the dipeptidyl peptidase IV (DPPIV) inhibitor P32/98 (Ile-thiazolidide) influenced the mRNA expression of the GLP-1R, DPPIV, or sodium transporter molecules: NHE3, sodium-dependent glucose transporter slc5al, slc5a2 (SGLT1,2). However, there was a weak suggestion that sodium reabsorption is inhibited by GLP-1 but this effect was not replicated by Exendin-4. These findings are not robust enough to conclude that there is an interaction of GLP-1 with active sodium transport mechanisms involved in blood pressure regulation *in vivo*. A weakness in Schlatter's results may have been due to varying species-specific expression patterns between isoforms of Na^+/H^+ exchangers (Schlatter *et al,* 2007). It may be worthwhile to remember that GLP-1 has been postulated to increase intracellular messenger, cyclic adenosine monophosphate (cAMP). For instance in the glucoregulatory pathways of the beta cell GLP-1 has been postulated to increase cAMP dependent protein kinase A (PKA); the signalling cascade which is also implicated in the inhibition of NHE3 depending on the presence of co-regulators, most notably Na^+ or H^+ regulatory factor (NHERF) (**Figure, 2.3.1**) (Zizak *et al*, 1999; Weinman *et al,* 2001). (Thorens *et al,* 1993;

Shigeto *et al,* 2008). The inability of exendin-4 to inhibit sodium reabsorption in the porcine model may be due to its clearance being solely a result of glomerular filtration rate (GFR) as exemplified in a previous study (Simonsen *et al,* 2006). In the same study, renal clearance of GLP-1 was shown to involve other but not identified mechanisms in conjunction with GFR. The expression of GLP-1R in kidney proximal tubules is also closely associated with DPPIV and therefore it might be expected that this is the site where GLP-1 is broken down and the constituent amino acids recycled. Exendin-4 has recently been demonstrated to modulate NHE3 but these experiments utilised higher doses (500pM Exendin-4) (Carraro-Lacroix & Girardi, 2008).

Figure, 2.3.1- Signal complex regulation of NHE3 by PKA. It is suggested that the complex of scaffolding proteins are required for activated PKA to phosphorylate specific residues in the c-terminus region of NHE3. This leads to inhibition of NHE3 activity. (Taken from Weinman *et al*, (2001). *Kidney Int.* Aug; 60(2):450

Hirata and co-workers attempted to elucidate the effects of exendin-4 in type 2 diabetic (*db/db;* C57BLK/s; BLKs) mice and after angiotensin II (ANG II) infusion in non-diabetic C57BLK/6J wild type littermates (Hirata *et al,* 2009). A characteristic of the BLKs mouse is a significant increase in blood pressure. Salt-induced hypertension was produced in the BLKs mice by infusion of ANG II, which is proposed to be a key mediator of hypertension in diabetes (Chin *et al,* 2005; Shibuya *et al*, 2005; Crowley *et al*, 2005). These experiments demonstrate that daily injection of exendin-4 significantly attenuates hypertension in BLKs mice at 12 weeks. It was also shown that the blunted urinary excretion of sodium seen in the BLKs mouse model in response to salt-loading is significantly reduced after administration of exendin-4 conferring in this animal model the natriuresis described in man. Furthermore, exendin- 4 significantly increased natriuresis and attenuated high salt- sensitivity in mice with salt-induced hypertension. Exendin- 4 was also shown to inhibit ANG II phosphorylation of ERK1/2 (a mediator of intracellular ANG II signalling in renal cells) in a dose dependent manner. Real- time PCR confirmed the expression of GLP-1 receptor (GLP-1R) in tissues including the kidney and that urinary excretion of cAMP was also elevated, suggesting that exendin-4 functionally interacts with its receptor in the kidneys of these mice to direct inhibition of sodium at the tubules (Hirata *et al*, 2009).

Interestingly and in agreement with previous studies in rodents and to develop Schlatter's findings further, Crajoinas and colleagues have recently demonstrated GLP-1 infusions into rodents to increase glomerular filtration rate (GFR) and renal plasma flow (RPF). These researchers also

demonstrated inhibition of proximal sodium, bicarbonate and water reabsorption in part by inhibiting NHE3-mediated Na^+/H^+ exchange. Crajoinas demonstrated inhibition of NHE3 to be via an increase in cAMP and phosphorylation of the exchangers COOH-terminal region at the PKA consensus sites, serines 552 and 605 (Crajoinas *et al,* 2010). Interestingly urinary excretion of both sodium and potassium occurred suggesting involvement of the distal tubule and collecting duct although this research group failed to confirm the presence of GLP-1R in this portion of the nephron (Crajoinas *et al,* 2010).

Various reports offer conflicting information with regards to blood pressure regulation by GLP-1 highlighting the limitations to using many different species in exploratory studies (Yamamoto *et al*, 2002; Barragan *et al*, 1999) For example, intravenous GLP-1 (7-37) has been reported to increase heart-rate in calves, but not arterial blood pressure in this species (Edwards *et al*, 1997). However, the same research group also demonstrated that in fasted healthy human subjects subcutaneous injection of GLP-1 (7-37) increased both blood pressure and heart rate (Edwards *et al*, 1998). With regards to this latter finding, there is much evidence in man to suggest that blood pressure is reduced but heart rate is increased (Gutzwiller *et al*, 2004; Madsbad, 2004; Buse *et al*, 2004). For instance, clinical trial data shows a statistically significant decrease in blood pressure from baseline (Apovian, 2010; Derosa, 2010; Nauck, 2009; Russell & Jones, 2009; Diamant 2010).

2.3.1 Central control of blood pressure homeostasis

Interestingly, GLP-1 receptors (and secreted GLP-1) have been identified in the hypothalamus and there is strong supporting evidence to this effect. A

provocative theory is that in rodents, GLP-1 is involved in the stress (fight or flight) response, linking taste aversion mechanisms with that of blood pressure control. This contradicts findings whereby blood pressure is decreased upon administration of GLP-1 derivatives. Experimental techniques such as route of administration, use of anaesthesia and species differences, may hinder direct comparison of results. It is likely that this hormone is also able to perform opposing roles via central and peripheral mechanisms or that there could be unidentified receptor types or metabolites. Often hormones display opposing or dual actions. Moreno and co-workers demonstrated GLP-1 to exert dual effect on the kidney in experiments whereby the effects of GLP-1 and an antagonist were compared when administered either intravenously (I.V.) or intracerebroventricularly (I.C.V.) (Moreno *et al*, 2002).

With regards to blood pressure centres, GLP-1 receptors have been located in hypothalamic magnocellular neurons (Bojanowska & Stempniak, 2002), and the neurohypophysis and neurons of the caudal region of nucleus of the solitary tract in rodents (Yamamoto *et al*, 2002). These brain centres are implicated in control of diuresis and the baroreflex. Administration of GLP-1 or Exendin-4 into these animals has been shown to increase Fos-like immunoreactivity (Fos-IR) in these and related brain centres indicating that certain GLP-1 derivatives activate these neurons. Areas of the brain which were activated included the paraventricular nucleus of the hypothalamus which is responsible for regulating diuretic responses by the kidney. Secretion of vasopressin by PVH acts on V_2 receptors which are mainly located in the renal collecting ducts and initiates adenyl cyclase, resulting in

an increase of cytosolic cAMP. Vasopressin acts as an antidiuretic hormone through the V2 receptor whereby it increases water permeability of the apical membrane of the distal nephron, hence increasing water absorption. This results in a decrease in plasma osmolarity causing an increase in atrial natriuretic peptide (ANP) secretion (Marunaka *et al*, 1997). Other areas involved in activation by GLP-1 are, the locus coeruleus and A5 catecholamine group, responsible for synthesis of noradrenaline and hence initiation of the “fight or flight” response; and the rostral ventrolateral medulla, principally involved in regulation of blood pressure via innervation of the sympathetic nervous system and baroreceptors. Neurons containing corticotropin- releasing hormone in PVH are also stimulated, increasing plasma corticosterone and the chronotropic and pressor response. GLP-1 (7-36) has been shown to increase basal levels of neurohypophysial hormones in normotensive rats and rats undergoing hypovolemia (Larson *et al*, 1997, Bojanowska & Stepniak 2000; Barragan *et al*, 1999).

Yamamoto and co-workers showed that centrally and peripherally administered GLP-1 increases blood pressure and heart rate and appears to be mediated through sympathetic preganglionic neurons via activation of c-fos expression in the adrenal medulla and catecholamine receptors in the rat brain. These results suggest an adrenergic response to GLP-1 (Yamamoto *et al*, 2002). However, contrary to this, Barragan and co-workers suggest a noradrenergic response (Barragan *et al*, 1994). It was later proposed that GLP-1 may produce opposing effects because it was demonstrated in rats that vasoconstriction mediated by β-adrenoceptor and tachycardia occurs as a result of sympatho-adrenal activation. It was suggested that both α- and β-

adrenoceptors are involved except during mesenteric vasoconstriction or the receptors for vasopressin or angiotensin (Gardiner *et al*, 2006). It is a strong possibility that GLP-1 displays a dual response and is able to contribute to a decrease blood pressure via the kidneys, and finely tune this action through the central nervous system responses. In this way, blood pressure is regulated within a narrow index. Considering this, it appears likely that contradictory findings in animals and humans are in part a result of limitations of animal models or experimental differences, such as condition or preparation of the subject or animal before the treatment or the effect of variables, not controlled for in the experiment.

3.0 Aims of the thesis

The ability of GLP-1R agonists to normalise post-prandial blood glucose excursions, with a low incidence of hypoglycaemia offers great promise in the treatment of T2DM. Of special interest is the potential for this class of agent to decrease body weight and blood pressure. The aims of this thesis are to:

1. Explore the effects of GLP-1 agonists on blood pressure heart rate and body weight through conducting a meta-analysis of clinical trials.

2. To evaluate the effects of obesity and diabetes- like conditions on GLP-1R expression in the murine kidney.

3. To establish that GLP-1R is functional in human kidney cell lines.

4. To ascertain whether hyperglycaemia influences renal GLP-1R in whole kidney and in human renal cells.

5. Perform a mechanistic study to assess the effects of GLP-1 on α-ENaC mRNA and protein expression and signalling in cells of the human collecting duct.

4.0 Effects of Exenatide and Liraglutide on heart rate, blood pressure and body weight: Systematic review and meta-analysis

4.1 Introduction

In contrast to the weight increasing effects of several traditional anti-diabetic drug classes, GLP-1 analogues have been shown to reduce both body weight and blood pressure (Nathan *et al*, 2009; Vilsbøll *et al*, 2009). The mechanisms producing weight loss have been extensively investigated, and involve improved satiety and reduced calorie ingestion both through effects on the central nervous system and through delayed gastric emptying (Flint *et al*, 1998; MacDonald *et al*, 2001; Williams *et al*, 1996; Meier & Nauck, 2005). Mechanisms leading to reduced blood pressure are less adequately understood, but this effect has been shown to occur as early as two weeks after commencing therapy, preceding significant weight loss, suggesting that a direct hypotensive effect is at least partly responsible (Varanassi *et al*, 2011). The blood pressure response to GLP-1 agonists is more modest than effects on weight loss but has been reported to be beneficial. Experimental studies of GLP-1 analogues have also reported direct effects on blood pressure, possibly via interaction with the autonomic nervous system (Bojanowska & Stepniak, 2003).

Whilst a number of studies have reported heart rate increases, the majority of published reports show only very small overall increases of up to 3 bpm and given the safety implications attributed to raised heart rate in other contexts, there is a surprising lack of concern over its possible implications in long-term treatment of diabetes patients (Levine, 1997; Aronow, 1996; Hozawa *et al*, 2004; Anselmino *et al*, 2010). A recent review of liraglutide by

Buse acknowledges the effect, but a meta-analysis on safety of incretin based therapies published in 2010 did not mention it, nor does an overview of the LEAD trials of liraglutide by Blonde and Russell-Jones (Bode *et al*, 2012; Fakhoury *et al*, 2010; Blonde & Russell- Jones, 2009). A large nationwide audit of exenatide designed by the Association of British Clinical Diabetologists (ABCD) and supported by Eli Lilly did not include it as an outcome despite citing evidence for the effect in the main published report (Ryder et al, 2010; Gill et al, 2010). A subsequent (on-going) ABCD audit of liraglutide also aims to identify unknown safety issues but has similarly omitted this outcome from the protocol (ABCD audit, 2012).

GLP-1 analogues are an expanding drug class, with recent development of longer acting agents including the once weekly form of exenatide, Bydureon. This drug has recently obtained approval from the National Institute of Health and Clinical Excellence for use in type 2 diabetes and its use is likely to increase (NICE, 2012). A review of trial data from five long acting GLP-1 agonists (exenatide once weekly, taspoglutide, albiglutide, LY2189265 and CJC-1134-PC) concluded that they were more likely than shorter acting formulations to raise heart rate (Madsbad et al, 2011). A more recently published study of the long acting GLP-1 agent PF-04603629 reported a substantial rise in heart rate (mean increase 23 bpm at 24 hours after injection of the higher dose studied), together with a rise in diastolic blood pressure (Gustarson *et al*, 2011). Mechanisms to an increase in heart rate are not fully understood, but may be a direct result of autonomic activation with antagonism of vagus tone or secondary to metabolic and/ or blood pressure effects of the agents.

Whilst there is no evidence to date that these agents (short or long acting) increase cardiovascular event rates, safety data are limited by short follow up duration (Ratner *et al*, 2011). Longer term follow up is underway but will take a number of years to complete.

We aimed to identify and synthesise all available heart rate data from both published and unpublished sources, to quantify the effect of GLP-1 analogues on this outcome, as well as that on blood pressure and body weight.

4.2 Methods

4.2.1 Literature searches

The following resources were systematically searched to identify completed, new or on-going controlled trials: Clinical Trials Gov (www.clinicaltrials.gov); Entertrials.co.uk; Clinicaltrialssearch.org; Centerwatch; Drugsontrial; WebMD; MEDLINE (1960- date); EMBASE (1960- date); The Cochrane Library Central Register of Controlled Trials (CENTRAL). We used a search strategy to capture “exenatide”, “liraglutide” or “glucagon-like peptide-1” in any field, limited to “Randomised” or “Controlled Clinical Trial”. Conference proceedings (British Endocrinology Society, Diabetes UK European Association for the Study of Diabetes) and websites (American Diabetes Association, Federal Drug Agency and European Medicines Agency) were examined, and the reference lists of trials, meta-analyses and reviews were searched for further studies. Novo Nordisk and Ely Lilly were contacted directly to request unpublished data.

4.2.2 Inclusion and exclusion criteria

a) Participants

We included only trials involving participants with type 2 diabetes. Diagnosis of T2DM should have been established using up-to-date standard diagnostic criteria from the beginning of the trial (e.g. ADA, 2008), evidence of HBA1c indicative of T2DM using standard diagnostic criteria, or patients using oral anti-diabetic therapy (OADs) for at least 3 months prior to screening.

b) Study designs

Randomised trials with >12 weeks. Long term trials were excluded based on the design not comprising a comparator arm.

c) Intervention(s)

Trials of clinically relevant doses of liraglutide (1.2 or 1.8 mg daily), exenatide (5 or 10 µg twice a day), or exenatide once- weekly, either alone or in combination with an oral anti-diabetic drug (OAD) were included.

c) Comparison groups(s)

Comparators included placebo, continuous OAD, lifestyle intervention, or basal insulin.

d) Outcomes

We included all phase 2 and 3 studies (not including open-label extensions) reporting blood pressure, body weight and/or heart rate outcomes.

4.2.3 Data extraction

Studies were retrieved by two researchers independently and both researchers contacted trial organisers for missing data. We assessed studies for inclusion using the above criteria. Mean effect data from cross-over trials were extracted at the end of the initial phase.

4.2.4 Risk of Bias

Risk of bias was assessed for each included study according to criteria described in Cochrane Handbook for Systematic reviews version 5 (Higgins, 2011), below:

A) **Randomisation**: A trial was considered to be of “low risk of bias”, if there was evidence of random sequence generation by a computer or web based automated system, or referring to a random number table. The trial met the criteria of “high risk of bias” when random sequence generation was based on a series of laboratory tests or a rule based on treatment centre, clinician judgement or similar.

B) **Allocation concealment**: Trials were assigned “low risk” when a central allocation method was used (a computer or web based automated system), opaque coded envelopes or other similar approach. The trial met the criteria of “high risk of bias” when an open allocation schedule or other such method was employed.

C) **Blinding of participants/ investigators/ sponsors**: Trials were considered to be of “low risk of bias” when blinding of the participants and investigators were described and it was considered unlikely that blinding had been broken; or when the study was not blinded but it was unlikely that the outcome was influenced by lack of blinding. Trials met the criteria of “high risk of bias” when no blinding or incomplete blinding occurred; there was a high risk of the outcome being influenced by lack of blinding or that blinding had been broken.

D) **Blinding outcome assessment**: Trials met the criteria of “low risk of bias” when blinding of outcome was described, or in trials where blinding did not occur, this was not considered to influence the outcome measurement.

Trials met the criteria of “high risk of bias” when no blinding occurred or there was evidence that blinding was broken.

E) **Incomplete outcome data**: The trials met the criteria of “low risk of bias” when there were no missing outcome data, where attrition was well balanced between trial arms, and where effect size was not altered by the missing outcome (results of the trials were plausible) or that reasons for missing data were not related to true outcome. The trial met the criteria of “high risk of bias” when reasons for the missing outcome data are related to true outcome (for example, participant drop-outs due to nausea when assessing the effects on patients’ tolerability), with an imbalance of numbers across groups; the effect size is directly related to missing outcome and introduces “favourable” results; application of an unsuitable imputation or other plausible reason for high risk criteria to be met.

F) **Selective outcome reporting**: The trials met the criteria of “low risk of bias” when the study protocols were available and all outcome data was reported within a pre-specified criteria; or a protocol was not available but it is clear that the trials report the outcomes of interest in a pre-specified way. The criteria of “high risk of bias” was met when the trials failed to report all pre-specified outcomes; the reported outcome measurement, analysis differed from those pre-specified; negative results or results lacking statistical significance were not reported or that data was incomplete, preventing assessment by meta-analysis.

G) **Other bias**: Any other bias which I considered might influence the outcomes of the trials.

The criteria of “unclear risk of bias” was met when trial authors failed to describe the methods employed or the trials could not be classified as either “low” or “high” risk of bias.

Where appropriate, the Cochrane risk of bias tool was used to assess risk of bias items at the level of the specific outcomes were performed. I assessed bias using funnel plots. A funnel plot is able to detect publication bias, poor methodological design of small studies or true heterogeneity of effect due to trial size which is depicted by asymmetry of the funnel plot (Higgins, 2011). Due to the subjective approach to the analysis, minimal emphasis was placed on findings from analysis of funnel plots. Prior to undertaking the analysis, I planned to compare pooled mean differences and 95%CI of the completer groups against intention-to treat populations. Intention-to treat group numbers will be shown in forest plots and described in the results section

4.2.5 Analysis

I undertook all data analyses. For our quantitative meta-analyses, we excluded ‘open-label’ extension studies of phase 3 trials because such trials are less well controlled and do not include a comparator arm. Means and standard deviations for baseline and outcome values for blood pressure, heart rate and body weight were extracted. Where standard deviations for the outcome were not available they were imputed according to Cochrane Handbook for Systematic reviews version 5 (Higgins, 2011). Where change from baseline measurements and the corresponding standard deviations were not available, these were calculated, taking into account the correlation of baseline to follow up measurements within individuals. In instances where

the correlation coefficient could not be calculated, methods were employed as recommended by Follman (Follman, 1992).

Study results were combined using RevMan version 5.2. Heterogeneity was estimated using the χ^2- test and I^2 statistic (95%CI) and repeated for 90%CI to enhance detection of heterogeneity. Interaction effects were evaluated using pre-specified subgroup analyses (comparing various doses of study drug to active control or placebo) and type of GLP-1 agonist (liraglutide once daily, exenatide twice daily and exenatide once weekly (ExLAR) preparations). Sensitivity analyses were planned only to investigate clinical, methodological or statistical heterogeneity.

A synthesis of all available studies for each outcome was conducted. For these analyses, multiple comparison trials such as those featuring placebo and active control arms, data from placebo were preferentially entered into the analysis. Similarly, highest dose of GLP-1 agonist; and active comparators considered to be most “neutral” with respect to outcome were preferred.

Initially, both the fixed and random effects models were applied using the inverse variance statistical approach for combining studies with 95% confidence intervals. Results using the random effects model are reported. This approach was considered appropriate as high heterogeneity were expected due to differences in background therapy, patients’ characteristics and other clinical variables. If the results from random and fixed effects differed, results from fixed effects analyses are also described. Where there was evidence of high inter-trial heterogeneity, pooled results are reported with appropriate caution.

4.3 Results

We identified 520 articles through the initial searches. Of these, 471 were excluded on the basis of the title or abstract being irrelevant to the aims of this review. Forty-nine studies were examined full text. Out of these studies, 8 studies were open-label extension trials, 6 were excluded either because the comparator was another form of GLP-1 (2 studies), the doses were not as specified in our inclusion criteria (2 studies), or because the study was a duplicate of an included trial, in which case we chose the primary source (2 studies). This left 35 trials included in our review (**Table 4.3.0.1 Characteristics of included studies**). Of the included trial publications, the preferred method was to describe data which was derived using intention-to treat population with last observation carried forward (LOCF) with 2 studies describing per protocol or full analysis set. Eleven of the thirty- five included trials measured heart rate as a primary or secondary outcome. We were able to gain access to heart rate data from 5 Liraglutide studies (Lead 1-5) and also 4 Exenatide trials (equating to 82% of the total heart rate data), either through publication searches or direct contact with the trial organisers. Similarly, we obtained data from 85% (17 out of 20 studies) which measured systolic and diastolic blood pressure; and 19 studies measuring body weight. The majority of missing data was kindly supplied to us by trial coordinators.

Heart rate and blood pressure data from a further 8 exenatide trials were kindly requested for the study (Barnett *et al*, 2007; Buse *et al*, 2004; Davies *et al*, 2009; Defronzo *et al*, 2005; Defronzo *et al*, 2010; Gao *et al*, 2009; Gallwitz *et al*, 2011; Kendall *et al*, 2005). However, data had been collected as part of trial safety assessments, and were not designated primary or

secondary outcomes. Appropriate power calculations had not been incorporated into the trial designs for these measurements.

Of the included studies, there was a high amount of clinical heterogeneity (e.g. differences in patients' characteristics, background OAD therapy) and methodological heterogeneity (e.g. blinding, allocation concealment) (**Table 4.3.0.1 PRISMA flow diagram; Table 4.3.0.2 Characteristics of included studies; Table 4.3.1.1 Risk of bias across included studies**).

Table 4.3.0.1 PRISMA flow diagram

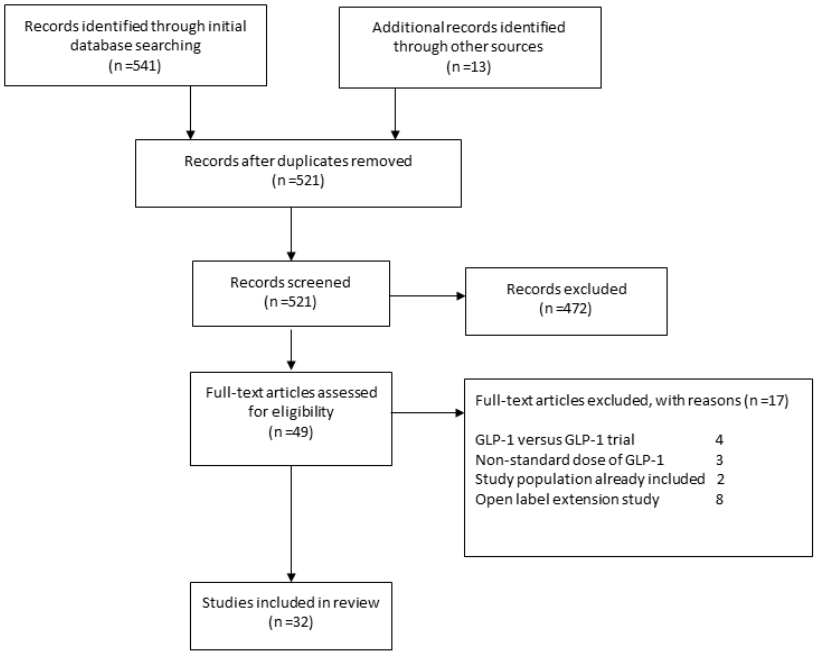


Table 4.3.0.2. Characteristics of included studies

Study	Comparisons	Duration (weeks)	Study population /ethnicity	Country	Body weight groups included	Balanced Male/ Female?	Mean age	Standardised diet/ exercise	Background OAD
Apovian, 2010	EX/PLAC	24	MR	US	OW	>60% F	54.8	Y	MET and/or SU
Barnett, 2007	EX/IG	16	MR	Multi-national	N/OW/ OB	Y	54.9	N	MET or SU
Bergenstal, 2009	EX/BIAsp	24	MR	US	N/OW	Y	52.6	N	MET and SU
Bergenstal, 2010	EX LAR vs PIO EX LAR vs SITA	26	MR	Multi-national	N/OW/ OB	Y	52.3	N	MET
Buse, 2004	EX/PLAC	30	MR	US	OW/OB	60% M	55.3	N	SU
Buse, 2011	IG+EX/ IG+PLAC	30	MR	Multi-national	N/OW/ OB	Y	59.0	N	MET or PIO
Davies, 2009	EX/IG	26	MR	GB	OW/OB	>60% M	56.5	N	Two or three OADS: MET, SU, or TZD
Defronzo, 2005	EX/PLAC	30	MR	US	OW/OB	Y	53.0	N	MET
Defronzo, 2010	EX vs ROSI	20	MR	US	OW/OB	Y	56.0	N	MET

Study	Comparisons	Duration (weeks)	Study population /ethnicity	Country	Body weight groups included	Balanced Male/ Female?	Mean age	Standardised diet/ exercise	Background OAD
Derosa, 2010	EX/GLIB	52	W	IT	OW/OB	Y	56.5	Y	MET
Derosa, 2011	EX/GLIM	52	CAUC	IT	OW/OB	Y	55.5	Y	MET
Diamant, 2010	EX LAR/IG	26	MR	Multi-national	OW/OB	Y	58.0	N	MET
Gallwitz, 2011	EX/BIAsp	26	MR	GER	OW/OB	Not reported	57.0	N	MET
Gallwitz 2012	EX/GLIM	Up to 4.5 years	MR	Multi-national	OW/OB	Y	56.0	N	MET
Gao, 2009	EX/PLAC	12	C/I/K/T	Multi-national	N/OW/ OB	Y	54.0	N	MET and/or SU
Garber, 2009	LIR/GLIM	52	MR	US/MEX	N/OW/ OB	Y	53.0	N	Nil - previous OAD withdrawn
Gill, 2010	EX/PLAC	12	MR	CAN/NL	OW/OB	Y	55.6	N	MET and/or TZD
Heine, 2005	EX/IG	26	MR	Multi-national	OW/OB	Y	58.9	N	MET and SU
Kadowaki , 2009	EX/PLAC	12	JP	JP	N/OW/ OB	>60% M	60.3	N	SU, with or without either BG or TZD

Study	Comparisons	Duration (weeks)	Study population /ethnicity	Country	Body weight groups included	Balanced Male/ Female?	Mean age	Standardised diet/ exercise	Background OAD
Kendall, 2005	EX/PLAC	30	MR	US	OW/OB	Y	55.3	Y	MET and SU
Kim, 2007	EX LAR/PLAC	15	MR	US	OW/OB	60% M	53.7	Y	MET
Liutkus, 2010	EX/PLAC	26	MR	Multi-national	OW/OB	Y	54.7	N	TZD with or without MET
Marre, 2009	LIR/PLAC/ROSI	26	MR	Multi-national	N/OW/ OB	Y	56.0	N	SU
Moretto, 2008	EX/PLAC	24	MR	Multi-national	OW/OB	Y	54.0	Y	DRUG NAIVE
Nauck, 2007	EX/PIA	52	MR	Multi-national	OW/OB	Y	58.5	N	SU and MET
Nauck, 2009	LIR/GLIM/PLAC	26	MR	Multi-national	N/OW/ OB	Y	56.7	N	MET
Pratley, 2010	LIR/SIT	26	MR	Multi-national	N- OW-OB	Y	55.3	N	MET
Russell-Jones, 2009	LIR/IG/PLAC	26	MR	Multi-national	N/OW/ OB	Y	57.5	N	MET and SU
Russell-Jones, 2012	EX LAR/MET EX LAR/PIO EX LAR/SITA	26	MR	Multi-national	N/OW/ OB	Y	54.0	N	DRUG NAIVE
Yang, 2011	LIR/GLIM	16	C/K/I	Multi-national	N/OW/ OB	Y	53.3	N	MET
Zinman, 2007	EX/PLAC	16	MR	Multi-national	OW/OB	Y	56.1	N	TZD with or without MET

Study	Comparisons	Duration (weeks)	Study population /ethnicity	Country	Body weight groups included	Balanced Male/ Female?	Mean age	Standardised diet/ exercise	Background OAD
Zinman, 2009	LIR/PLAC	26	MR	US/CA N	N/OW/ OB	Y	55.0	N	MET and ROSI

EX, Exenatide; EX LAR, Exenatide long acting release; PLAC, placebo; IG, insulin glargine; BIAsp, biphasic insulin aspart; PIO, pioglitazone; SITA, sitagliptin; ROSI, rosiglitazone; GLIB, glibenclamide; GLIM, glimepiride; LIR, liraglutide; MET, metformin, BG, Biguanide.
MR, Multi-racial; C, Chinese; K, Korean; I, Indian; T, Taiwanese; JP, Japanese; W, White; CAUC, Caucasian.
GB, Great Britain; US, United States; GER, Germany; CAN, Canada; JP, Japan; NL, Netherlands; MEX, Mexico; IT, Italy.
N, normal weight; OW, overweight; OB, obese.

4.3.1 Methodological quality and risk of bias

The assessment of funnel plots showed satisfactory symmetry. Risk of bias for each of the included studies is shown in **Table 4.3.1.1 Risk of bias across included studies.** None of the trials were terminated prematurely and all trials included sample size calculations relevant to primary and/or secondary outcomes.

Assessment of bias of the 32 included studies showed 31 studies to describe randomisation in sufficient detail and 1 study was allocated unclear risk; All studies reported allocation concealment in sufficient detail; 19 studies were blind and 13 studies were either partially blind (e.g. to investigator or statistician) or open label. Of these studies, blinding of outcome assessment was adequate for 20 but not sufficiently described for 12 studies; Losses to follow up were adequately described for 23 studies, 9 studies were considered “unclear risk” due to attrition rates being higher than the generally accepted value of 20%; 23 studies reported all outcomes numerically. A further 9 studies either did not report all outcomes, for instance, those which were not significant or the trial arms were not balanced; 3 studies showed potential for “other biases” which were, changes to maximum dose regimes due to country where the investigation took place, higher weight loss due to nausea and diet and exercise regime not standardised.

Table 4.3.1.1 Risk of bias across included studies.

Included studies were assessed using the Cochrane Risk of Bias Tool for factors which may cause bias in the trial outcomes and subsequent evaluation by meta-analysis: A) Randomisation, B) Allocation concealment, C) Blinding of participants/investigators/sponsors, D) Blinding outcome assessment, E) Incomplete outcome data, F) Selective outcome reporting, G) Other bias.

No.	Study	A	B	C	D	E	F	G	Comments
1	**Apovian, 2010**[‡]								Greater than 20% attrition.
2	**Barnett, 2007***[‡]								Open label cross-over study.
3	**Bergenstal, 2009**[*‡]								Open label. Greater than 20% attrition and higher attrition in exenatide group.
4	**Bergenstal, 2010**[‡]								Greater than 20% attrition. Blinding removed after finalisation of analysis plan.
5	**Buse, 2004**								Greater than 20% attrition. Higher attrition in the placebo arm.
6	**Buse, 2011**[‡]								Groups not balanced for sex and concomitant medication.
7	**Davies, 2009**[*]								Open label.
8	**Defronzo, 2005**								
9	**Defronzo, 2010**[*‡]								Open label. Greater than 20% attrition.

No.	Study	A	B	C	D	E	F	G	Comments
10	**Derosa, 2010**								Single blind.
11	**Derosa, 2011**[ŧ]								Single blind.
12	**Diamant, 2010**[*8]								Open label. Higher attrition in the exenatide arm.
13	**Gallwitz, 2011**[*]								Open label.
14	**Gallwitz 2012**[*]								Open label. Greater than 20% attrition. Higher attrition in the exenatide arm.
15	**Gao, 2009**[ǂ]								
16	**Garber, 2009**[ǂ]								Greater than 20% attrition.
17	**Gill, 2010**								
18	**Heine, 2005**[*]								Open label. Higher attrition in the exenatide arm.
19	**Kadowaki,**[¥] **2009**								
20	**Kendall, 2005**								
21	**Kim, 2007**								
22	**Liutkus, 2010**[¥]								

No.	Study	A	B	C	D	E	F	G	Comments
23	**Marre, 2009**								Higher attrition in the placebo arm. Restriction of glimipiride and rosiglitazone in some countries precluded maximal dose regimes.
24	**Moretto, 2008**								Diet and exercise regimes not standardised.
25	**Nauck, 2007*‡**								Open label.
26	**Nauck, 2009‡**								Higher attrition in Liraglutide 1.8 mg and placebo arms.
27	**Pratley, 2010***								Open label, but statistician was masked to the allocation.
28	**Russell-Jones, 2009*‡**								Insulin glargine arm-open label.
29	**Russell-Jones 2012‡**								
30	**Yang, 2011**								Higher attrition in the liraglutide groups.
31	**Zinman, 2007**								Greater than 20% attrition. Higher attrition in exenatide group.
32	**Zinman, 2009**								Greater than 20% attrition. Higher attrition in placebo group.

* Open label; ǂ method of randomisation/allocation concealment consisted of a computer random-number generator and voice-response or telephone system; ȣ permuted block randomisation; ¥ randomised according to baseline biochemical values or background pharmacological agent; ŧ randomised according to coded envelopes designed by a statistician high risk; low risk; unclear risk.

4.3.2 Blood pressure and heart rate

We were able to obtain heart rate data directly from Novo Nordisk for 5 out of 8 liraglutide studies (62.5% of trials) (Garber *et al*, 2009; Marre *et al*, 2010; Nauck *et al*, 2009; Russell-Jones *et al*, 2009; Zinman *et al*, 2009; Feinglos *et al*, 2005; Yang *et al*, 2011; Pratley *et al*, 2010). It is not known whether the remainder of the studies measured a significant heart rate effect. Novo Nordisk provided the data grouped into quartiles of baseline heart rate (**Figure 4.3.2.1 Effect of GLP-1 agonists on heart rate in patients with type 2 diabetes**). Pooled results show liraglutide to produce an increase in heart-rate versus placebo, weighted mean difference 2.71 beats per minute [N= 2056, 95% CI, 1.45, 3.97], heterogeneity, random effects: $Chi^2 = 13.34$, df = 6 (P = 0.04); $I^2 = 55\%$, versus placebo. Heterogeneity was reduced to acceptable limits when Marre, 2009 (Lead 1) was removed from the analysis. However, removal of this study did not significantly alter the strength or direction of effect.

Similarly, and although sponsors were very willing to assist this meta-analysis, we were only able to obtain data from 88% of trials measuring the effects of exenatide on heart rate. Pooling of exenatide trials did not result in a significant increase in heart rate, -0.88 bpm [N= 1652, 95%CI, -0.47, 2.22; I^2= 40%], versus placebo. Slight statistically significant increases were seen compared to active control, 1.36 bpm [N= 4803, 95%CI, 0.57, 2.14, I^2= 37%], with exenatide once- weekly formulations (ExLAR) showing the highest increases in heart rate, 2.14 bpm [N=1776, 95%CI, 1.11, 3.17] (**Figure 4.3.2.1 Effect of GLP-1 agonists on heart rate in patients with type 2 diabetes)**.

Overall, GLP-1 agonists' raised heart rate by a small but statistically significant extent, 1.86 bpm [N=3708, 95%CI, 0.85, 2.87, I^2= 57%], versus placebo. No effect on pooled mean differences or confidence intervals were observed due to patients lost- to- follow- up, and also when fixed effects approach was employed.

We included 25 (90%) of trials reporting systolic blood pressure and 22 (78%) of trials reporting diastolic blood pressure outcomes. GLP-1 agonists show a favourable effect on blood pressure. Liraglutide and exenatide produced comparable reductions in systolic blood pressure of 2-4 mmHg. Heterogeneity was fairly high (I^2=45%, I^2=57% GLP-1 agonists' vs placebo and active control groups respectively. In the GLP-1 agonists' versus placebo group inter-trial heterogeneity was reduced to acceptable levels after removal of Gao, 2009 and Kadowaki, 2009. Similarly, the source of heterogeneity in the GLP-1 agonists' versus active control groups appeared to be Barnett, 2007 and Defronzo, 2010. Removal of these studies did not significantly alter the strength or direction of the effect. (**Figure 4.3.2.2 GLP-1 agonists' effect on systolic blood pressure in patients with type 2 diabetes).**

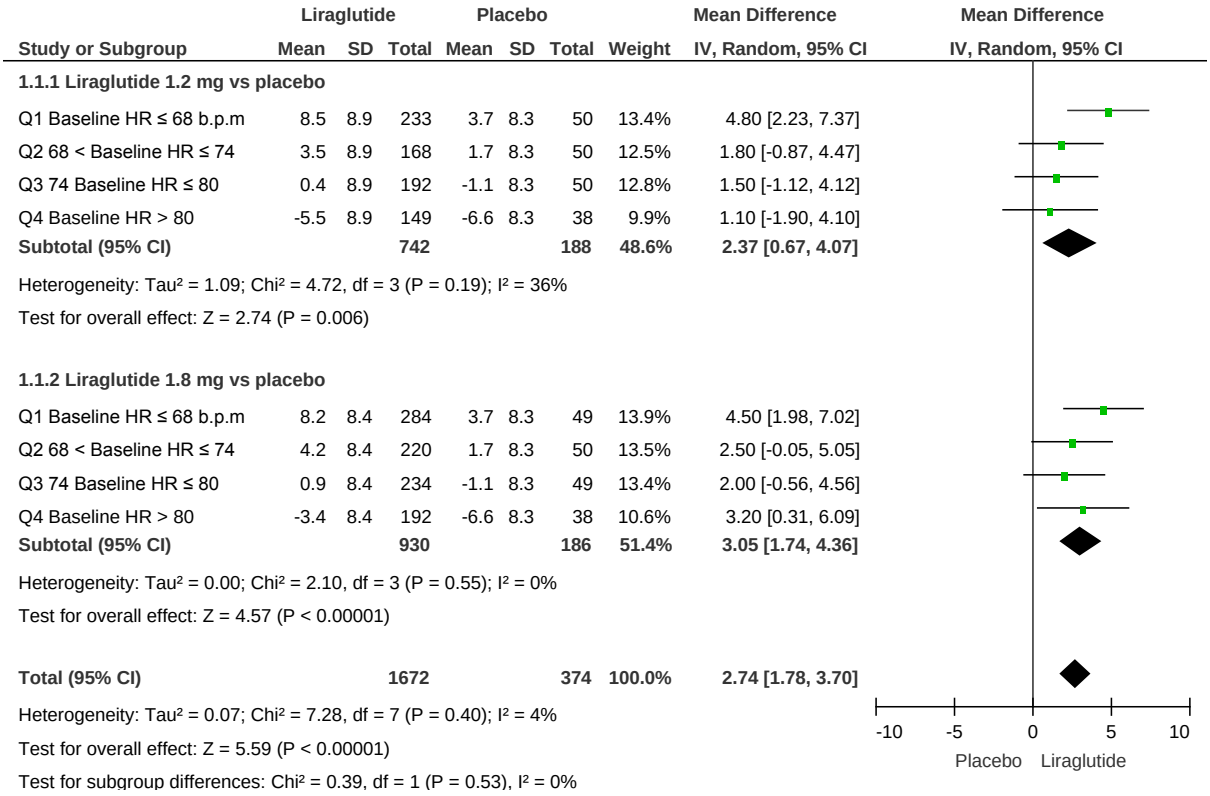
Sensitivity analysis to assess inclusion of included studies whereby blood pressure and heart rate was measured for patient safety did not alter the strength or direction of these outcomes (Barnett, 2007; Buse, 2004; Davies, 2009; Defronzo, 2005; Gao, 2009; Gallwitz, 2011; Kendall, 2005).

Figure 4.3.2.1 Effect of GLP-1 agonists on heart rate in patients with type 2 diabetes:

i) Heart rate change from baseline to endpoint in patients undergoing Liraglutide therapy for <30 weeks, stratified by baseline blood pressure (from Novo Nordisk)

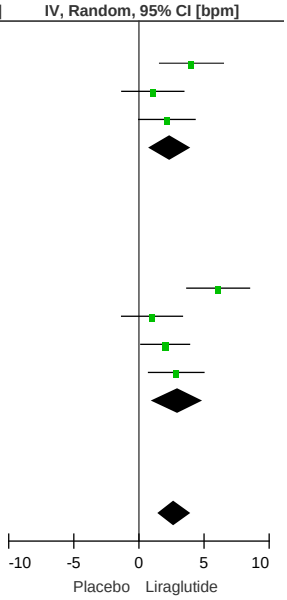
a) Liraglutide versus placebo using a random effects model

ii) Data from Lead1-5 studies: Liraglutide versus placebo in T2DM patients stratified by baseline heart rate.



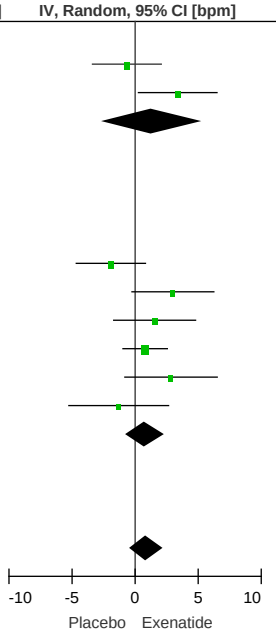
iii) Liraglutide versus placebo including dose subgroups

Study or Subgroup	Liraglutide Mean [bpm]	SD [bpm]	Total	Placebo Mean [bpm]	SD [bpm]	Total	Weight	Mean Difference IV, Random, 95% CI [bpm]
1.2.1 Liraglutide 1.2 mg vs placebo								
Marre, 2009	2.49	8.9	228	-1.55	8.3	57	13.2%	4.04 [1.60, 6.48]
Nauck 2009	2.13	8.9	240	1.06	8.3	60	13.5%	1.07 [-1.31, 3.45]
Zinman 2009	2.4	8.9	178	0.25	8.3	89	14.7%	2.15 [-0.01, 4.31]
Subtotal (95% CI)			**646**			**206**	**41.4%**	**2.39 [0.75, 4.03]**
Heterogeneity: Tau² = 0.69; Chi² = 2.98, df = 2 (P = 0.23); I² = 33%								
Test for overall effect: Z = 2.86 (P = 0.004)								
1.2.2 Liraglutide 1.8 mg vs placebo								
Marre, 2009	4.54	8.4	234	-1.55	8.3	57	13.4%	6.09 [3.68, 8.50]
Nauck 2009	2.07	8.4	242	1.06	8.3	61	13.7%	1.01 [-1.33, 3.35]
Russell- Jones, 2009	2.21	8.4	230	0.19	8.3	114	16.6%	2.02 [0.15, 3.89]
Zinman 2009	3.12	8.4	178	0.25	8.3	88	15.0%	2.87 [0.74, 5.00]
Subtotal (95% CI)			**884**			**320**	**58.6%**	**2.95 [0.96, 4.94]**
Heterogeneity: Tau² = 2.90; Chi² = 10.09, df = 3 (P = 0.02); I² = 70%								
Test for overall effect: Z = 2.90 (P = 0.004)								
Total (95% CI)			**1530**			**526**	**100.0%**	**2.71 [1.45, 3.97]**
Heterogeneity: Tau² = 1.58; Chi² = 13.34, df = 6 (P = 0.04); I² = 55%								
Test for overall effect: Z = 4.22 (P < 0.0001)								
Test for subgroup differences: Chi² = 0.18, df = 1 (P = 0.67), I² = 0%								



iv) Exenatide versus placebo including dose subgroups

Study or Subgroup	Exenatide Mean [bpm]	SD [bpm]	Total	Placebo Mean [bpm]	SD [bpm]	Total	Weight	Mean Difference IV, Random, 95% CI [bpm]
1.3.1 Exenatide 5mcg vs placebo								
Buse, 2004	0.47	9.054	125	1.12	8.911	62	13.8%	-0.65 [-3.38, 2.08]
DeFronzo, 2005	2.89	9.573	110	-0.49	9.815	57	11.7%	3.38 [0.27, 6.49]
Subtotal (95% CI)			**235**			**119**	**25.5%**	**1.29 [-2.65, 5.24]**
Heterogeneity: Tau² = 5.89; Chi² = 3.64, df = 1 (P = 0.06); I² = 73%								
Test for overall effect: Z = 0.64 (P = 0.52)								
1.3.2 Exenatide 10 mcg vs placebo								
Buse, 2004	-0.8	9.201	129	1.12	8.911	61	13.7%	-1.92 [-4.66, 0.82]
Buse, 2011	2.3	13.16	137	-0.7	13.42	122	11.1%	3.00 [-0.24, 6.24]
DeFronzo, 2005	1.06	10.746	113	-0.49	9.815	56	11.1%	1.55 [-1.70, 4.80]
Gao, 2009	0.1	9.82	234	-0.7	9.5	230	20.6%	0.80 [-0.96, 2.56]
Gill, 2010	2.14	6.52	26	-0.71	6.81	25	9.4%	2.85 [-0.81, 6.51]
Liutkus, 2010	0.5	13.72	111	1.8	11.31	54	8.5%	-1.30 [-5.25, 2.65]
Subtotal (95% CI)			**750**			**548**	**74.5%**	**0.76 [-0.76, 2.28]**
Heterogeneity: Tau² = 1.32; Chi² = 8.02, df = 5 (P = 0.16); I² = 38%								
Test for overall effect: Z = 0.98 (P = 0.33)								
Total (95% CI)			**985**			**667**	**100.0%**	**0.88 [-0.47, 2.22]**
Heterogeneity: Tau² = 1.46; Chi² = 11.76, df = 7 (P = 0.11); I² = 40%								
Test for overall effect: Z = 1.28 (P = 0.20)								
Test for subgroup differences: Chi² = 0.06, df = 1 (P = 0.81), I² = 0%								

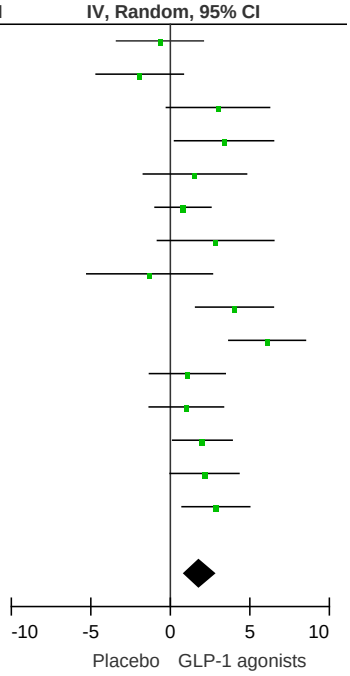


v) GLP-1 agonists versus placebo

	GLP-1 agonists			Placebo				Mean Difference	Mean Difference
Study or Subgroup	**Mean**	**SD**	**Total**	**Mean**	**SD**	**Total**	**Weight**	**IV, Random, 95% CI**	**IV, Random, 95% CI**
Buse, 2004	0.47	9.054	125	1.12	8.911	62	6.4%	-0.65 [-3.38, 2.08]	
Buse, 2004	-0.8	9.201	129	1.12	8.911	61	6.4%	-1.92 [-4.66, 0.82]	
Buse, 2011	2.3	13.16	137	-0.7	13.42	122	5.4%	3.00 [-0.24, 6.24]	
DeFronzo, 2005	2.89	9.573	110	-0.49	9.815	57	5.6%	3.38 [0.27, 6.49]	
DeFronzo, 2005	1.06	10.746	113	-0.49	9.815	56	5.4%	1.55 [-1.70, 4.80]	
Gao, 2009	0.1	9.82	234	-0.7	9.5	230	8.9%	0.80 [-0.96, 2.56]	
Gill, 2010	2.14	6.52	26	-0.71	6.81	25	4.7%	2.85 [-0.81, 6.51]	
Liutkus, 2010	0.5	13.72	111	1.8	11.31	54	4.2%	-1.30 [-5.25, 2.65]	
Marre, 2009	2.49	8.9	228	-1.55	8.3	57	7.1%	4.04 [1.60, 6.48]	
Marre, 2009	4.54	8.4	234	-1.55	8.3	57	7.2%	6.09 [3.68, 8.50]	
Nauck 2009	2.13	8.9	240	1.06	8.3	60	7.2%	1.07 [-1.31, 3.45]	
Nauck 2009	2.07	8.4	242	1.06	8.3	61	7.3%	1.01 [-1.33, 3.35]	
Russell- Jones, 2009	2.21	8.4	230	0.19	8.3	114	8.6%	2.02 [0.15, 3.89]	
Zinman 2009	2.4	8.9	178	0.25	8.3	89	7.8%	2.15 [-0.01, 4.31]	
Zinman 2009	3.12	8.4	178	0.25	8.3	88	7.9%	2.87 [0.74, 5.00]	
Total (95% CI)			**2515**			**1193**	**100.0%**	**1.86 [0.85, 2.87]**	

Heterogeneity: Tau² = 2.19; Chi² = 32.90, df = 14 (P = 0.003); I² = 57%

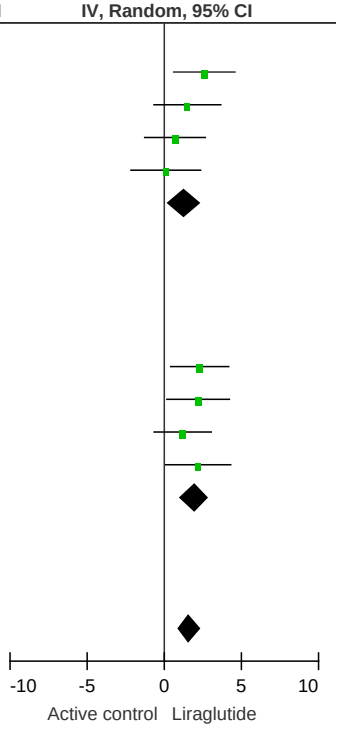
Test for overall effect: Z = 3.61 (P = 0.0003)



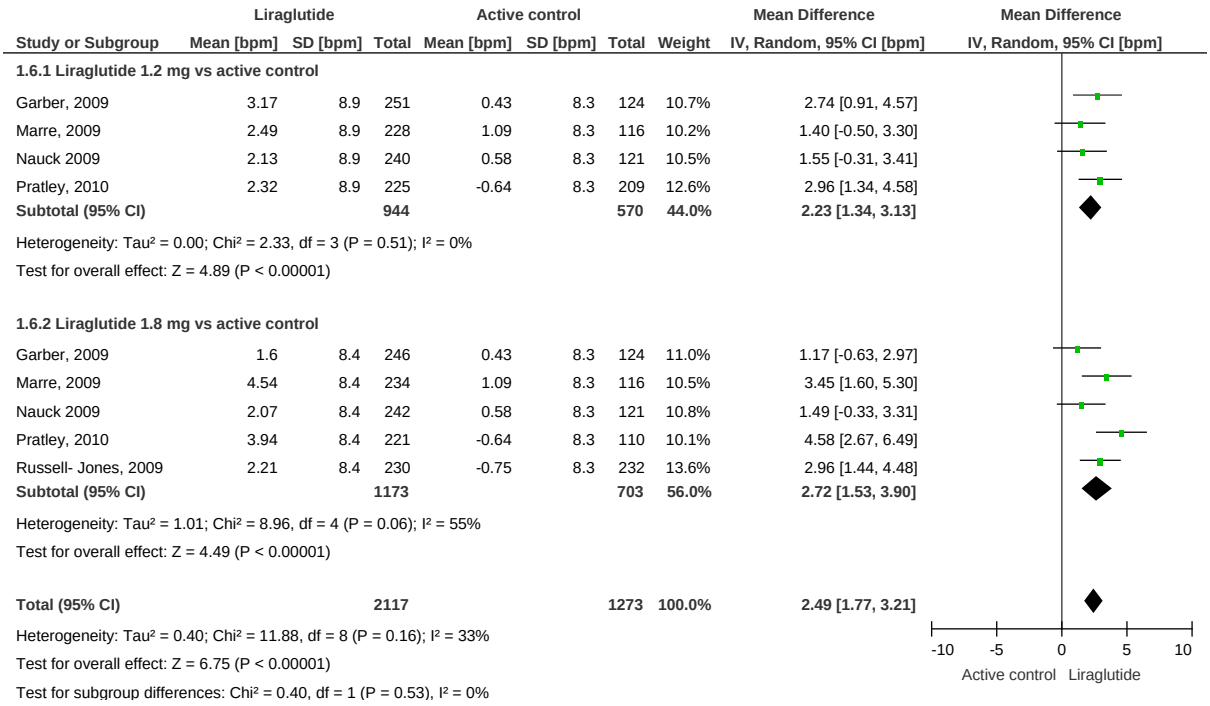
b) Effect of GLP-1 versus active control on heart rate in patients with type 2 diabetes using a random effects model

i) Data from Lead1-5 studies: Liraglutide versus active control in T2DM patients, stratified by baseline heart rate

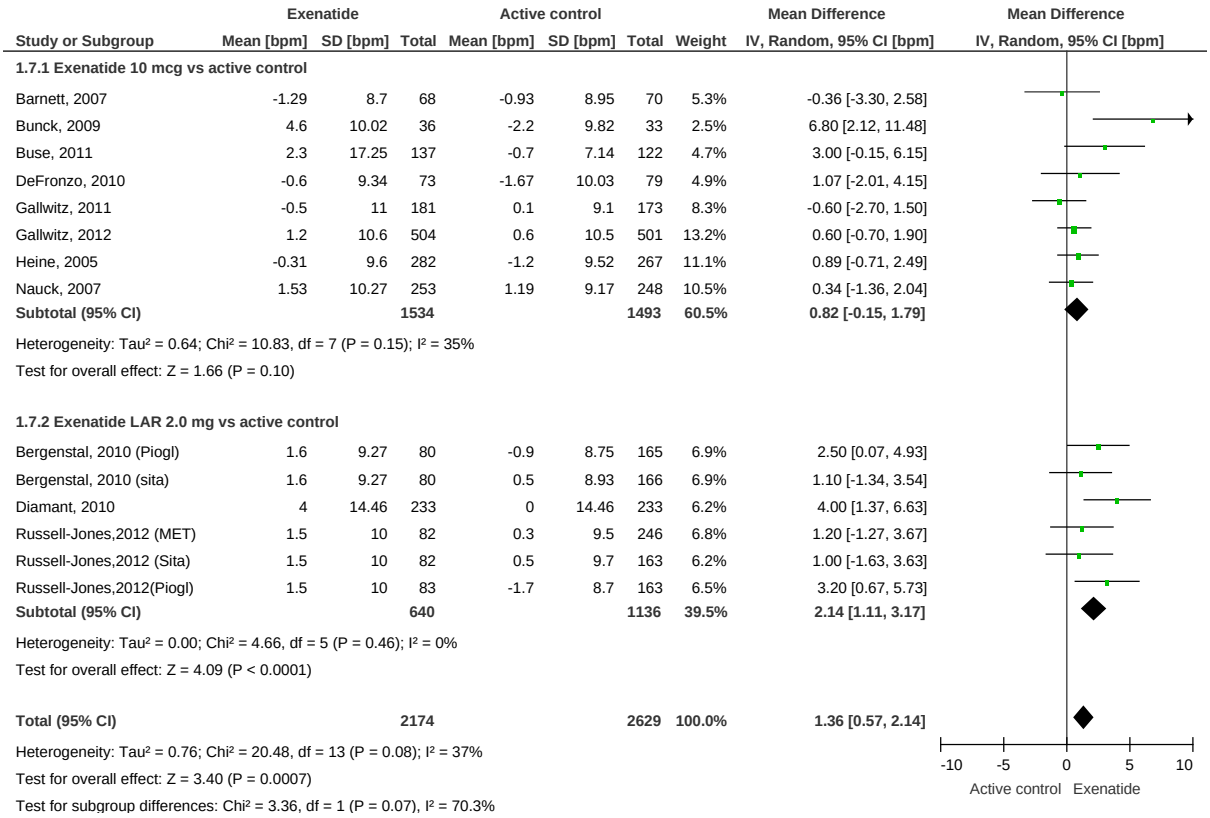
	Liraglutide			Active control				Mean Difference	Mean Difference
Study or Subgroup	**Mean**	**SD**	**Total**	**Mean**	**SD**	**Total**	**Weight**	**IV, Random, 95% CI**	**IV, Random, 95% CI**
1.5.1 Liraglutide 1.2 mg vs active control									
Q1 Baseline HR ≤ 68 b.p.m	8.5	8.9	223	5.9	8.3	103	12.9%	2.60 [0.62, 4.58]	
Q2 68 < Baseline HR ≤ 74	3.5	8.9	168	2	8.3	92	10.9%	1.50 [-0.67, 3.67]	
Q3 74 Baseline HR ≤ 80	0.4	8.9	192	-0.3	8.3	116	13.2%	0.70 [-1.27, 2.67]	
Q4 Baseline HR > 80	-5.5	8.9	149	-5.6	8.3	87	10.0%	0.10 [-2.15, 2.35]	
Subtotal (95% CI)			**732**			**398**	**47.0%**	**1.28 [0.22, 2.34]**	
Heterogeneity: Tau² = 0.05; Chi² = 3.13, df = 3 (P = 0.37); I² = 4%									
Test for overall effect: Z = 2.36 (P = 0.02)									
1.5.2 Liraglutide 1.8 mg vs active control									
Q1 Baseline HR ≤ 68 b.p.m	8.2	8.4	284	5.9	8.3	103	14.4%	2.30 [0.42, 4.18]	
Q2 68 < Baseline HR ≤ 74	4.2	8.4	220	2	8.3	92	12.4%	2.20 [0.17, 4.23]	
Q3 74 Baseline HR ≤ 80	0.9	8.4	234	-0.3	8.3	116	14.8%	1.20 [-0.65, 3.05]	
Q4 Baseline HR > 80	-3.4	8.4	192	-5.6	8.3	87	11.4%	2.20 [0.09, 4.31]	
Subtotal (95% CI)			**930**			**398**	**53.0%**	**1.95 [0.97, 2.93]**	
Heterogeneity: Tau² = 0.00; Chi² = 0.87, df = 3 (P = 0.83); I² = 0%									
Test for overall effect: Z = 3.90 (P < 0.0001)									
Total (95% CI)			**1662**			**796**	**100.0%**	**1.63 [0.92, 2.35]**	
Heterogeneity: Tau² = 0.00; Chi² = 4.84, df = 7 (P = 0.68); I² = 0%									
Test for overall effect: Z = 4.49 (P < 0.00001)									
Test for subgroup differences: Chi² = 0.83, df = 1 (P = 0.36), I² = 0%									



ii) Liraglutide versus active control displaying dose subgroups



iii) Exenatide versus active control displaying dose subgroups



Study or Subgroup	Exenatide Mean [bpm]	Exenatide SD [bpm]	Exenatide Total	Active control Mean [bpm]	Active control SD [bpm]	Active control Total	Weight	Mean Difference IV, Random, 95% CI [bpm]
1.7.1 Exenatide 10 mcg vs active control								
Barnett, 2007	-1.29	8.7	68	-0.93	8.95	70	5.3%	-0.36 [-3.30, 2.58]
Bunck, 2009	4.6	10.02	36	-2.2	9.82	33	2.5%	6.80 [2.12, 11.48]
Buse, 2011	2.3	17.25	137	-0.7	7.14	122	4.7%	3.00 [-0.15, 6.15]
DeFronzo, 2010	-0.6	9.34	73	-1.67	10.03	79	4.9%	1.07 [-2.01, 4.15]
Gallwitz, 2011	-0.5	11	181	0.1	9.1	173	8.3%	-0.60 [-2.70, 1.50]
Gallwitz, 2012	1.2	10.6	504	0.6	10.5	501	13.2%	0.60 [-0.70, 1.90]
Heine, 2005	-0.31	9.6	282	-1.2	9.52	267	11.1%	0.89 [-0.71, 2.49]
Nauck, 2007	1.53	10.27	253	1.19	9.17	248	10.5%	0.34 [-1.36, 2.04]
Subtotal (95% CI)			**1534**			**1493**	**60.5%**	**0.82 [-0.15, 1.79]**
Heterogeneity: Tau² = 0.64; Chi² = 10.83, df = 7 (P = 0.15); I² = 35%								
Test for overall effect: Z = 1.66 (P = 0.10)								
1.7.2 Exenatide LAR 2.0 mg vs active control								
Bergenstal, 2010 (Piogl)	1.6	9.27	80	-0.9	8.75	165	6.9%	2.50 [0.07, 4.93]
Bergenstal, 2010 (sita)	1.6	9.27	80	0.5	8.93	166	6.9%	1.10 [-1.34, 3.54]
Diamant, 2010	4	14.46	233	0	14.46	233	6.2%	4.00 [1.37, 6.63]
Russell-Jones,2012 (MET)	1.5	10	82	0.3	9.5	246	6.8%	1.20 [-1.27, 3.67]
Russell-Jones,2012 (Sita)	1.5	10	82	0.5	9.7	163	6.2%	1.00 [-1.63, 3.63]
Russell-Jones,2012(Piogl)	1.5	10	83	-1.7	8.7	163	6.5%	3.20 [0.67, 5.73]
Subtotal (95% CI)			**640**			**1136**	**39.5%**	**2.14 [1.11, 3.17]**
Heterogeneity: Tau² = 0.00; Chi² = 4.66, df = 5 (P = 0.46); I² = 0%								
Test for overall effect: Z = 4.09 (P < 0.0001)								
Total (95% CI)			**2174**			**2629**	**100.0%**	**1.36 [0.57, 2.14]**
Heterogeneity: Tau² = 0.76; Chi² = 20.48, df = 13 (P = 0.08); I² = 37%								
Test for overall effect: Z = 3.40 (P = 0.0007)								
Test for subgroup differences: Chi² = 3.36, df = 1 (P = 0.07), I² = 70.3%								

iv) GLP-1 versus active control

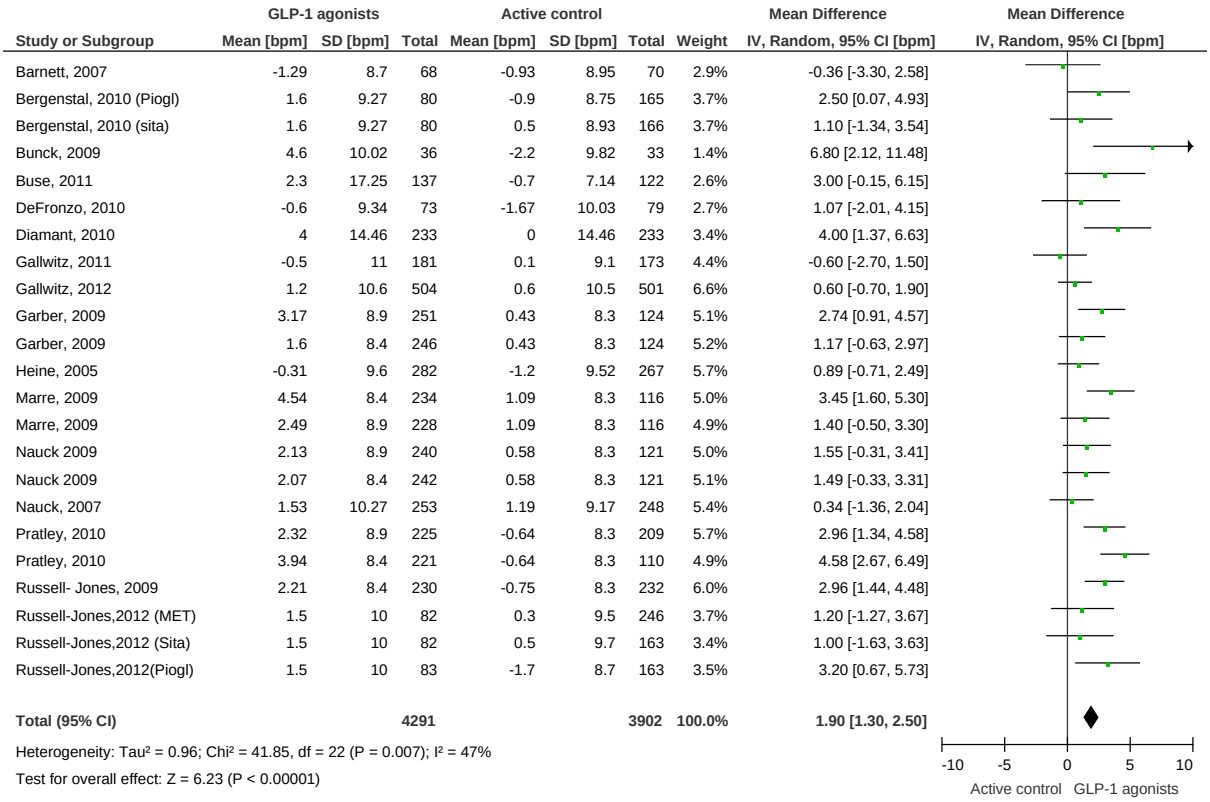
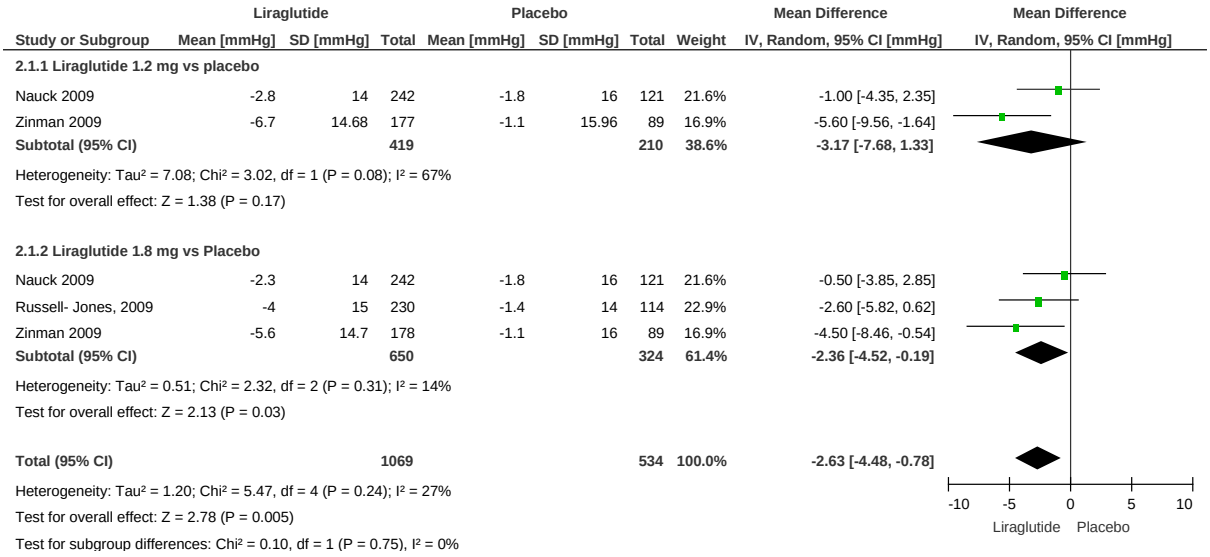


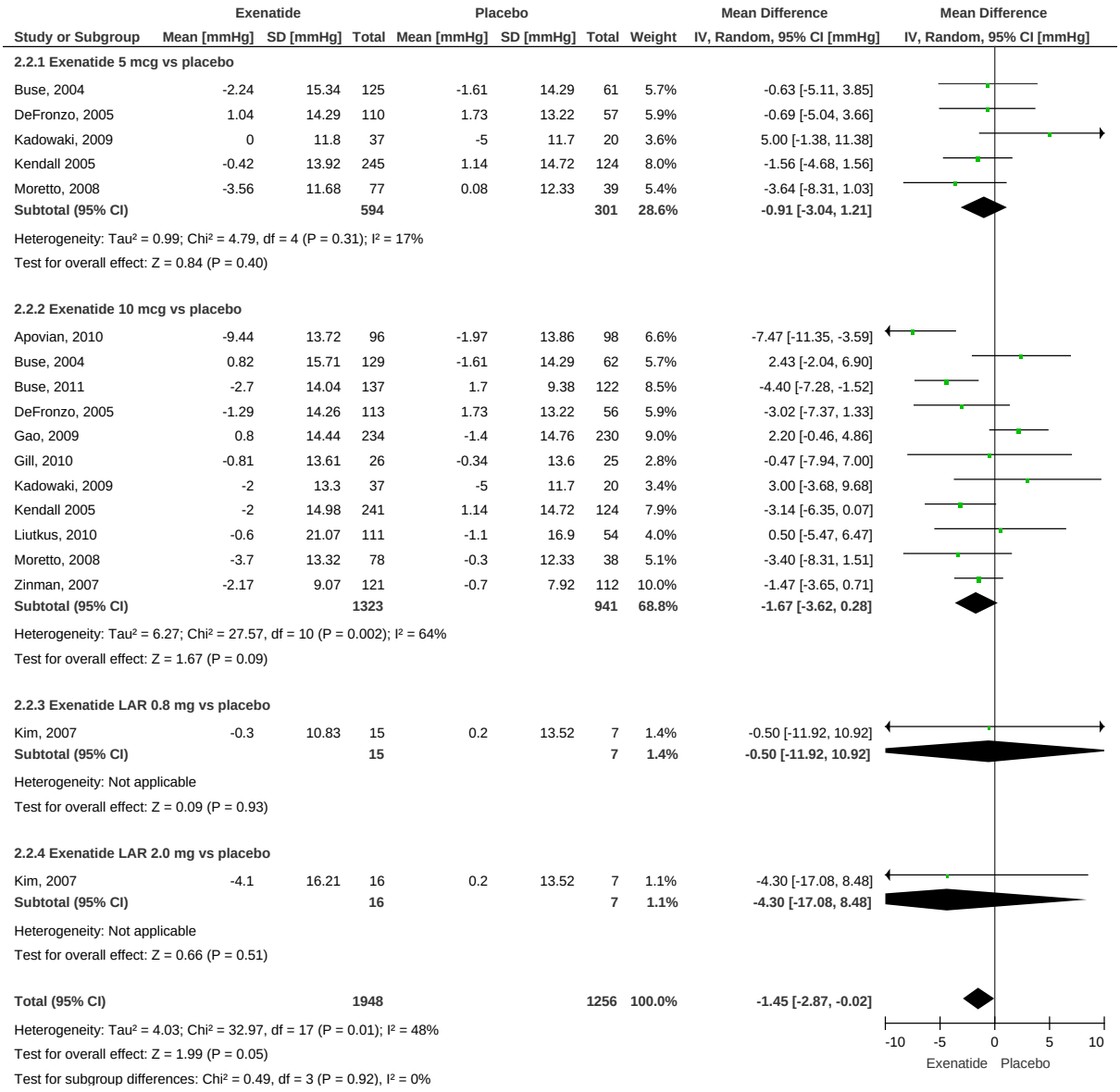
Figure 4.3.2.2 GLP-1 agonists' effect on systolic blood pressure in patients with type 2 diabetes:

a) Effect of GLP-1 versus placebo on systolic blood pressure in patients with type 2 diabetes using a random effects model

i) Liraglutide versus placebo including dose subgroups



ii) Exenatide versus placebo including dose subgroups

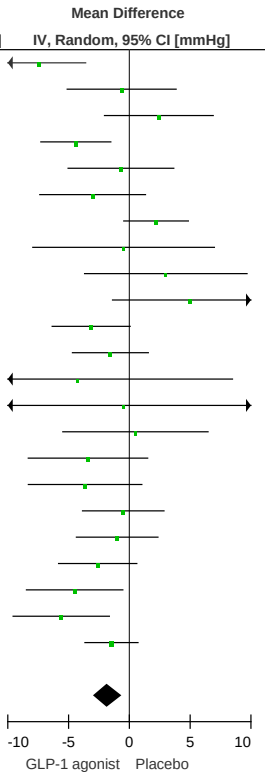


iii) GLP-1 agonists versus placebo

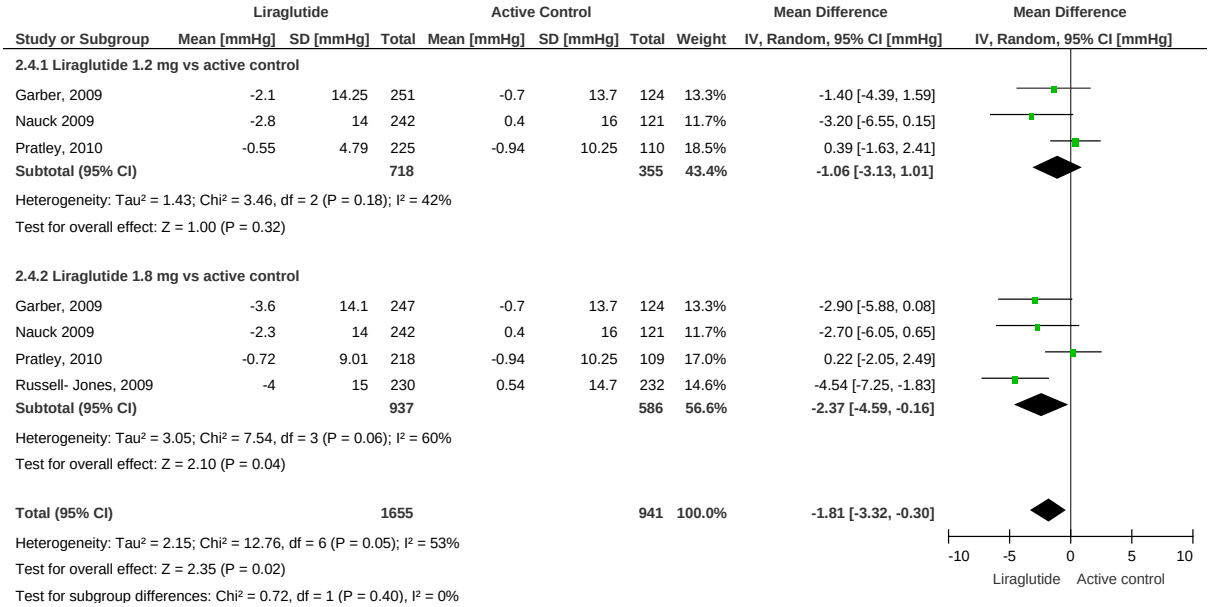
Study or Subgroup	GLP-1 agonists Mean [mmHg]	SD [mmHg]	Total	Placebo Mean [mmHg]	SD [mmHg]	Total	Weight	Mean Difference IV, Random, 95% CI [mmHg]
Apovian, 2010	-9.44	13.72	96	-1.97	13.86	98	4.9%	-7.47 [-11.35, -3.59]
Buse, 2004	-2.24	15.34	125	-1.61	14.29	61	4.1%	-0.63 [-5.11, 3.85]
Buse, 2004	0.82	15.71	129	-1.61	14.29	62	4.1%	2.43 [-2.04, 6.90]
Buse, 2011	-2.7	14.04	137	1.7	9.38	122	6.5%	-4.40 [-7.28, -1.52]
DeFronzo, 2005	1.04	14.29	110	1.73	13.22	57	4.3%	-0.69 [-5.04, 3.66]
DeFronzo, 2005	-1.29	14.26	113	1.73	13.22	56	4.3%	-3.02 [-7.37, 1.33]
Gao, 2009	0.8	14.44	234	-1.4	14.76	230	6.9%	2.20 [-0.46, 4.86]
Gill, 2010	-0.81	13.61	26	-0.34	13.6	25	1.9%	-0.47 [-7.94, 7.00]
Kadowaki, 2009	-2	13.3	37	-5	11.7	20	2.3%	3.00 [-3.68, 9.68]
Kadowaki, 2009	0	11.8	37	-5	11.7	20	2.5%	5.00 [-1.38, 11.38]
Kendall 2005	-2	14.98	241	1.14	14.72	124	5.9%	-3.14 [-6.35, 0.07]
Kendall 2005	-0.42	13.92	245	1.14	14.72	124	6.0%	-1.56 [-4.68, 1.56]
Kim, 2007	-4.1	16.21	16	0.2	13.52	7	0.8%	-4.30 [-17.08, 8.48]
Kim, 2007	-0.3	10.83	15	0.2	13.52	7	0.9%	-0.50 [-11.92, 10.92]
Liutkus, 2010	-0.6	21.07	111	-1.1	16.9	54	2.8%	0.50 [-5.47, 6.47]
Moretto, 2008	-3.7	13.32	78	-0.3	12.33	38	3.6%	-3.40 [-8.31, 1.51]
Moretto, 2008	-3.56	11.68	77	0.08	12.33	39	3.9%	-3.64 [-8.31, 1.03]
Nauck 2009	-2.3	14	242	-1.8	16	121	5.6%	-0.50 [-3.85, 2.85]
Nauck 2009	-2.8	14	242	-1.8	16	121	5.6%	-1.00 [-4.35, 2.35]
Russell- Jones, 2009	-4	15	230	-1.4	14	114	5.9%	-2.60 [-5.82, 0.62]
Zinman 2009	-5.6	14.7	178	-1.1	16	89	4.7%	-4.50 [-8.46, -0.54]
Zinman 2009	-6.7	14.68	177	-1.1	15.96	89	4.7%	-5.60 [-9.56, -1.64]
Zinman, 2007	-2.17	9.07	121	-0.7	7.92	112	7.8%	-1.47 [-3.65, 0.71]
Total (95% CI)			**3017**			**1790**	**100.0%**	**-1.79 [-2.94, -0.64]**

Heterogeneity: Tau² = 3.19; Chi² = 39.65, df = 22 (P = 0.01); I² = 45%

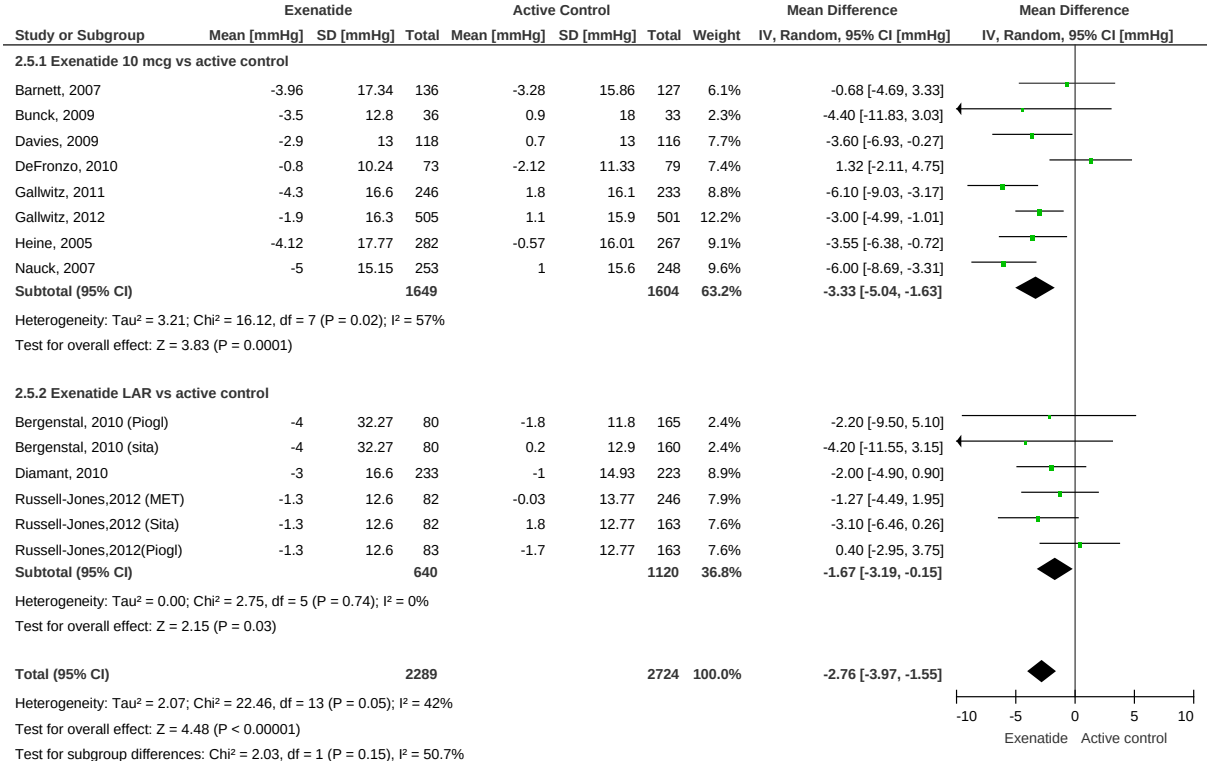
Test for overall effect: Z = 3.05 (P = 0.002)



b) Effect of GLP-1 versus active control on systolic blood pressure in patients with type 2 diabetes using a random effects model



ii) Exenatide versus active control displaying dose subgroups



iii) GLP-1 agonists' versus active control

	GLP-1 agonist			Active Control				Mean Difference	Mean Difference
Study or Subgroup	**Mean [mmHg]**	**SD [mmHg]**	**Total**	**Mean [mmHg]**	**SD [mmHg]**	**Total**	**Weight**	**IV, Random, 95% CI [mmHg]**	**IV, Random, 95% CI [mmHg]**
Barnett, 2007	-3.96	17.34	136	-3.28	15.86	127	3.7%	-0.68 [-4.69, 3.33]	
Bergenstal, 2010 (Piogl)	-4	32.27	80	-1.8	11.8	165	1.5%	-2.20 [-9.50, 5.10]	
Bergenstal, 2010 (sita)	-4	32.27	80	0.2	12.9	160	1.5%	-4.20 [-11.55, 3.15]	
Bunck, 2009	-3.5	12.8	36	0.9	18	33	1.5%	-4.40 [-11.83, 3.03]	
Davies, 2009	-2.9	13	118	0.7	13	116	4.7%	-3.60 [-6.93, -0.27]	
DeFronzo, 2010	-0.8	10.24	73	-2.12	11.33	79	4.5%	1.32 [-2.11, 4.75]	
Diamant, 2010	-3	16.6	233	-1	14.93	223	5.4%	-2.00 [-4.90, 0.90]	
Gallwitz, 2011	-4.3	16.6	246	1.8	16.1	233	5.3%	-6.10 [-9.03, -3.17]	
Gallwitz, 2012	-1.9	16.3	505	1.1	15.9	501	7.2%	-3.00 [-4.99, -1.01]	
Garber, 2009	-3.6	14.1	247	-0.7	13.7	124	5.2%	-2.90 [-5.88, 0.08]	
Garber, 2009	-2.1	14.25	251	-0.7	13.7	124	5.2%	-1.40 [-4.39, 1.59]	
Heine, 2005	-4.12	17.77	282	-0.57	16.01	267	5.5%	-3.55 [-6.38, -0.72]	
Nauck 2009	-2.8	14	242	0.4	16	121	4.6%	-3.20 [-6.55, 0.15]	
Nauck 2009	-2.3	14	242	0.4	16	121	4.6%	-2.70 [-6.05, 0.65]	
Nauck, 2007	-5	15.15	253	1	15.6	248	5.8%	-6.00 [-8.69, -3.31]	
Pratley, 2010	-0.55	4.79	225	-0.94	10.25	110	7.2%	0.39 [-1.63, 2.41]	
Pratley, 2010	-0.72	9.01	218	-0.94	10.25	109	6.6%	0.22 [-2.05, 2.49]	
Russell- Jones, 2009	-4	15	230	0.54	14.7	232	5.7%	-4.54 [-7.25, -1.83]	
Russell-Jones,2012 (MET)	-1.3	12.6	82	-0.03	13.77	246	4.8%	-1.27 [-4.49, 1.95]	
Russell-Jones,2012 (Sita)	-1.3	12.6	82	1.8	12.77	163	4.6%	-3.10 [-6.46, 0.26]	
Russell-Jones,2012(Piogl)	-1.3	12.6	83	-1.7	12.77	163	4.6%	0.40 [-2.95, 3.75]	
Total (95% CI)			**3944**			**3665**	**100.0%**	**-2.39 [-3.35, -1.42]**	

Heterogeneity: Tau² = 2.32; Chi² = 39.21, df = 20 (P = 0.006); I² = 49%

Test for overall effect: Z = 4.85 (P < 0.00001)

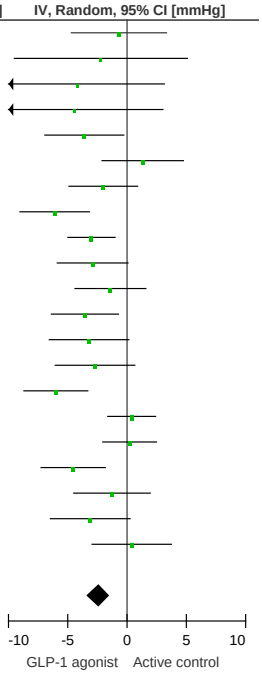
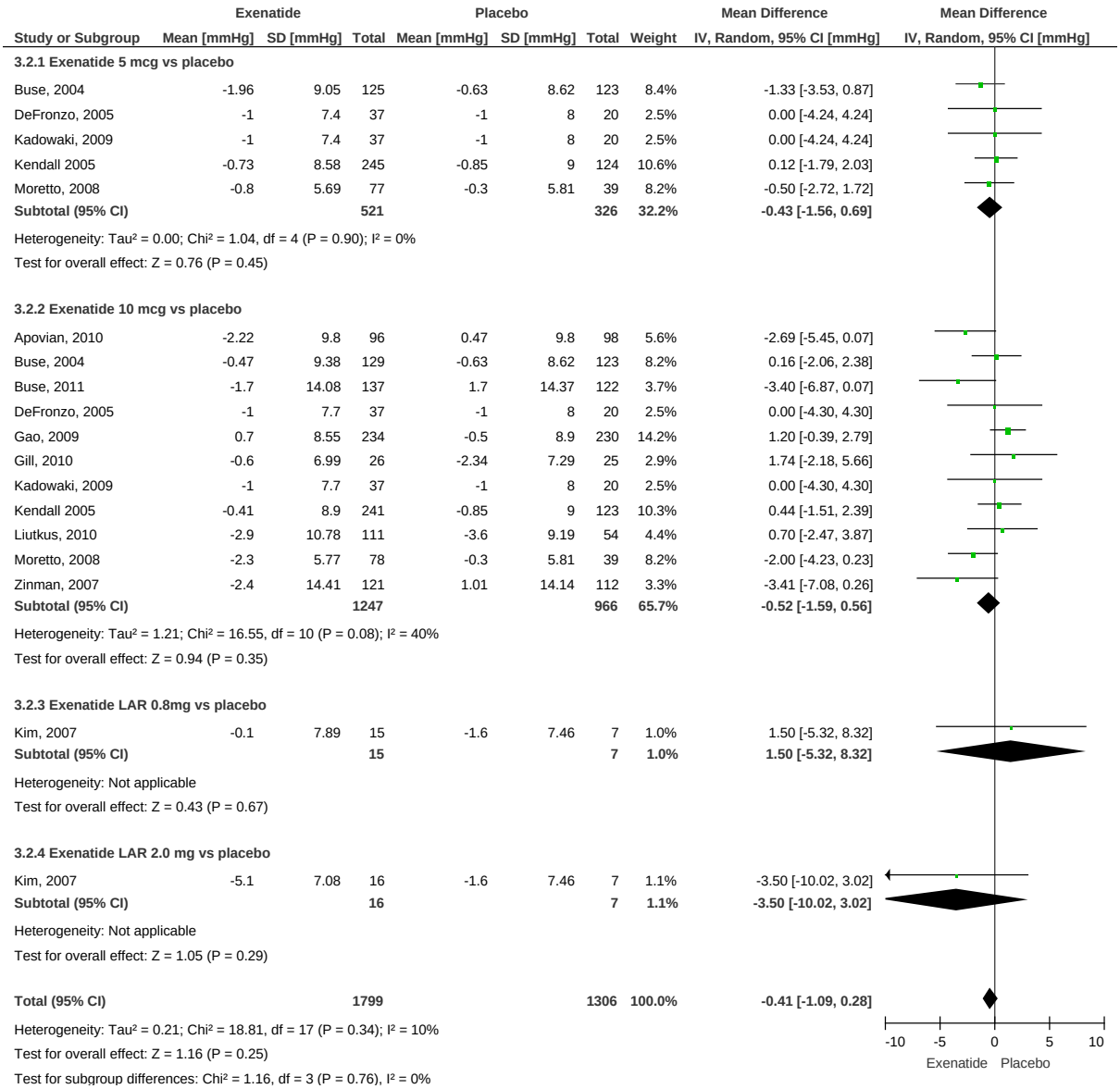


Figure 4.3.2.3 GLP-1 agonists' effect on diastolic blood pressure in patients with type 2 diabetes:

a) Effect of GLP-1 versus active control on diastolic blood pressure in patients with type 2 diabetes using a random effects model

i) Exenatide versus placebo displaying dose subgroups

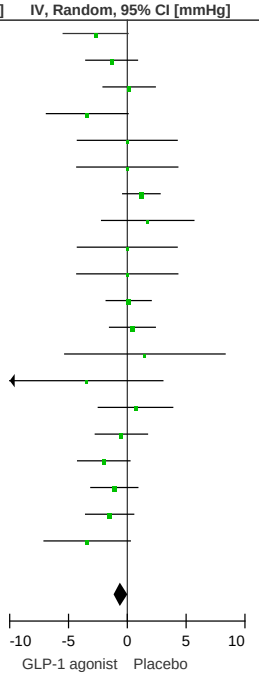


ii) GLP-1 agonists' versus placebo

	GLP-1 agonists			Placebo				Mean Difference	Mean Difference
Study or Subgroup	**Mean [mmHg]**	**SD [mmHg]**	**Total**	**Mean [mmHg]**	**SD [mmHg]**	**Total**	**Weight**	**IV, Random, 95% CI [mmHg]**	**IV, Random, 95% CI [mmHg]**
Apovian, 2010	-2.22	9.8	96	0.47	9.8	98	4.6%	-2.69 [-5.45, 0.07]	
Buse, 2004	-1.96	9.05	125	-0.63	8.62	123	7.0%	-1.33 [-3.53, 0.87]	
Buse, 2004	-0.47	9.38	129	-0.63	8.62	123	6.9%	0.16 [-2.06, 2.38]	
Buse, 2011	-1.7	14.08	137	1.7	14.37	122	3.0%	-3.40 [-6.87, 0.07]	
DeFronzo, 2005	-1	7.4	37	-1	8	20	2.0%	0.00 [-4.24, 4.24]	
DeFronzo, 2005	-1	7.7	37	-1	8	20	2.0%	0.00 [-4.30, 4.30]	
Gao, 2009	0.7	8.55	234	-0.5	8.9	230	12.5%	1.20 [-0.39, 2.79]	
Gill, 2010	-0.6	6.99	26	-2.34	7.29	25	2.3%	1.74 [-2.18, 5.66]	
Kadowaki, 2009	-1	7.4	37	-1	8	20	2.0%	0.00 [-4.24, 4.24]	
Kadowaki, 2009	-1	7.7	37	-1	8	20	2.0%	0.00 [-4.30, 4.30]	
Kendall 2005	-0.73	8.58	245	-0.85	9	124	9.0%	0.12 [-1.79, 2.03]	
Kendall 2005	-0.41	8.9	241	-0.85	9	123	8.7%	0.44 [-1.51, 2.39]	
Kim, 2007	-0.1	7.89	15	-1.6	7.46	7	0.8%	1.50 [-5.32, 8.32]	
Kim, 2007	-5.1	7.08	16	-1.6	7.46	7	0.9%	-3.50 [-10.02, 3.02]	
Liutkus, 2010	-2.9	10.78	111	-3.6	9.19	54	3.5%	0.70 [-2.47, 3.87]	
Moretto, 2008	-0.8	5.69	77	-0.3	5.81	39	6.9%	-0.50 [-2.72, 1.72]	
Moretto, 2008	-2.3	5.77	78	-0.3	5.81	39	6.9%	-2.00 [-4.23, 0.23]	
Zinman 2009	-1.9	8.07	178	-0.8	7.7	88	8.3%	-1.10 [-3.10, 0.90]	
Zinman 2009	-2.3	8.66	178	-0.8	7.7	89	8.0%	-1.50 [-3.54, 0.54]	
Zinman, 2007	-2.4	14.41	121	1.01	14.14	112	2.7%	-3.41 [-7.08, 0.26]	
Total (95% CI)			**2155**			**1483**	**100.0%**	**-0.54 [-1.15, 0.07]**	

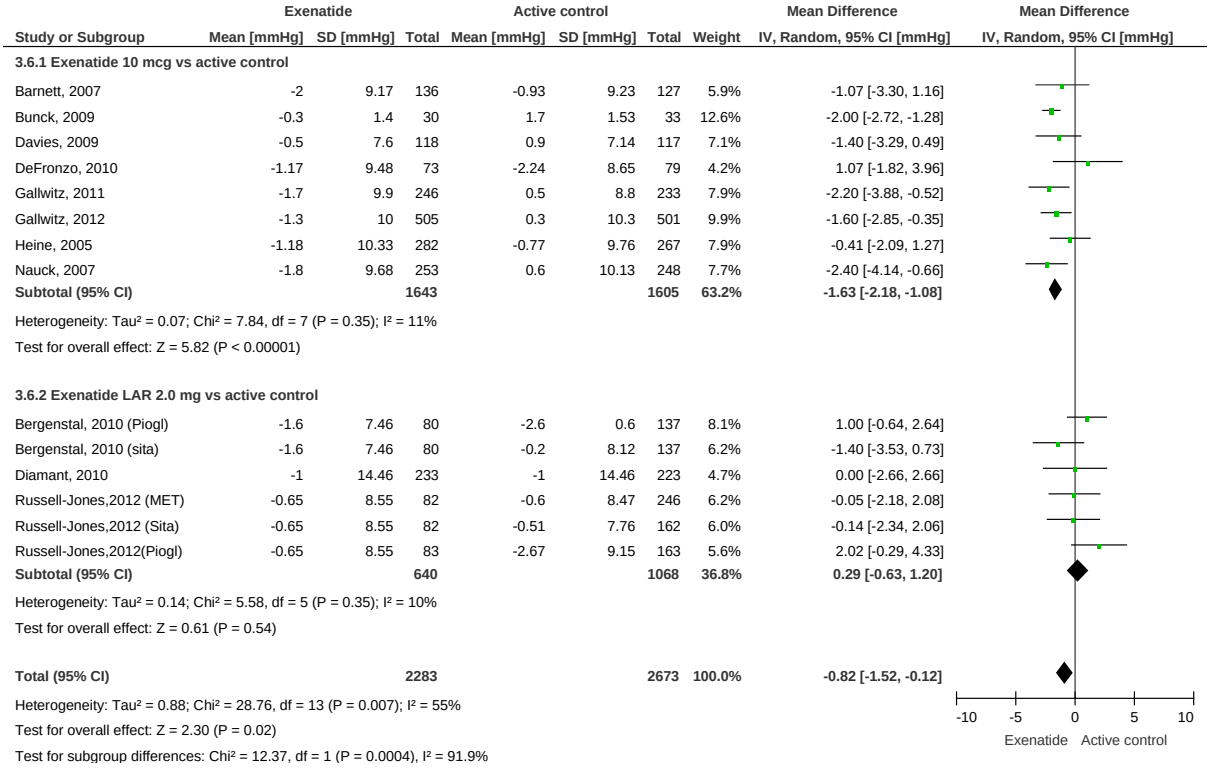
Heterogeneity: Tau² = 0.12; Chi² = 20.27, df = 19 (P = 0.38); I² = 6%

Test for overall effect: Z = 1.72 (P = 0.08)



b) Effect of GLP-1 versus active control on diastolic blood pressure in patients with type 2 diabetes using a random effects model

i) Exenatide versus active control displaying dose subgroups

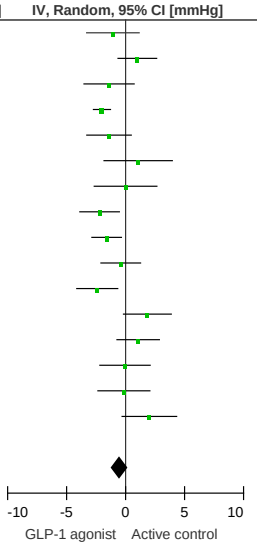


ii) GLP-1 agonists' versus active control

	GLP-1 agonists			Active control				Mean Difference	Mean Difference
Study or Subgroup	**Mean [mmHg]**	**SD [mmHg]**	**Total**	**Mean [mmHg]**	**SD [mmHg]**	**Total**	**Weight**	**IV, Random, 95% CI [mmHg]**	**IV, Random, 95% CI [mmHg]**
Barnett, 2007	-2	9.17	136	-0.93	9.23	127	5.4%	-1.07 [-3.30, 1.16]	
Bergenstal, 2010 (Piogl)	-1.6	7.46	80	-2.6	0.6	137	7.0%	1.00 [-0.64, 2.64]	
Bergenstal, 2010 (sita)	-1.6	7.46	80	-0.2	8.12	137	5.7%	-1.40 [-3.53, 0.73]	
Bunck, 2009	-0.3	1.4	30	1.7	1.53	33	9.6%	-2.00 [-2.72, -1.28]	
Davies, 2009	-0.5	7.6	118	0.9	7.14	117	6.3%	-1.40 [-3.29, 0.49]	
DeFronzo, 2010	-1.17	9.48	73	-2.24	8.65	79	4.1%	1.07 [-1.82, 3.96]	
Diamant, 2010	-1	14.46	233	-1	14.46	223	4.5%	0.00 [-2.66, 2.66]	
Gallwitz, 2011	-1.7	9.9	246	0.5	8.8	233	6.9%	-2.20 [-3.88, -0.52]	
Gallwitz, 2012	-1.3	10	505	0.3	10.3	501	8.1%	-1.60 [-2.85, -0.35]	
Heine, 2005	-1.18	10.33	282	-0.77	9.76	267	6.9%	-0.41 [-2.09, 1.27]	
Nauck, 2007	-1.8	9.68	253	0.6	10.13	248	6.7%	-2.40 [-4.14, -0.66]	
Pratley, 2010	0.07	8.84	221	-1.78	8.83	109	5.9%	1.85 [-0.18, 3.88]	
Pratley, 2010	-0.71	5.43	225	-1.78	8.83	110	6.5%	1.07 [-0.73, 2.87]	
Russell-Jones,2012 (MET)	-0.65	8.55	82	-0.6	8.47	246	5.7%	-0.05 [-2.18, 2.08]	
Russell-Jones,2012 (Sita)	-0.65	8.55	82	-0.51	7.76	162	5.5%	-0.14 [-2.34, 2.06]	
Russell-Jones,2012(Piogl)	-0.65	8.55	83	-2.67	9.15	163	5.2%	2.02 [-0.29, 4.33]	
Total (95% CI)			**2729**			**2892**	**100.0%**	**-0.50 [-1.24, 0.24]**	

Heterogeneity: Tau² = 1.35; Chi² = 42.27, df = 15 (P = 0.0002); I² = 65%

Test for overall effect: Z = 1.33 (P = 0.18)



4.3.3 Body weight

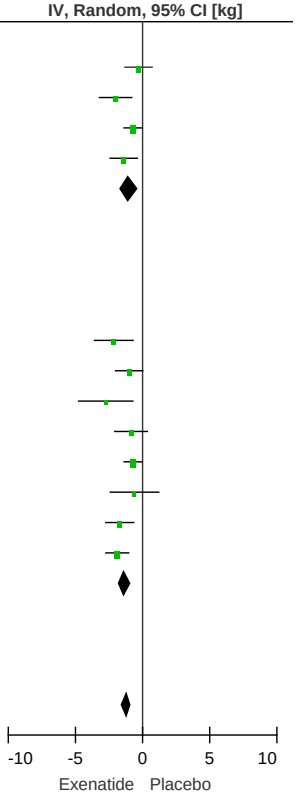
This study confirms GLP-1 agonists to display a clinically beneficial effect on body weight, WMD -1.22 kg [N=3128, 95%CI -0.93, -1.51, I^2= 26%] versus placebo. Liraglutide reduced body weight by -2.46 kg [N=1825, 95%CI, -2.04, -2.87, I^2= 65%] compared to active control. Removal of Russell-Jones, 2009 from this analyses abolished heterogeneity in the Liraglutide 1.8 mg subgroup and brought the overall inter-trial heterogeneity to acceptable limits. Heterogeneity was I^2= ≥95% for trials comparing exenatide versus active control. Results were unchanged when using a fixed effects model or examining the number of completers rather than ITT. Overall, mean (SD) body weight decreased by between -0.39 kg (2.38) and -6.22 kg (5.09) (**Figure 4.3.3.1 GLP-1 agonists' effects on weight loss in type 2 diabetes patients**).

Figure 4.3.3.1 GLP-1 agonists effects on weight loss in type 2 diabetes patients:

a) GLP-1 agonists versus placebo for included studies using the random effects model

i) Exenatide versus placebo displaying dose subgroups

	Exenatide			Placebo				Mean Difference	Mean Difference
Study or Subgroup	**Mean [kg]**	**SD [kg]**	**Total**	**Mean [kg]**	**SD [kg]**	**Total**	**Weight**	**IV, Random, 95% CI [kg]**	**IV, Random, 95% CI [kg]**
4.2.1 Exenatide 5 mcg vs placebo									
Buse, 2004	-0.9	3.35	125	-0.6	3.3	62	8.9%	-0.30 [-1.31, 0.71]	
Kadowaki, 2009	-1.54	2.31	73	0.47	2.34	18	7.0%	-2.01 [-3.21, -0.81]	
Kendall 2005	-1.6	3.13	245	-0.9	3.14	124	13.8%	-0.70 [-1.38, -0.02]	
Moretto, 2008	-2.8	2.63	77	-1.4	2.63	39	8.9%	-1.40 [-2.41, -0.39]	
Subtotal (95% CI)			**520**			**243**	**38.7%**	**-1.02 [-1.69, -0.36]**	
Heterogeneity: Tau² = 0.22; Chi² = 5.85, df = 3 (P = 0.12); I² = 49%									
Test for overall effect: Z = 3.01 (P = 0.003)									
4.2.2 Exenatide 10 mcg vs placebo									
Apovian, 2010	-6.22	5.09	96	-4.08	5.15	98	5.4%	-2.14 [-3.58, -0.70]	
Buse, 2004	-1.6	3.4	129	-0.6	3.3	61	8.9%	-1.00 [-2.01, 0.01]	
Buse, 2011	-1.78	8.31	137	0.96	8.28	122	3.0%	-2.74 [-4.76, -0.72]	
Kadowaki, 2009	-0.39	2.38	72	0.47	2.34	18	7.0%	-0.86 [-2.07, 0.35]	
Kendall 2005	-1.6	3.1	241	-0.9	3.14	123	13.8%	-0.70 [-1.38, -0.02]	
Liutkus, 2010	-1.4	6.32	111	-0.8	5.14	54	3.7%	-0.60 [-2.41, 1.21]	
Moretto, 2008	-3.1	2.65	78	-1.4	2.7	38	8.6%	-1.70 [-2.74, -0.66]	
Zinman, 2007	-2.12	2.75	121	-0.24	2.75	60	11.0%	-1.88 [-2.73, -1.03]	
Subtotal (95% CI)			**985**			**574**	**61.3%**	**-1.33 [-1.80, -0.86]**	
Heterogeneity: Tau² = 0.13; Chi² = 10.00, df = 7 (P = 0.19); I² = 30%									
Test for overall effect: Z = 5.52 (P < 0.00001)									
Total (95% CI)			**1505**			**817**	**100.0%**	**-1.21 [-1.58, -0.83]**	
Heterogeneity: Tau² = 0.15; Chi² = 17.04, df = 11 (P = 0.11); I² = 35%									
Test for overall effect: Z = 6.28 (P < 0.00001)									
Test for subgroup differences: Chi² = 0.55, df = 1 (P = 0.46), I² = 0%									

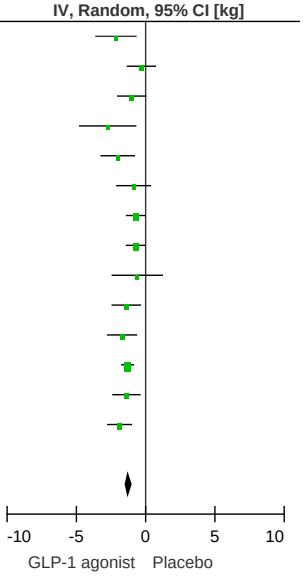


ii) GLP-1 agonists' versus placebo (includes 2 liraglutide studies: Russell-Jones, 2009, Nauck, 2009)

	GLP-1 agonist			Placebo				Mean Difference	Mean Difference
Study or Subgroup	**Mean [kg]**	**SD [kg]**	**Total**	**Mean [kg]**	**SD [kg]**	**Total**	**Weight**	**IV, Random, 95% CI [kg]**	**IV, Random, 95% CI [kg]**
Apovian, 2010	-6.22	5.09	96	-4.08	5.15	98	3.6%	-2.14 [-3.58, -0.70]	
Buse, 2004	-0.9	3.35	125	-0.6	3.3	62	6.5%	-0.30 [-1.31, 0.71]	
Buse, 2004	-1.6	3.4	129	-0.6	3.3	61	6.5%	-1.00 [-2.01, 0.01]	
Buse, 2011	-1.78	8.31	137	0.96	8.28	122	1.9%	-2.74 [-4.76, -0.72]	
Kadowaki, 2009	-1.54	2.31	73	0.47	2.34	18	4.9%	-2.01 [-3.21, -0.81]	
Kadowaki, 2009	-0.39	2.38	72	0.47	2.34	18	4.8%	-0.86 [-2.07, 0.35]	
Kendall 2005	-1.6	3.13	245	-0.9	3.14	124	11.4%	-0.70 [-1.38, -0.02]	
Kendall 2005	-1.6	3.1	241	-0.9	3.14	123	11.4%	-0.70 [-1.38, -0.02]	
Liutkus, 2010	-1.4	6.32	111	-0.8	5.14	54	2.4%	-0.60 [-2.41, 1.21]	
Moretto, 2008	-2.8	2.63	77	-1.4	2.63	39	6.5%	-1.40 [-2.41, -0.39]	
Moretto, 2008	-3.1	2.65	78	-1.4	2.7	38	6.2%	-1.70 [-2.74, -0.66]	
Nauck 2009	-2.8	2.76	230	-1.5	1.72	232	18.5%	-1.30 [-1.72, -0.88]	
Russell- Jones, 2009	-1.8	4.75	230	-0.42	4.1	114	6.9%	-1.38 [-2.35, -0.41]	
Zinman, 2007	-2.12	2.75	121	-0.24	2.75	60	8.4%	-1.88 [-2.73, -1.03]	
Total (95% CI)			**1965**			**1163**	**100.0%**	**-1.22 [-1.51, -0.93]**	

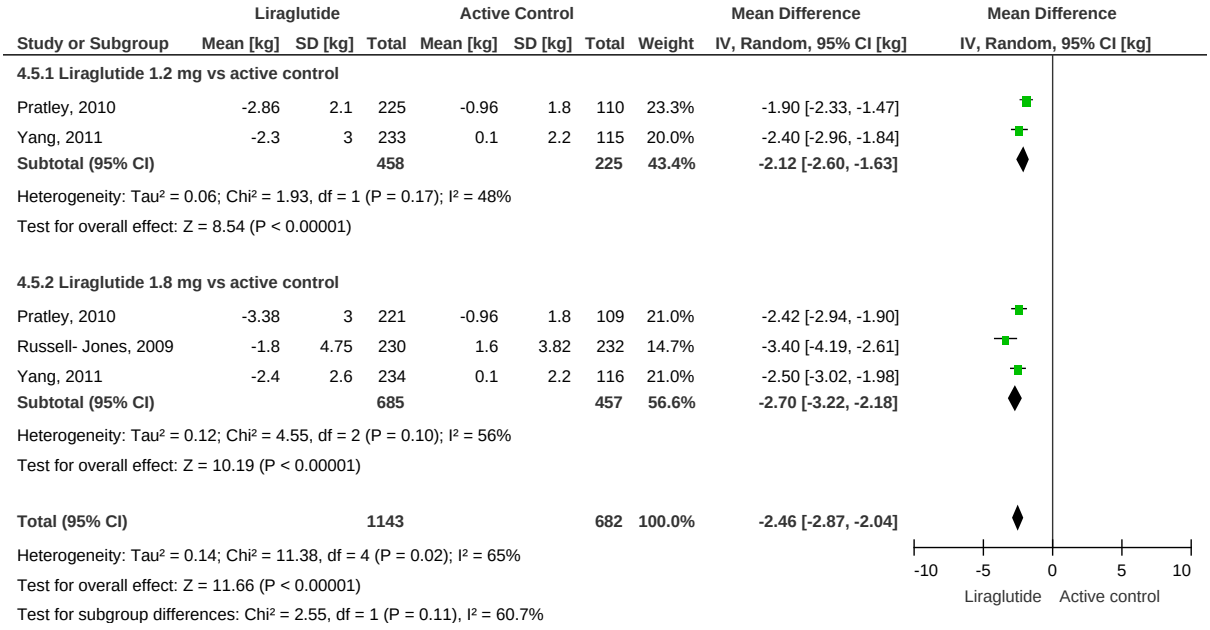
Heterogeneity: Tau² = 0.07; Chi² = 17.54, df = 13 (P = 0.18); I² = 26%

Test for overall effect: Z = 8.20 (P < 0.00001)

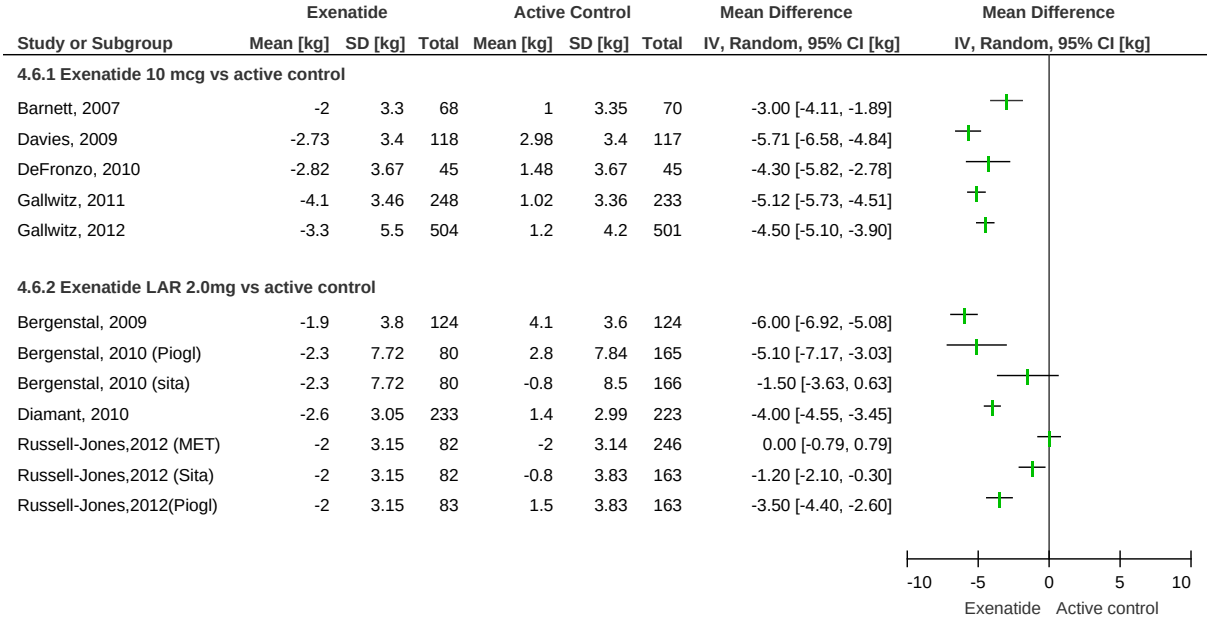


b) GLP-1 agonists versus active control for included studies using the random effects model (95%CI)

ii) Liraglutide versus active control effects on body weight, displaying dose subgroups

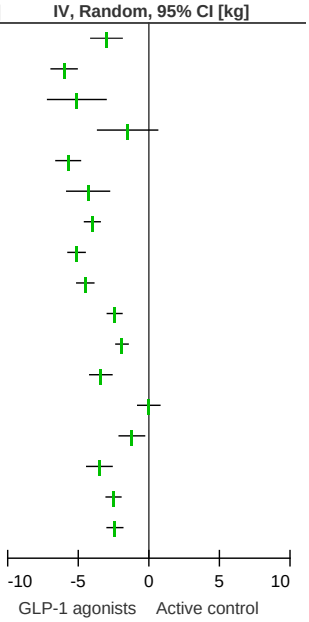


ii) Exenatide versus active control effects on body weight, displaying dose subgroups



iii) GLP-1 agonists' versus active control

	GLP-1 agonists			Active Control			Mean Difference	Mean Difference
Study or Subgroup	Mean [kg]	SD [kg]	Total	Mean [kg]	SD [kg]	Total	IV, Random, 95% CI [kg]	IV, Random, 95% CI [kg]
Barnett, 2007	-2	3.3	68	1	3.35	70	-3.00 [-4.11, -1.89]	
Bergenstal, 2009	-1.9	3.8	124	4.1	3.6	124	-6.00 [-6.92, -5.08]	
Bergenstal, 2010 (Piogl)	-2.3	7.72	80	2.8	7.84	165	-5.10 [-7.17, -3.03]	
Bergenstal, 2010 (sita)	-2.3	7.72	80	-0.8	8.5	166	-1.50 [-3.63, 0.63]	
Davies, 2009	-2.73	3.4	118	2.98	3.4	117	-5.71 [-6.58, -4.84]	
DeFronzo, 2010	-2.82	3.67	45	1.48	3.67	45	-4.30 [-5.82, -2.78]	
Diamant, 2010	-2.6	3.05	233	1.4	2.99	223	-4.00 [-4.55, -3.45]	
Gallwitz, 2011	-4.1	3.46	248	1.02	3.36	233	-5.12 [-5.73, -4.51]	
Gallwitz, 2012	-3.3	5.5	504	1.2	4.2	501	-4.50 [-5.10, -3.90]	
Pratley, 2010	-3.38	3	221	-0.96	1.8	109	-2.42 [-2.94, -1.90]	
Pratley, 2010	-2.86	2.1	225	-0.96	1.8	110	-1.90 [-2.33, -1.47]	
Russell- Jones, 2009	-1.8	4.75	230	1.6	3.82	232	-3.40 [-4.19, -2.61]	
Russell-Jones,2012 (MET)	-2	3.15	82	-2	3.14	246	0.00 [-0.79, 0.79]	
Russell-Jones,2012 (Sita)	-2	3.15	82	-0.8	3.83	163	-1.20 [-2.10, -0.30]	
Russell-Jones,2012(Piogl)	-2	3.15	83	1.5	3.83	163	-3.50 [-4.40, -2.60]	
Yang, 2011	-2.4	2.6	234	0.1	2.2	116	-2.50 [-3.02, -1.98]	
Yang, 2011	-2.3	3	233	0.1	2.2	115	-2.40 [-2.96, -1.84]	



4.4 Discussion

In agreement with recent literature, GLP-1 agonists' display a beneficial effect on blood pressure and also a clinically significant reduction in body weight (Vilsbøll, 2012). The effectiveness of GLP-1 agonists in improving diabetes co-morbidities such as raised blood pressure and body weight is clearly of importance in clinical decision making. This small but favourable effect on blood pressure might confer significant clinical benefit evident at the population level, particularly in patients with diabetes and associated cardiovascular morbidity (Stäessen & Wang, 2001). Studies in these high risk patients show that as little as a 2-5mmHg lowering of blood pressure can reduce the relative risk of cardiovascular events by 20-40 % (Stäessen & Wang, 2001). This assumes that the mechanism behind this change in blood pressure, which is not yet understood, is itself beneficial. Interestingly, this meta-analysis highlights that inter-trial heterogeneity was reduced to acceptable limits (I^2 <20%) when Gao, 2009 and Kadowaki, 2009 were removed from the GLP-1 agonists versus placebo groups. This suggests that there might be ethnic differences in systolic blood pressure response as the patients from these trials were of Asian descent. Possible reasons for heterogeneity in other comparisons were not identified. However, it could be argued that statistical heterogeneity will always exist in a meta-analysis as it is not possible to fully account for such differences. Identification of the sources of heterogeneity and subsequent removal did not alter the strength or direction of the effects for blood pressure and heart rate and so further exploration using meta-regression was not deemed appropriate. However, it would have been interesting to compare further the potential ethnic

differences had there been a higher number of trials using Asian patients. Interestingly, patients undergoing therapy with exenatide long acting release formulations displayed slightly less blood pressure reductions over all than patients administered shorter-acting GLP-1 agonists compared to active comparators. The suggestion of reduced blood pressure gains and increased pulse rate is of clear concern for the safety evaluation of these long-acting formulations. Further explorations into the possible mechanisms behind this effect would be beneficial.

The inclusion of trials consisting of active comparators impacted on the strength and direction of effect on body weight and also led to substantial between-trial heterogeneity, which is at least in- part due to the tendency for traditional oral antidiabetic agents to increase body weight. However, I am able to demonstrate the reduction of body weight by GLP-1 agonists remain apparent when the control arm consisted of patients receiving placebo. Reports in the literature show a large variation in response to exenatide with roughly 30% of patients displaying high response to short term treatment (>5% weight loss), 39% being moderate responders (<5% weight loss) and 31% being non responders (Dushay *et al,* 2012; Ryder *et al,* 2010) which may have contributed to heterogeneity seen in this study. Physiological actions of GLP-1 agonists leading to body weight reduction are understood to be through activation of taste aversion via GLP-1s actions in the amygdala, decreased gastric emptying and development of conditioned taste aversion as well as increased energy expenditure (Grieve, 2009).

While there is indication that these drugs improve blood pressure as well as to offer clinically significant reductions in body weight, clinicians need to continue to be cautious and careful about the potential harm that a drug that increases heart rate might produce. It is likely that the increased heart rate responses to GLP-1 agonists are produced via similar neuronal mechanisms to those which regulate glycaemia and body weight. GLP-1R has been located in vagal afferent fibers, pancreatic β-cells and within CNS neurons (Hayes, 2012; Griffion *et al,* 2011; Cabou *et al,* 2008), the significance of which is GLP-1's involvement in the co-ordinated control of metabolic and cardiovascular function (Hayes, 2012; Griffion *et al,* 2011; Cabou *et al,* 2008). Recently, personal communications with Novo Nordisk researchers highlight the presence of GLP-1R on sino-atrial tissue. This might prove to be a significant side effect for individuals sensitive to heart rate effects of GLP-1 agonists.

Interpretation of the impact of GLP-1 agonists' on heart rate is limited due to the paucity of available data. The tendency for reports to only publish significant data could result in (reporting) bias towards an increase in overall heart rate for patients taking liraglutide. The inclusion of unpublished data for exenatide enables some indication of the effect this drug has on heart rate. However, differences in the included trial designs with respect to this particular outcome might potentially weaken the findings of this meta-analysis. In keeping with current reports, liraglutide displayed a slightly more pronounced increase in heart rate to exenatide but was comparable to exenatide once weekly suggesting that the prolonged half life of exenatide long acting release formulation increases the risk of chronotropic side

effects. Interestingly, NovoNordisk quartile data highlight the possibility that liraglutide causes greater heart rate increases in T2DM patients with a lower baseline heart rate and one assumes higher vagal tone. Although this data could be interpreted as being biased statistically by the regression to the mean phenomenon, a physiological mechanism is another valid explanation particularly as this effect is being reported as enhanced in the longer acting agents.

GLP-1R has been located in a variety of extra-pancreatic tissues including vascular smooth muscle, cardiomyocytes, endocardium and coronary endothelium/ smooth muscle suggestive of direct cardiovascular action of GLP-1 (Grieve, 2009). As mentioned, GLP-1R has also been discovered in autonomic nervous tissue (vagal and sympathetic) and GLP-1R exists in the cardiac sinus node. Supportive studies in animals indicate that GLP-1 acts as a neuropeptide and has been shown consistently to raise heart rate and also blood pressure in rodents within the picomolar and nanomolar range, but disparity exists in studies of large animals and humans. Furthermore, these effects have not yet been clearly demonstrated to be species- specific (Hirata *et al,* 2009; Bojanowska & Stempniak, 2000; 2003; Barragan *et al*, 1999). Interestingly, and which has been highlighted in a comprehensive review by Grieve (Grieve, 2009), studies in animals and humans show a biphasic response to infusions of GLP-1(7-36) with an initial rise in blood pressure followed by a prolonged hypotensive effect which could be the result of the action of DPPIV truncated GLP-1(9-36) (Barragan, 1994; Bojanowska & Stepniak, 2000; Edwards, 1998). Liraglutide is also understood to be converted to truncated GLP-1 (9-36) but after a prolonged

half-life due to resistance to DPPIV (Malm-Erjefalt, 2010). However, this study confirms comparable blood pressure reductions in patients taking exenatide suggestive that more complex mechanisms relating to blood pressure reduction exist. With regards to heart rate both animal and human studies suggest that this is a GLP-1R-mediated action and thus might be expected to be a consequence of action of GLP-1(7-36) and GLP-1(7-37) and exenatide. It might be valuable to conduct clinical trials into the effects of other GLP-1 metabolites such as GLP-1(9-36) as there is increasing evidence that this metabolite might play a beneficial role in the cardiovascular system as has been shown to display vasorelaxant effects as well as to improve LV, systolic and diastolic function (Grieve *et al,* 2009).

A limitation to this meta-analysis is that I was not able to compare GLP-1 agonists to specific oral antidiabetic drugs (OADs) due to the limited number of trials with similar therapeutic strategies to directly compare. Furthermore, meta-regression could not be conducted for all comparisons due to some comparisons having less than 10 trials to compare. A minimum of 10 trials is needed for meta-regression to increase statistical power. Therefore, we chose not to perform meta-regression for this study. Other types of meta-analyses such as network meta-analysis would not have been appropriate due to limited number of trials with multiple intervention and comparator arms. We therefore chose a simpler approach that allowed for stratification of the different drugs and doses allowing the potential differences in effect to be easily compared. Further research needs to be conducted to improve our understanding of the physiological mechanisms through which these drugs act and this particularly needs to be addressed for the long acting

GLP-1 agonists, whilst the results of longer term safety studies are awaited. There is also a clear need to improve the comprehensive reporting of all outcome data measured during clinical trials of anti-diabetic agents, particularly those relevant to cardiovascular outcomes.

5.0 Reversible glucotoxicity as a possible causal mechanism for blunted GLP-1R expression in diabetic and obese transgenic mice

5.1 Introduction

The presence of GLP-1R in renal tissue suggests that GLP-1 might play an important role in either renal control of glucose or sodium. GLP-1 has been shown to inhibit hepatic gluconeogenesis and so it is logical that GLP-1 might regulate renal glucose by similar mechanisms (Tomas & Habener, 2009). As previously discussed GLP-1 has been shown to increase natriuresis and hence is speculated to inhibit renal sodium reabsorption (Hirata *et al*, 2009). Therefore, it might be pertinent to elucidate and dissect possible involvement of renal GLP-1R -dependent and -independent regulation of core metabolic processes. I hypothesize that extended exposure to high glucose (such as during the metabolic syndrome (obesity) and T2DM), will result in blunted renal GLP-1R expression in models of obesity and diabetes.

Circulating GLP-1 has been shown to be reduced in obesity and T2DM in humans (Muscilli *et al*, 2007; Manucci *et al*, 2000; Holst & Gromada 2004; Vilsbøll *et al*, 2003; Vaag *et al*, 1996). Furthermore, Xu and co-workers have shown that gene expression of GLP-1R to be significantly reduced in islets of 90% pancreatectomised hyperglycaemic rats. This effect was reversed when glucose levels were normalised by sodium glucose transporter (SGLT) inhibitor, phlorizin which prevents both intestinal glucose absorption and induces glycosuria. GLP-1 receptor expression was also shown to be blunted in rats using the hyperglycaemic clamp technique for 96 hours and further replicated in cells cultured with high glucose for 48 hours (Xu *et al*,

2007). In addition, Cheong and colleagues describe fluctuating exposure to high glucose to result in blunted GLP-1R expression in cultured cells (Cheong *et al*, 2011). GLP-1R mediated insulinotropic effects are highly dependent on ambient glucose which can be exemplified by the protection of GLP-1 agonists against hypoglycaemia (Grieve *et al*, 2009). GLP-1R is dependent on ambient glucose concentrations and is present in the kidney. It is thus possible that GLP-1R-mediated actions could contribute to renal glucose regulation and sodium transport under normal conditions and that during pathological states GLP-1R signalling is disrupted. The following study shows GLP-1R expression to be altered in the kidneys of *ob/ob* (C57BL/6; BL/6), *db/db* (C57BLK/s; BLK/s) and Tamoxifen-induced diabetic mice (MycER) animal models of obesity and T2DM; and that hyperglycaemia might, at least in part, be a driving force behind abnormal GLP-1R expression in these mice.

5.1 Materials and methods

5.1.1 Transgenic mice

Ob/ob and db/db mice: Adult *Db/db* mice with a genetic background C57BL/Ks and *ob/ob* transgenic mouse strains with genetic background C57BLK/6 as well as wild-type C57BLK/6J controls (n=9) were obtained from Harlan Ltd (Loughborough, UK). Both mice strains were congenic mutants and were maintained as an inbred model via full sib matings at the Harlen facility before the mice were passed to researchers at the University of Warwick. Animal husbandry and sacrifice described herein was performed by post doctoral researchers at University of Warwick. All mice were housed individually and maintained under pathogen-free conditions with controlled

lighting (0700- 1900 h), 12 hr dark (1900- 0700 h) cycle with controlled temperature and humidity. Mice had free access to standard laboratory chow consisting of 70% carbohydrate, 10% fat, and 20% protein with an energy density of 3.85 kcal/ g. At 11 weeks, mice were killed by CO_2 exposure for 10 minutes and kidneys dissected, ensuring complete removal of encapsulating adipose tissue. The overall weights for all mice used in this study were C57BL/6J: 30 ± 5g; C57BL/6 *ob/ob*; 55 ± 5g; C57BL/Ks *db/db*: 48 ± 4g.

Tamoxifen- induced diabetic mice: Whole kidneys from the right hand side of heterozygous transgenic pIns-c-MycER$^{TAM+/-}$ (MycER) mice were kindly donated by Professor Michael Kahn (University of Warwick). Dr. Manjunath Ramanjanaya killed and I subsequently dissected kidneys from matched control (wild type) mice. I performed this procedure under the kind supervision of Dr. Manjunath Ramanjanaya, University of Warwick. Kidneys from these mice were transferred immediately to storage at -80 C until use.

pIns-c-MycER^{TAM} mice express, under the control of the *Ppi* promoter, human c-MYC fused to the hormone binding domain of 4-OH-tamoxifen (TAM)-responsive mutant oestrogen receptor (Littlewood, 1995). Pascal and co-workers have previously described their characteristics and the effect of *in vivo* activation and deactivation of the MycER^{TAM} construct on β-cell apoptosis and proliferation, β-cell mass, and glucose tolerance (Pascal, 2008). Professor Mike Khan's research group maintained these mice on a mixed genetic background (C57BL/6J x CBA F1 mice) and genotyped by PCR analysis (using MycER^{TAM}-specific primers (sense 5'-CCA AAG GTT

GGC AGC CCT CAT GTC-3'; antisense 5'-AGG GTC AAG TTG GAC AGT GTC AGA GT-3')). All mice (N = 12) had previously been housed under controlled lighting (12:12-h light-dark cycle) and temperature conditions and received a common laboratory chow (Carfil Quality; Pavan, Oud-Turnhout, Belgium) and water ad libitum. Professor Michael Khan's group subsequently killed the mice by cervical dislocation and decapitation and measured their body weight and blood glucose concentration in the fed state (Ascensia Elite glucometer; Bayer Healthcare, Leverkusen, Germany), before excision of organs and storage in -80 C. The whole kidneys from the right hand sides of the animals were then kindly donated to me for analysis. All animal procedures had been performed under UK guidance on the Operation of Animals Scientific Procedures) Act (1986).

5.1.2 Preparation of kidney homogenates

For the analyses and regulation of GLP-1R protein, kidneys were removed from -80 C storage and thawed at room temperature. A coronal section of each kidney was removed and homogenised. All fresh kidney samples were kept on ice to minimise degradation. Samples were separated and prepared either for analysis by RT-PCR or western blot.

5.1.3 RT-PCR.

Primers against murine GLP-1R were purchased from Primer design (**Figure 5.1.3.1**). Quantitative PCR of murine GLP-1 receptor were performed on an ABI 7000 system (Applied Biosystems). PCRs were carried out in a reaction mixture consisting of 10 µl Precision^{TM} master mix containing syber green and ROX (Primerdesign, UK), 1.0 µl of each primer (300 nM per 20 µl reaction), 5 µl cDNA (≈5 ng/ µl), and 4 µl RNAse/DNAse free water. Protocol

conditions consisted of denaturation for 95 C for 10 minutes, followed by 40 cycles of 95 C for 15 sec, 57 C for 60 sec, followed by melting curve analysis. The RNA levels were expressed as a ratio, using δ-δ method which is an approximation method for comparing relative expression results between measurements in real-time PCR (Pfaffl, 2001). Ten microlitres of the reaction mixture were subsequently eletrophoresed on a 1% agarose gel and visualised by ethidium bromide, using a 1-kb DNA ladder (Invitrogen) to estimate band sizes. RNAs were assayed from three independent biological replicates.

Table 5.1.3.1

Gene	Forward primer	Reverse Primer	Size (bp)
Mus musculus GLP-1R*	5-TGGGGTACGCACTTTCTTTC-3	5'-GCAAACAGGTTCAGGTGGAT-3	106

*, Pre-validated primers, patent protection (Primer Design, UK)

5.1.4 Western blot

Protein lysates were prepared by mixing equal amounts of Laemmli buffer [5 M urea, 0.17 M sodium dodecyl sulphate, 0.4 M dithiothreitol, and 50 mM Tris-HCL (pH 8.0)] and placing on a hot plate at 95 Celsius for 5 minutes. All samples were then allowed to cool at room temperature. The proteins in protein lysates (35 µg/lane) were separated by SDS-PAGE (10% resolving gel) and transferred to polyvinylidene diflouride (PVDF) membranes at 100 V for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. PVDF membranes were then blocked in tris-buffered saline containing 0.1% Tween 20 and 5% BSA overnight. Membranes were then incubated in primary murine GLP-1R antibody or murine GLP-1 peptide antibody (1:2000 +0.5% B.S.A TBST) for 1 hr before undergoing 5

consecutive 15 minute washes under gentle agitation. Membranes were then incubated in secondary anti-rabbit peroxidise (1:8000 +0.5% BSA TBST) or secondary anti-mouse peroxidise (1:2000 +0.5% BSA, TBST) for GLP-1R and GLP-1 respectively before a further 5, 15 minute washes and subsequent visualisation by ECL chemiluminescence.

5.2 Results

5.2.1 Decreased GLP-1R mRNA expression in the kidneys of obese (db/db and ob/ob) mice

I initially examined GLP-1R mRNA expression in whole kidneys from wild-type (WT) BL/6J, obese BL/6 and diabetic BLK/s male mice. The kidneys were separated according to the three comparator groups before preparation of cDNA and subsequent analysis by PCR. One- way analysis of variance showed an approximate 4-fold decrease in GLP-1R mRNA expression in BL/6 (P = 0.05) and 5-fold decrease in GLP-1R expression in BLK/s mice (P = 0.001) compared to wild-type littermates (**Figure, 5.2.1.1 Differences in GLP-1R mRNA expression in kidneys of wild-type (C57BL/6J), *ob/ob* (C57BL/6) and *db/db* (C57BL/Ks) mice**). Evaluation of the GLP-1R expression on the protein level by western blot showed a corresponding 2-fold decrease in GLP-1R expression in the kidneys of these mice (P= 0.01) (**Figure 5.2.1.2 Differences in GLP-1R protein expression in kidneys of wild-type (C57BL/6J), *ob/ob* (C57BL/6) and *db/db* (C57BL/Ks) mice**). Protein characteristic of GLP-1 peptide was not found to be present in murine renal tissue. Results confirm my hypothesis that GLP-1 R mRNA expression is down-regulated in obesity and diabetes.

5.2.2 Decreased GLP-1R mRNA expression in kidneys from tamoxifen-induced hyperglycaemic mice

To dissociate obesity and leptin mediated factors and to assess the influence of hyperglycaemia on renal GLP-1 receptor expression *in vivo*, I then explored GLP-1R expression in Tamoxifen-induced diabetic mice. Kidneys were separated according to stage of hyperglycaemia initiated by activation

of the beta-cell c-myc promoter by tamoxifen, (Day 0: baseline normoglycaemia (mean ± S.E.M glucose levels: 77.48 ± 8.83 mg/dL), Day 7: severe hyperglycaemia (426.49 ± 22.88 mg/dL) and recovery day 6 showing a reduction of hyperglycaemia towards normal levels (285.23 ± 95.32 mg/dL). Statistical analysis showed the overall effect to be an inverse relationship of GLP-1R mRNA expression with circulating glucose levels in these mice. A 3- fold reduction of GLP-1R expression was seen at day 7 hyperglycaemia compared to day 0 control (*P*= 0.021) and this returned towards normal values by day 6 of the recovery phase (*P*= N.S.) (**Figure, 5.2.2.1 GLP-1R mRNA expression in tamoxifen-induced hyperglycaemic mice**).

Figure 5.2.1.1- Differences in GLP-1R mRNA expression in kidneys of wild-type (C57BL/6J), *ob/ob* (C57BL/6) and *db/db* (C57BL/Ks) mice. Coronal sections from the kidneys of BL/6J, BL/6 and BL/Ks transgenic mouse strains (N=9) were homogenised and extracted for total RNA as described previously. Complimentary cDNA was then prepared before analysis by RT-PCR for GLP-1R mRNA expression. Ubiquitously expressed GapDH was used as control. Data are expressed as relative difference of median of control (WT). Nine independent experiments were performed and each experiment was carried out in three replicates. Results were analysed using ANOVA and expressed as means ± S.E.M.

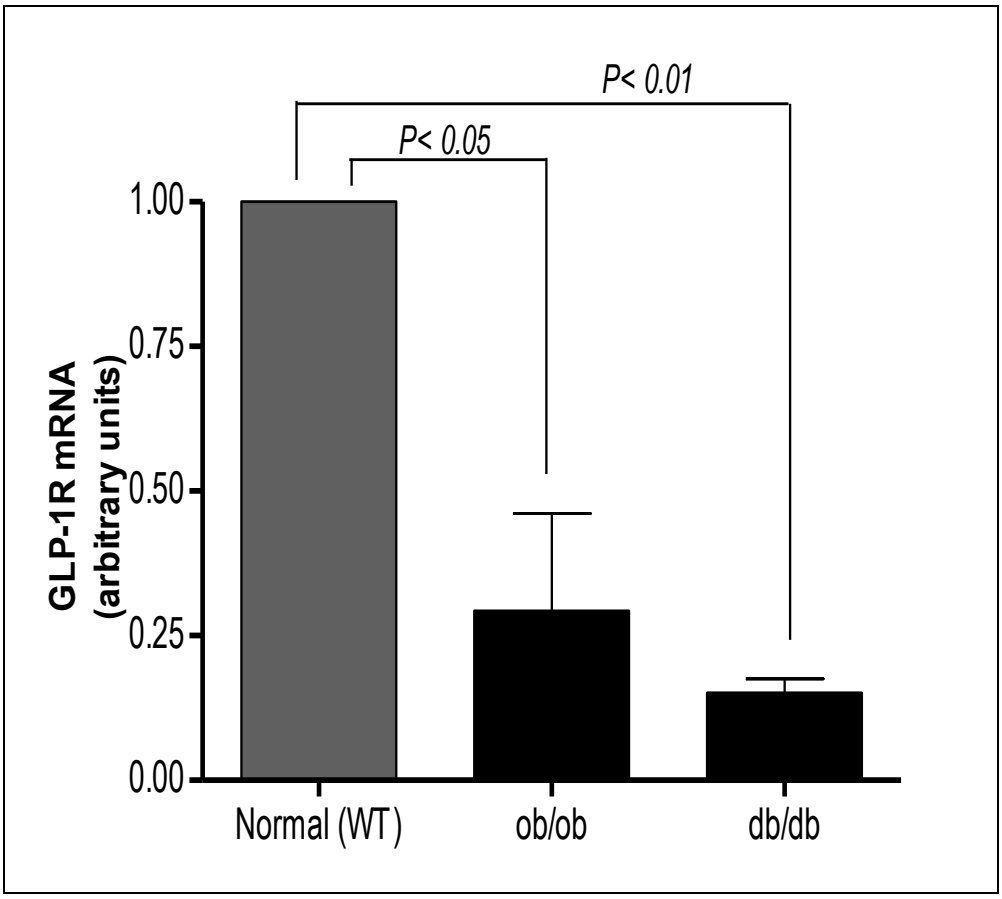


Figure 5.2.1.2- Differences in GLP-1R protein expression in kidneys of wild-type (C57BL/6J), *ob/ob* (C57BL/6) and *db/db* (C57BL/Ks) mice. BL/6J, BL/6 and BL/Ks transgenic mouse strains were culled at 11 weeks. Right kidneys from each animal (N=9) were homogenised and proteins separated by western blot and probed for anti-GLP-1R antibody. Glucagon-like peptide receptor (GLP-1R) protein levels relative to β actin were significantly decreased in BL/6 and BL/Ks mice compared to WT using western blotting. Data are expressed relative to median of control (WT). Nine independent experiments were performed and each experiment was carried out in three replicates. Group comparison were analysed using ANOVA with Dunnett's Multiple Comparison test. Results are expressed as means ± S.E.M.

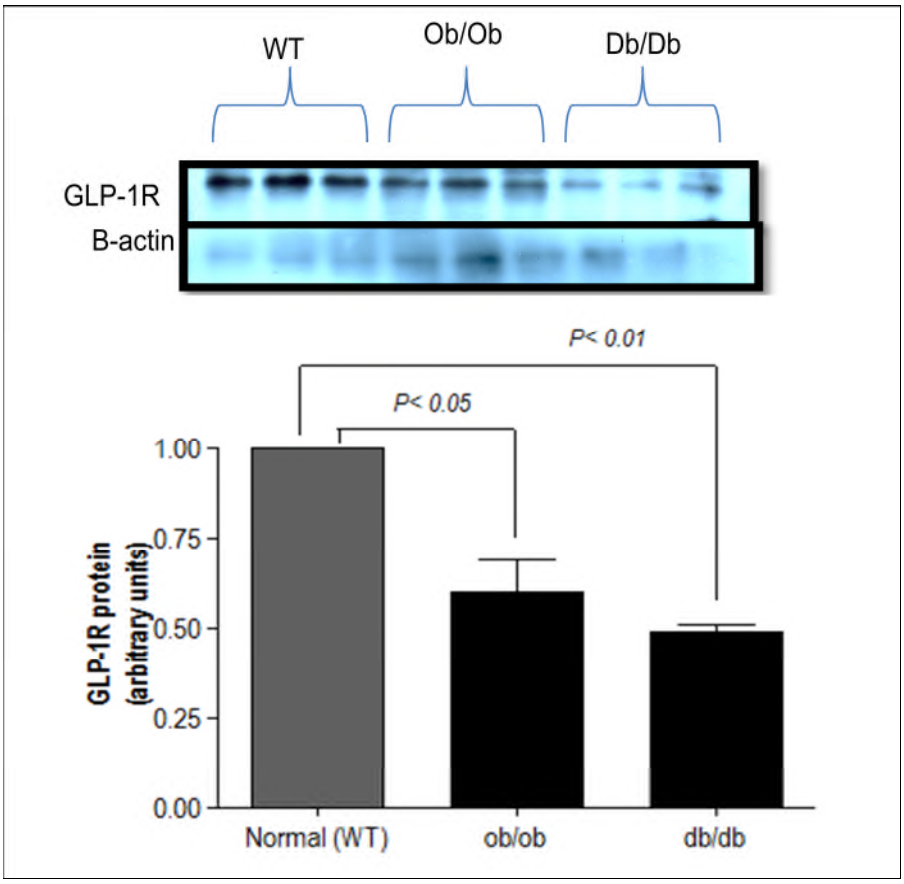
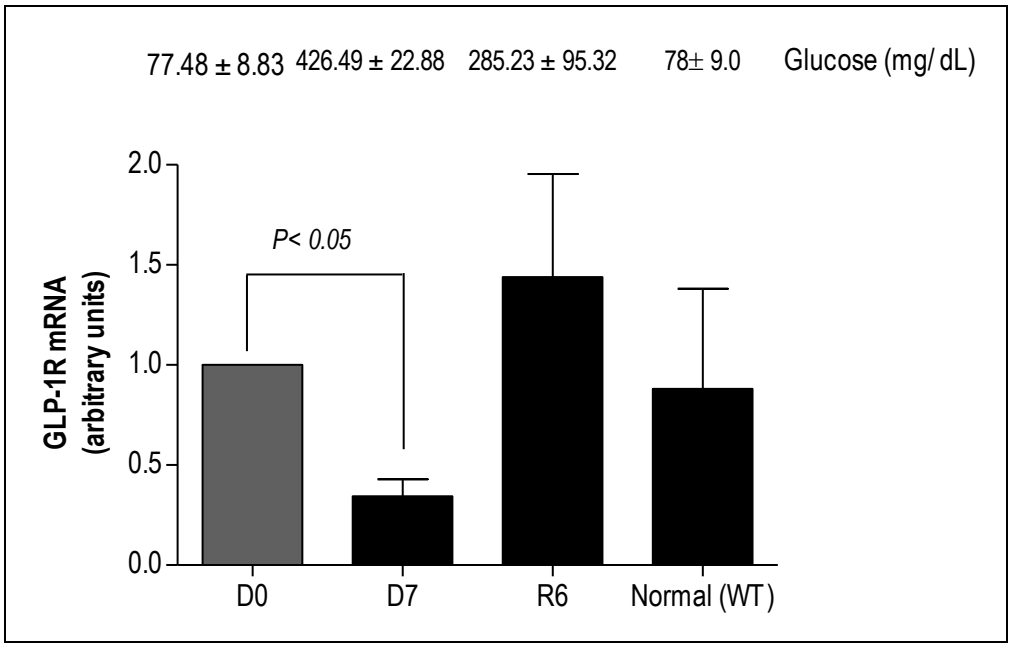


Figure 5.2.2.1- GLP-1R mRNA expression in tamoxifen-induced hyperglycaemic mice (MycER). Blood glucose was measured at baseline (D0), and during hyperglycaemia (D7) and recovery phase (R6) in tamoxifen-induced MycER mice. GLP-1R mRNA was quantified using RT-PCR at corresponding days to show 3-fold decrease in GLP-1R mRNA expression with a corresponding rise in glucose levels (above graph). Data are expressed relative to median of control (WT). Twelve independent experiments were carried out and each experiment was performed in three replicates. Group comparison by repeated measures ANOVA with Tukey's Multiple Comparisons test.



5.3 Discussion

GLP-1 signalling pathways are well understood to be influenced by plasma glucose concentrations. That is to say that GLP-1 is secreted in response to glucose and plays a role in glucose regulation and insulin secretion as well as certain GLP-1 metabolites displaying insulin-like action in peripheral tissues (Thomas & Habener, 2009). Furthermore, insulinotropic actions of GLP-1 are dependent on ambient glucose concentrations which can be exemplified by the inability of GLP-1 agonists to induce hypoglycaemia

(Grieve *et al,* 2009). A release of GLP-1 in response to increasing glucose levels stimulates insulin, an effect which is halted subsequent to normalisation of ambient glucose concentrations by insulin. This phenomenon is of clinical benefit in the treatment of T2DM by GLP-1 agonists which, unlike traditional anti-diabetes therapy protects against hypoglycaemic episodes.

The animals in the following chapter were chosen primarily to assess GLP-1R expression in response to differing levels of derangement in insulin signalling, with consequent hyperglycaemia. Following this, I planned to focus specifically on GLP-1R expression during hyperglycaemia after reversible *Ppi* gene knock-out and hence, β-cell apoptosis and depletion of insulin secretion. Benefits of the latter mouse model, MycER (Tamoxifen-induced diabetes mouse), is that the direct effects of insulin depletion and subsequent marked hyperglycaemia can be observed *in vivo* with dissociation of obesity and leptin factors which are present in the other experimental models. The use of spontaneous or genetically derived T2DM mice such as *ob/ob* (C57BL/6; BL/6) and *db/db* (C57BLK/s; BLK/s) mice have various advantages and disadvantages over nutrient or chemically induced species (e.g. C57BL/6J mouse, similar to BL/6 strain but which are normal weights until administered high fat feed; or GTG treated obese mice). These mice are genetically homogenous and thus only a small number of animals are required for experimentation and environmental factors are easily controlled making it simpler to tease out genetic influences from this multifactoral disease (Srinivasan & Ramarao, 2007). However, these mice are not similar to human obesity-induced diabetes which is highly

heterogenous. Transgenic/knock-out diabetic mice such as the MycER mice are also highly homogenous but the complexity of this experimental model means that it is a highly costly option. Nevertheless, these animal models were chosen due to their genomic constitutions being derived from BL/6 which makes these mice highly comparable despite differing physiological profiles. This enabled me to study the effects of moderate to frank hyperglycaemia (and hence the corresponding relationship of insulin secretion and β-cell function) in the BL/6 and BLK/s mice against their wild type litter mates. I was then able to look closely at the pattern of response of renal GLP-1R expression to hyperglycaemia (with respect to insulin depletion and feedback to the gut-brain axis) in the MycER^{TAM} model without the associated factors related to obesity and leptin signalling.

Renal disease is more pronounced in the BLK/s than in the BL/6 mice possibly due to high circulating leptin levels and the ability of leptin to stimulate matrix production (BL/6 mice display reduced leptin secretion as a core mechanism in the development of obesity whereas BLK/s metabolic dysfunction arises from disrupted leptin receptor signalling). Renal disease may also be dependent on genetic background and hence the marked hyperglycaemia seen in this mouse strain (Sharma *et al*, 2003). GLP-1R mRNA and protein expression are markedly lower in BLK/s mice as compared to WT and BL/6 strains which may be in part, a reflection of more severe hyperglycaemia and hence diabetes in these mice or deregulated insulin secretion and β-cell apoptosis. However, before any inferences can be made as to whether disrupted neuronal signalling from pancreatic β-cells to the CNS and/ or subsequent hyperglycaemia is the driving force behind

the blunted receptor expression, we must first consider other factors that might lead to disruption of GLP-1R signalling in the kidney. Leptin and GLP-1 share interrelated actions, both opposing and complimentary, with respect to glucose homeostasis and satiety regulation. For instance, GLP-1 has been demonstrated to reside downstream of leptin action in the CNS pathways regulating food intake (Goldstone *et al*, 1997). GLP-1R antagonist exendin (9-39), has been shown to inhibit the leptin-induced reduction of food intake and weight loss (Goldstone *et al*, 1997) and leptin is understood to upregulate brainstem proglucagon RNA and hypothalamic GLP-1 content in rats (Goldstone *et al*, 2000). Furthermore, GLP-1 and leptin both activate *c-fos* expression in a subset of neurons in the nucleus of the solitary tract of the brain stem (Elias *et al*, 2000) and leptin up-regulates the GLP-1 receptor (Sanz *et al*, 2008; Schrocchi *et al,* 2000). Despite the ability of leptin to influence central GLP-1 production and brain GLP-1R expression it is unlikely that in BLK/s, circulating leptin directly down-regulates peripheral GLP-1R such as that in the kidney because the BL/6 model is deficient in leptin but also displayed down-regulation of renal GLP-1R.

It might therefore be hypothesized that down-regulation of renal GLP-1R might be correlated with the degree of β-cell destruction and hence insulin signalling to the hypothalamus via the gut-brain axis or a direct down-regulation by chronic hyperglycaemia. Hyperglycaemia is much greater and sustained in BLKs mice than in the BL/6 strain which might contribute to the more pronounced inhibition of GLP-1R in the BLK/s mice as compared to the BL/6 mice.

To explore the contribution of β-cell destruction and subsequent depletion of insulin/ hyperglycaemia to the down-regulation of renal GLP-1R I assessed GLP-1R mRNA expression in an *in vivo* model of hyperglycaemia. MycER mice were utilised in this part of the study. These mice are genetically manipulated to upregulate c-MYC after treatment with tamoxifen triggering cell apoptosis and decreased preproinsulin gene expression in c-MYC islets. This results in marked β-cell destruction, depletion of insulin secretion and hyperglycaemia while upon withdrawal of tamoxifen the glycaemic response returns to normal values. Mice were mostly male and were treated with tamoxifen for 7 days. Tamoxifen was then withdrawn and the mice were monitored for 50 days. Results show an inverse relationship between GLP-1R mRNA expression and circulating glucose levels which is indicative that these changes may be due to the effect of hyperglycaemia on GLP-1R expression. However, it might also be postulated that renal GLP-1R mRNA expression would also correlate with the degree of β-cell destruction and insulin secretion. Therefore, hypothalamic-regulatory neurons might relay signals from CNS to renal GLP-1R to down regulate the receptor. When considering this from the perspective of feedback from the brain to the periphery, it is highly unlikely that chronic hyperglycaemia *per se* would down regulate brain GLP-1R and subsequently relay messages to down-regulate renal GLP-1R. It would be more likely that, in order to protect the brain against pathologically high levels of glucose, chronically high brain glucose would “switch on” GLP-1R response and subsequently lead to hyperinsulinemia via CNS and vagal efferents for pancreatic β-cells. However, the reduction of brain insulin due to advanced diabetes and hence

β-cell destruction might trigger feedback to the periphery to down-regulate GLP-1R and also during advanced uncontrolled hyperglycaemia, GLP-1R might be directly influenced by glucose generation of reactive oxygen species and glycation end products in both the brain regions and also the kidney. This might subsequently be reversed by generation of nitric oxide (NO) and other free radical scavenging molecules by restoration of insulin signalling and GLP-1R response (Cabou *et al,* 2008; Ojima *et al,* 2013).

The MycER^{TAM} model reduces *Ppi* mRNA expression as a consequence of interaction with tamoxifen and therefore is important to consider whether tamoxifen administration interacts directly with GLP-1R function as well as the preproinsulin promoter and thus directly inhibits GLP-1R mRNA expression. As mentioned previously, activation of the MycER^{TAM} construct interferes with glucose homeostasis via disruption of insulin secretion and beta cell apoptosis (Pascal *et al*, 2008). GLP-1 is involved in glucose-dependent insulin secretion and stimulates pro-insulin biosynthesis and has been shown to increase insulin mRNA levels and insulin content (Doyle *et al*, 2007). GLP-1R activation is also understood to be involved in β-cell growth and proliferation through induction of WNT signalling in pancreatic β-cells, isolated islets and in INS-1 cells. Basal and GLP-1 agonist- induced proliferation of β-cells requires active WNT signalling cascade via GLP-1R-mediated activation of Akt and beta-cell independent GSK3β (Lui *et al*, 2008). This process involves activation of c-Myc (Lui *et al*, 2008) and so it is logical to consider that inactivation of c-Myc by tamoxifen might indirectly down-regulate GLP-1R by disruption of the GLP-1 signalling system. This may be tested by administering the animals with both tamoxifen and insulin

to restore glucose levels to normal values without restoring *Ppi* gene function and hence insulin secretion. This will allow more information as to whether hyperglycaemia is in fact a driving force for GLP-1R down-regulation and help to tease out the effects of direct neuronal feedback from pancreatic islets and insulin signalling to the hypothalamus. These results suggest that hyperglycaemia influences inhibition of the GLP-1R. The possible interaction between tamoxifen and pancreatic GLP-1R needs to be clarified.

Teasing out the relationship between dysregulated metabolic effects and the effects on GLP-1R expression is not an easy task and much research needs to be conducted to deduce what effects certain regulatory factors have on GLP-1 signalling and how restoration of GLP-1R activation by GLP-1 agonists might restore homeostatic mechanisms. Primarily there are two lines of thought when considering the reason as to why renal GLP-1R expression might be down-regulated in the animals utilised in this study, 1) Direct feedback from β-cells as a consequence of apoptosis and reduced insulin signalling, possibly due to neuronal feedback via the vagal nerve to the brain 2) direct glucose-dependent effects and possible disruption of GLP-1R signalling through generation of ROS. The following chapter will assess the latter hypothesis using isolated cells of the renal and proximal tubule. Further research is warranted into the neuropeptide effects of GLP-1 which links GLP-1 signalling to body weight, blood pressure and heart rate responses as will be discussed in the final chapter.

6.0 GLP-1R expression in human collecting duct and proximal tubule cells during hyperglycaemia-like conditions

6.1 Introduction

The primary purpose of the following chapter was to assess whether GLP-1R is present and functional in cells of the kidney in preparation for mechanistic studies into the effect of GLP-1 on α-ENaC expression studies (a surrogate marker for blood pressure regulation). The aim was also to continue work from the previous chapter to confirm whether high ambient glucose concentrations will down-regulate human GLP-1R expression. My rationale was to explore whether I could replicate down-regulation of GLP-1R in the absence of confounding factors present *in vivo* such as obesity- related leptin deficiency/ resistance and associated hypothalamic factors or insulin/ beta cell depletion seen in the animal models. I also wanted to assess whether my previous findings could be species specific and whether this effect can be replicated in human renal cells expressing GLP-1R. Information is scarce regarding mechanisms which influence GLP-1R in the kidney. I therefore mimicked hyperglycaemia in cell lines of the human kidney proximal tubule and collecting duct to assess the influence of high glucose on renal GLP-1R expression.

6.2 Materials and methods

6.1.1 Cell culture

Human collecting duct (HCD) and human proximal tubule (HKC8) cells were kindly donated by Dr. Rose Bland, University of Warwick. Cells were previously characterised and cloned and this is described elsewhere (Prie, 1995). Cells (passages 15-23) were maintained in DMEM/ Hams F-12 medium (GIBCO, Invitrogen), supplemented with 2% foetal calf serum (FCS), glutamine (2 mmol), 15 mmol/l HEPES, transferring (5 µg/ml), Na_2SeO_3 (5 ng/ml), insulin (5 µg/ml), and dexamethasone (5 x 10^{-8} M), 2 mL pen/strep. Normal growth media for HKC8 cells consisted of DMEM/Hams-F12 containing 5% foetal calf serum and 2 mL pen/strep. Subculturing and fasting media incorporated the same method for both cell lines (see '**appendix**'). Cells were grown to 80% confluence at 37^{o}C in a humidified atmosphere of 5% CO_2 in air.

My initial concern was in deciding upon glucose concentrations that constituted 'normal' and 'hyperglycaemia-like' conditions for HCD and HKC8 cell lines. In humans, two hour plasma glucose concentration of ≥11.1mM/L following ingestion of 75g anhydrous glucose in an oral glucose tolerance test (OGTT) is classed as (post-prandial) hyperglycaemia. Normal growth media for both HCD and HKC8 immortalised cell lines consisted of DMEM-F12 containing supraphysiological concentrations of approximately 18 mM/ L glucose. Therefore, the cell lines normal growth conditions consisted of ambient glucose concentrations which exceeded what would be considered pathological concentrations in humans. This highlighted several potential problems, a) What concentration would be considered to parallel human hyperglycaemia in these cells? b) How similar to human physiological

conditions are these cells? c) How do I control for the effects of background glucose levels on my experimental results? d) If I acclimatize the cells in low glucose containing media before performing my experiments, will this affect cellular integrity or viability? However, a common approach by researchers is to incubate the cells in serum-free media containing baseline values of 5 mM/ L D-glucose before subsequent experimentation (Hills, 2006). This baseline concentration is considered sufficient to maintain cell viability but also low enough to reduce the likelihood that baseline glucose will mask the effects of additive glucose levels on GLP-1R expression. To ensure that cell growth was not inhibited, a trypan blue assay was performed (data not shown). Subsequent concentrations of 10 mM/ L, 15 mM/ L and 25 mM/ L D-glucose were considered to be synonymous to varying degrees of hyperglycaemia for these cells.

Cells were seeded into 25 mL flasks until 80-90% confluence and passaged 1-2 times into 75 mL flasks before being aliquotted onto 6-well plates containing 2 mL media. Media was then replaced with serum-free media containing 5 mM/L D-glucose and fasted over night before subsequent incubation with 5 (no supplement), 10, 15, or 25 mM/ L D-glucose for either 4 or 24 hours. In separate samples, cells were incubated in 5 mM/L D-glucose plus 10, 15 or 25 mL D-Mannitol to control for osmotic effects. After 4h and 24 h respectively media was removed and replaced with ice cold PBS buffer to stop the cellular processes. Cells were kept on ice while removing PBS buffer and preparing cells for subsequent analysis by either RT-PCR or western blot.

6.1.2 Total RNA isolation and cDNA synthesis

Total cellular RNA was extracted from HCD and HKC8 cells using the RNeasy Mini Kit (Qiagen Ltd., UK) and first strand DNA synthesis was performed using qScript^{TM} reverse transcription kit (Primer Design, UK) according to the manufacturer's instructions.

6.1.3 RT-PCR

Quantitative PCR of human GLP-1 receptor were performed on an ABI 7000 system (Applied Biosystems). PCRs were carried out in a reaction mixture consisting of 10 µl Precision^{TM} master mix containing syber green and ROX (Primerdesign, UK), 1.0 µl of each primer (300 nM per 20 µl reaction), 5 µl cDNA (≈5 ng/ µl), and 4 µl RNAse/DNAse free water. Protocol conditions consisted of denaturation for 95 C for 10 minutes, followed by 40 cycles of 95 C for 15 sec, 57 C for 60 sec, followed by melting curve analysis. The RNA levels were expressed as a ratio, using δ-δ method for comparing relative expression results between measurements in real-time PCR (Pfaffl, 2001). Ten microlitres of the reaction mixture were subsequently eletrophoresed on a 1% agarose gel and visualised by ethidium bromide, using a 1-kb DNA ladder (Invitrogen) to estimate band sizes. As a negative control preparations lacking reverse transcriptase or water (blanks) were used in place of the cDNA. RNAs were assayed from three independent biological replicates. Quality of cDNA was assessed by electrophoresis using 1% agarose gel.

Table 6.1.3.1 Primer sequences

Gene	Forward primer	Reverse Primer	Size (bp)
Human GLP-1R*	5'-GTTTCTGGAAATGGCTGTAGGT-3	5'-CAGGCACCAAAACAACTCAAAT-3	94
Human GLP-1R	5'-GTTCCCCTGCTGTTTGTTGT-3	5'-CTTGGCAAGTCTGCATTTGA-3	227

*, Pre-validated primers, patent protection (Primer Design, UK)

6.1.4 Western blot analysis

For the analyses and regulation of GLP-1R protein, media was removed and protein lysates were prepared by mixing equal amounts of RIPA buffer and consequently adding Laemmli buffer [5 M urea, 0.17 M sodium dodecyl sulphate, 0.4 M dithiothreitol, and 50 mM Tris-HCL (pH 8.0)], mixed, and placed on a hot plate at 95 Celsius for 5 minutes. All samples were then allowed to cool at room temperature. The proteins in protein lysates (35 µg/lane) were separated by SDS-PAGE (10% resolving gel) and transferred to polyvinylidene diflouride (PVDF) membranes at 100 V for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. PVDF membranes were then blocked in tris-buffered saline containing 0.1% Tween 20 and 5% BSA overnight.

The PVDF membranes were incubated with primary GLP-1R antibody (dilution 1:2000) (Santa Cruz biotechnology, Middlesex, UK) for 1 h. The membranes were washed thoroughly for 60 min with TBS- 0.1% Tween 20 before incubation with the secondary antibody, anti-rabbit horseradish-peroxidase-conjugated Ig (1:10,000) (Dako Ltd., Cambridge, UK) for 1 h at room temperature. Membranes were also re-probed with the β-actin antibody (Cell Signalling Technology Inc., Beverly, MA, USA; 1 in 8000

dilution) to determine equal protein loading. Antibody complexes were visualised using chemiluminescence (ECL+; Amersham, Little Chalfont, UK). Membranes were subsequently re-probed with inclusion of antagonist Exendin (9-39) to confirm selectivity of primary antibody.

6.1.5 Statistical analysis

Data in the present study are expressed as means ± SEM. Comparisons among groups were made by repeated measures ANOVA. When significance ($P < 0.05$) was detected, a post hoc Dunnett's multiple-comparison test or Tukey's multiple comparison test was performed (Graph Pad software (version 4.0)).

6.2 Results

6.2.1 Expression of GLP-1 receptor in HCD and HKC8 cells

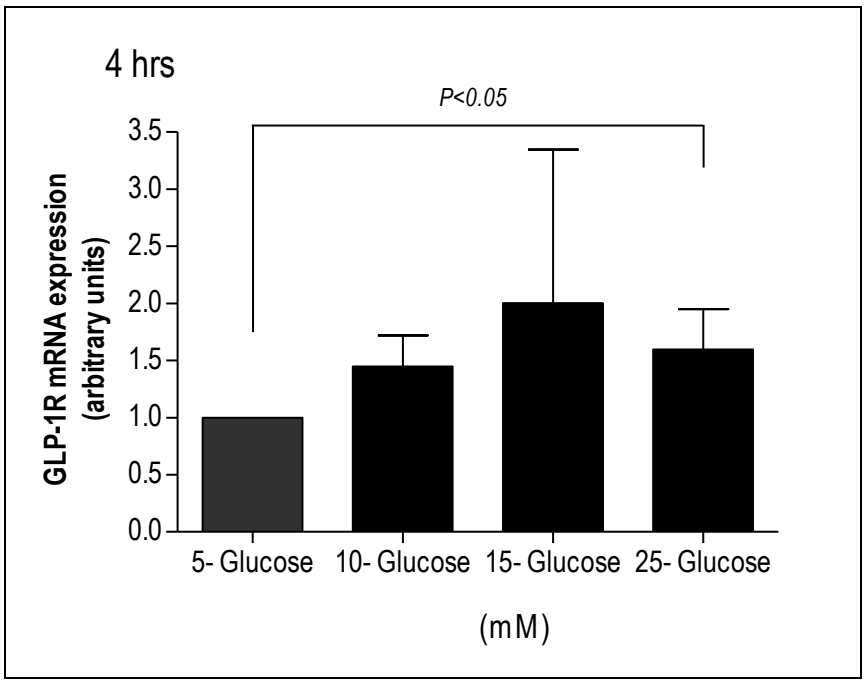
Preparations of both HKC8 and HCD samples underwent gel electrolysis which revealed the presence of PCR product which was characteristic of human *GLP-1R* mRNA. Specificity of primers to human *GLP-1R* was also confirmed using mouse kidney homogenates. Initially, primers were designed for end-point PCR with a product size of 227 bp (IDT Technology, UK). For quantitative RT-PCR separate pre-validated primers were purchased from Primer Design, UK with a product size of 95 bp. Standard deviations for RT-PCR Ct values ranged between 0.1- 1. To confirm that human GLP-1R mRNA was appropriately translated, protein expression was quantified using western blot analysis, revealing band at approximately 53 kDa. Addition of antagonist GLP-1(9-39) successfully blocked binding of GLP-1R antibody and hence confirmed its specificity to GLP-1R.

6.2.3 Human collecting duct cells

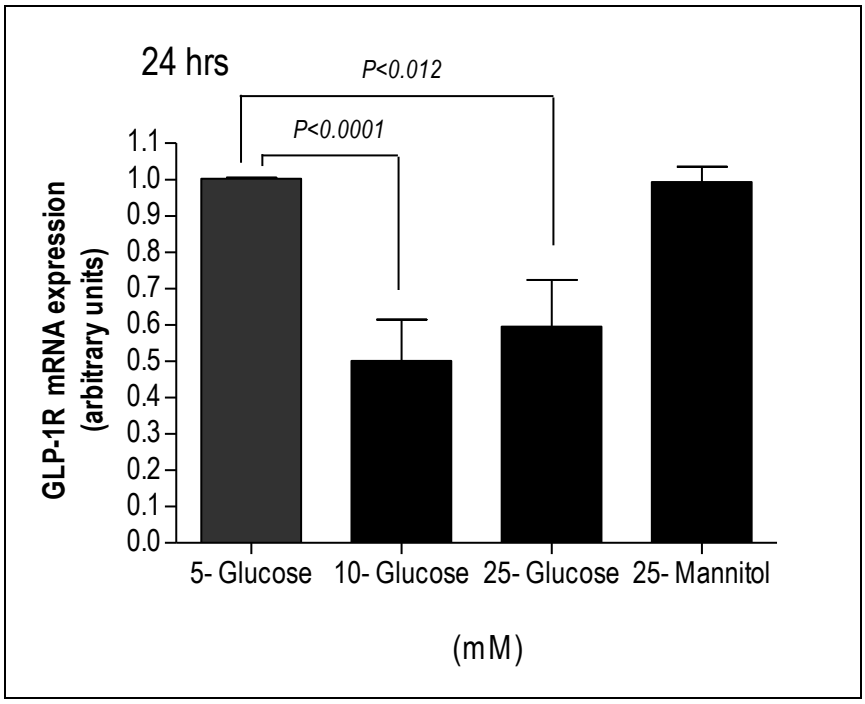
Timed experiments suggest that mRNA expression is down- regulated at 24 hrs by GLP-1R protein. One way ANOVA showed an overall decrease in GLP-1R mRNA expression [HCD, *P*= 0.001 (5 mM/ L versus 10 mM/ L, *P*= 0.005 *and 5* mM/ L versus 25 mM/ L *P*= 0.013); HKC8, *P*= 0.001 (5 mM/ L versus 10 mM/ L, *P*= 0.001 and 5 mM/ L versus 15 mM/ L, *P*= 0.012)]. GLP-1R mRNA was observed to be up-regulated 1.5- fold at 4 hrs in HCD cells (5 mM/ L versus 25 mM/ L D-glucose, *P*= 0.026). In HCD cells a concentration-dependent increase in GLP-1R expression was observed. This corresponded to a 3-fold increase (*P*= 0.053) in protein at 24 hours. Similarly, at 4hrs a 2-fold increase in GLP-1R mRNA was seen in HKC8 cells after incubation in 25 mM/ L D-glucose (*P*= 0.007). A corresponding 2-fold increase was seen for GLP-1R protein at 24 hrs (25 mM/ L, *P*= 0.041). No osmotic effects were observed for mRNA or protein expression in either cell line. Results show that GLP-1R expression is regulated by D-glucose in cells of the human collecting duct and proximal tubule. **Figure 6.2.3.1 Concentration-dependent effects of D-Glucose on GLP-1 mRNA expression in HCD cells A-D** and **Figure 6.2.3.2 Concentration- dependent effects of D-Glucose on GLP-1 protein expression A-B** shows the relative fold expression of GLP-1R with baseline (5 mM D-glucose) given an arbitrary value of 1.

Figure 6.2.3.1 - Concentration- dependent effects of D-Glucose on GLP-1 mRNA expression in HCD cells after **a)** 4 hr, **b)** 24 hr and in HKC8 cells after **c)** 4 hr and **d)** 24 hr, were assessed by RT-PCR, compared with basal (5 mM/ L Glucose). Ubiquitously expressed GapDH was used as control. Data are expressed as relative difference of median of basal. A minimum of three independent experiments were performed and each experiment was carried out in triplicates. No osmotic effects were observed in cells incubated in D-mannitol (+ 5 mM/ L glucose). Results are analysed using repeated measures ANOVA and Dunnett's multiple comparisons test. Data is expressed as mean ± S.E.M.

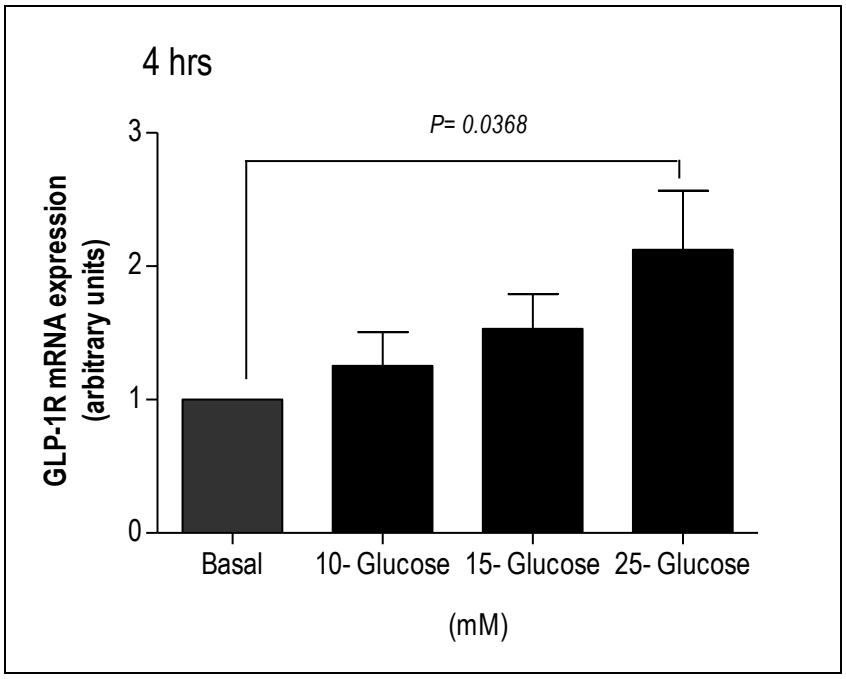
a) HCD cells incubated for 4 hours in various concentrations of glucose



b) HCD cells incubated for 24 hours in various concentrations of glucose



c) HKC8 cells incubated for 4 hours in various concentrations of glucose



d) HKC8 cells incubated for 24 hours in various concentrations of glucose

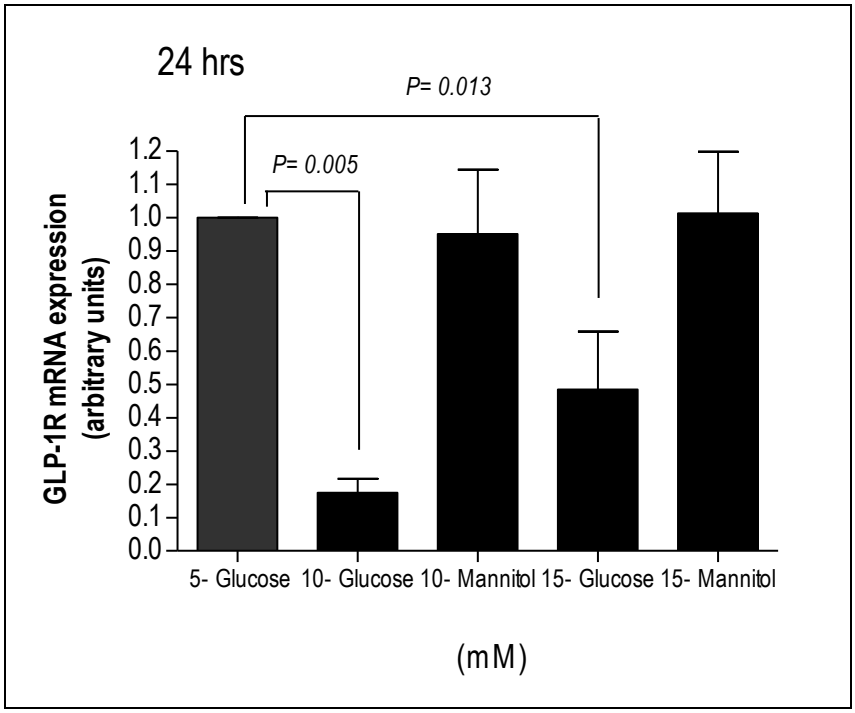
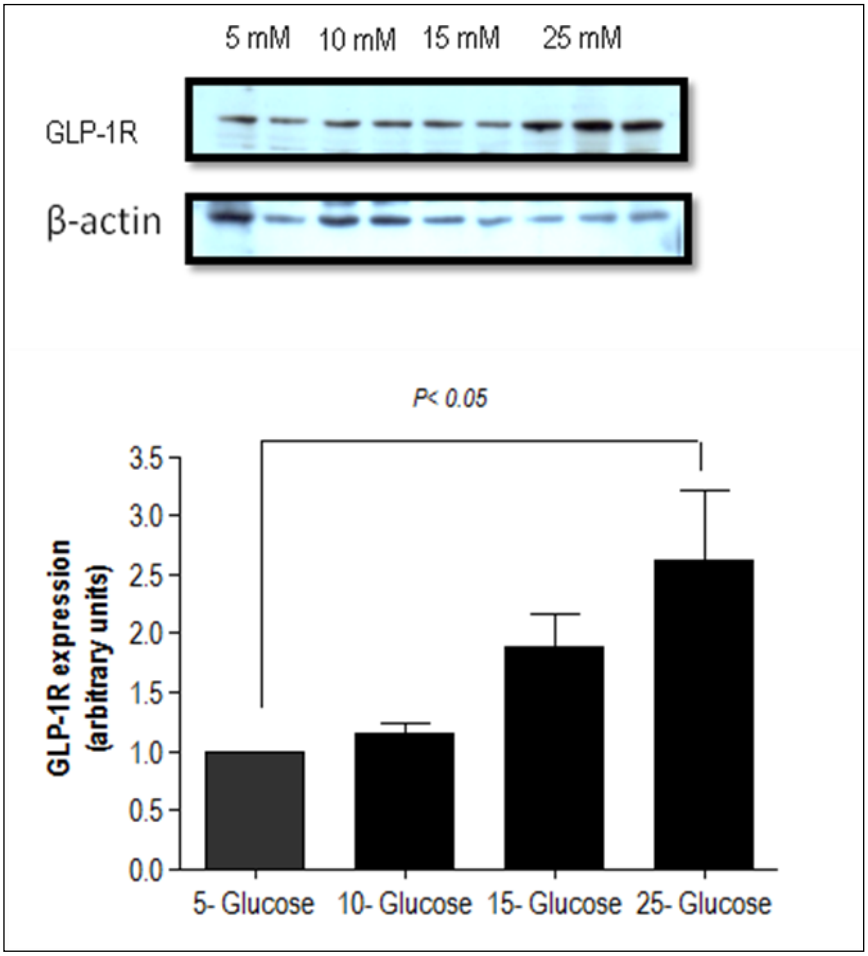
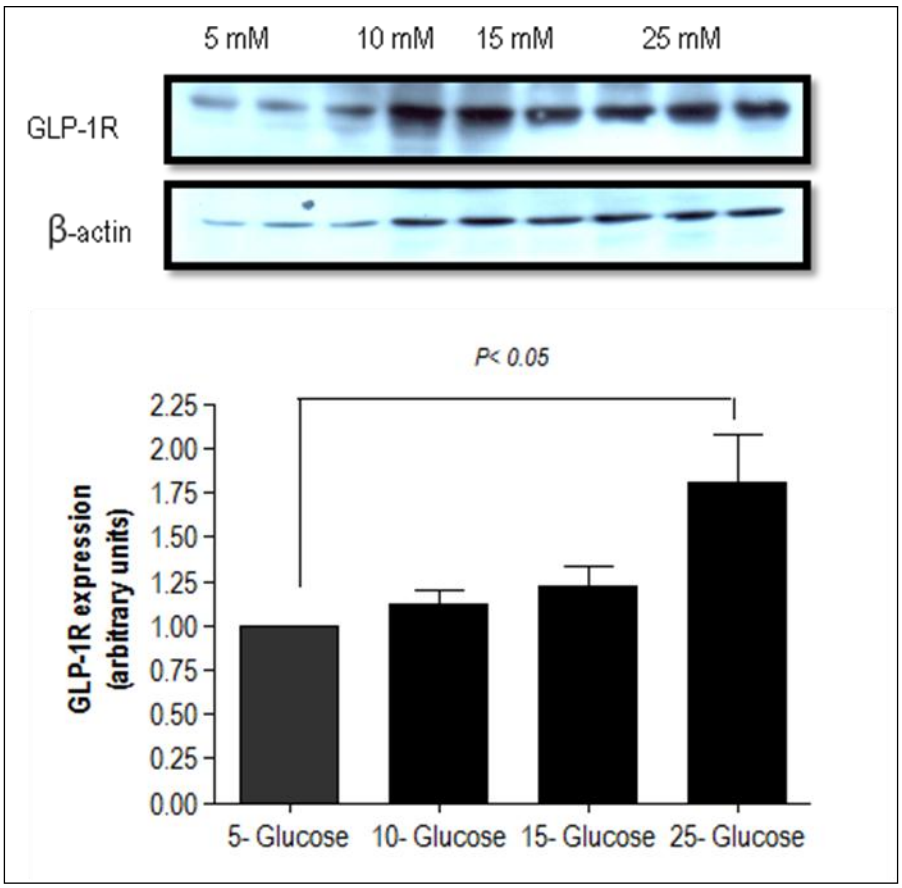


Figure 6.2.3.2- Concentration- dependent effects of D-Glucose on GLP-1 protein expression in **a)** HCD and **b)** HKC8 cells after 24 h incubation were assessed by western blot, compared with basal (5 mM/ L Glucose). Ubiquitously expressed GapDH was used as control. Data are expressed as relative difference of median of basal. Three independent experiments were performed and each experiment was carried out in triplicates. No osmotic effects were observed in cells incubated in D-mannitol (+ 5 mM/ L glucose). Results are analysed using repeated measures ANOVA and Dunnett's multiple comparisons test. Data is expressed as means ± S.E.M.

a) GLP-1R protein expression in HCD cells at 24 hours



b) GLP-1R protein expression in HKC8 cells at 24 hours



6.3 Discussion

I show for the first time that GLP-1R is present in human cortical collecting duct cells, which is contrary to studies in rodents suggesting species differences in renal GLP-1R expression. Furthermore, GLP-1R is functional in both human proximal tubule and collecting duct cells. I was not able to demonstrate inhibition of GLP-1R expression on mRNA or protein level due to chronic hyperglycaemia-like conditions. A limitation to this study is that the timing of my experiments might not have mimicked chronic hyperglycaemia (other studies assessing similar responses use 48h incubation times while cells described herein were incubated for 24 hrs).

Glucose absorption through the gastro-intestinal tract is well understood to stimulate GLP-1 release from L-cells (Gribble *et al,* 2003; Reinman & Gribble, 2002; Grieve, 2009). This phenomenon is dependent on the concentration of glucose and hence the amount of food ingested. It might be speculated that in renal cells, rises in ambient glucose might directly stimulate secretion of GLP-1. However, I was not able to detect secreted GLP-1 in renal cells by western blot. The increased renal GLP-1R expression seen in HCD and HKC8 cells might indicate that GLP-1R might be involved in the kidney regulation of glucose because the kidney regulates glucose by releasing glucose into the circulation (gluconeogenesis), uptake of glucose from the circulation to use as energy and reabsorption of glucose from glomerular filtrate to conserve glucose carbon (Gerich, 2009). It might also be speculated that GLP-1R activation might stimulate renal glucose uptake as approximately 10% of glucose is utilized by the kidney during the post-prandial glycaemic state (Gerich, 2009).

It would be interesting to assess GLP-1's effects on facilitated glucose uptake and sodium reabsorption hence, GLP-1R-mediated action on glucose transporters in the kidney, for example GLUT, and sodium-glucose co-transporter (SGLT 1/2), respectively. Previous research did not show GLP-1 to up-regulate renal glucose transporters in porcine proximal tubule cells but this may highlight species differences and so should be repeated in human cells (Schlatter, 2009). Further research into the possibility that GLP-1 might activate renal gluconeogenesis during post-prandial hyperglycaemia would be beneficial. GLP-1 is reported to suppress hepatic gluconeogenesis but it is not clear whether this is a direct GLP-1R-mediated action or non-GLP-1R-mediated action by GLP-1(9-36) or an indirect effect by suppression of insulin and glucagon (Larsson *et al,* 1997; Grieve, 2009). It is possible that GLP-1 might contribute to regulation of gluconeogenesis through hepatic renal glucoreciprocity and therefore future research is necessary to evaluate this and also to evaluate whether this is a GLP-1R-directed action.

I have been able to show GLP-1R expression to be down-regulated in diabetes and obesity in rodents and that the down-regulation of GLP-1R correlates with hyperglycaemia *in vivo.* This is the first study to show GLP-1R to be present and functional in cells of the human collecting duct. This study also shows GLP-1R expression to be up-regulated during acute hyperglycaemia-like conditions in both human proximal tubule and collecting duct cells. The significance of this in humans can only be speculated at this stage and further research is necessary. It might be postulated that restoration of GLP-1R expression in patients undergoing therapy with GLP-1 agonists might improve diabetes- related manifestations with regards to

sodium and glucose dysregulation and slow or prevent renal disease in susceptible individuals. Recently, GLP-1 and exendin-4 has been shown to reduce oxidative stress, improve histologic changes and suppress advanced glycation end product receptor (RAGE) gene expression in the human proximal tubule of streptozotocin- induced diabetic rats through GLP-1R activation of cAMP pathways (Ojima *et al,* 2013). However, studies into the effects of GLP-1 agonists in renal disease are lacking and further research is warranted. The significance of possible dysregulated GLP-1R during diabetes begs further research into the action of GLP-1 with regards to glucose regulation by the kidney. If GLP-1R does mediate glucose reabsorption and gluconeogenesis this would be of great importance in developing treatments to focus on slowing or improving diabetic nephropathy as many traditional oral anti-diabetes drugs are not suitable or contra-indicated in patients with renal disease. Presence of GLP-1R in renal cells might also highlight a beneficial pleiotropic action of GLP-1 agonists on the kidney.

It might be speculated that a down-regulation of GLP-1R expression in renal cells was not replicated in isolated cells of the proximal tubule and collecting duct due to the lack interaction with central autonomic control and the absence of insulin. It is necessary to perform further research into the effects of GLP-1R restoration through use of GLP-1 agonists with special focus on regulation by neural circuits and from the perspective of GLP-1 as a neuropeptide as this might be a primary factor linking the weight reducing, blood pressure and heart rate responses to GLP-1 agonists seen in clinical studies. It would be interesting to add to the work from the previous chapter

and to perform new experiments to study 1) the effects of bilateral vagotomy on renal GLP-1R expression in the MycER^{TAM} model before and after administration of endogenous insulin to assess whether central autonomic or hormonal influences are directly involved in the down regulation of renal GLP-1R and 2) how ablation of *Ppi* gene expression might affect free radical scavenging molecules in the renal tissue and 3) whether administration of insulin to isolated human kidney cells directly affects GLP-1R expression in the presence of glucose.

7.0 GLP-1 effects on Epithelial sodium channel – alpha in human collecting duct cells

The distal portion of the nephron is ultimately responsible for the rate of urine volume and composition, and also BP-induced changes in urine flow and Na^+ excretion via pressure natriuresis and diuresis (Peti-Peterdi, 2002). Furthermore, as I have already discussed, ENaC is strongly implicated in treatment-resistant hypertension commonly observed in obese and T2DM patients as it is dysregulated by hyperglycaemia, hyperinsulinemia and hyperaldosteronism. Research into the actions of GLP-1 on blood pressure has thus far focussed on renal and central nervous system effects using animal models and clinical findings from human trials (Hirata et al, 2009; Edwards, 1998). A small number of mechanistic studies have also been performed on a cellular level in the renal proximal tubule (Schlatter, 2006).

In animals and man there is strong suggestion that GLP-1 increases natriuresis at the kidney tubules (Yu, 2003; Gutzwiller, 2006). Thus far I have shown that renal GLP-1R expression is dependent on ambient glucose levels and might be down-regulated by prolonged hyperglycaemia. Sodium transport molecules such as ENaC are known to be “hyper”-activated by hyperglycaemia, hyperinsulinemia and hyperaldosteronism seen in the metabolic syndrome (obesity) and T2DM. I hypothesize that GLP-1 may play a role in regulating blood pressure through inhibition of ENaC in the renal tubules. Therefore, in the following chapter, I explore the effects of GLP-1 on *SCNN1A* (α- ENaC) mRNA and protein expression and potential involvement of rapid signalling cascades in cells of the human collecting duct.

7.1 Materials and Methods

7.1.1 Cell culture

HCD cells (passages 15-21) were used for this set of experiments. A detailed account of cell culture conditions is described in the '**appendix**' section of this document. The active metabolite of GLP-1 (GLP-1 (7-36) amide) is understood to have an IC50 of 0.5- 0.78 nM/ L (Greischel, 2010; Deacon, 1998). However, I planned to assess the effect of various concentrations of GLP-1 on α-ENaC and its' corresponding signalling molecules. Therefore, cells were treated with 0.1 nM/ L, 1 nM/ L, 10 nM/ L and 100 nM/ L GLP-1 (7-36) amide (Bachem, France) for 2 hr, 4 hr, 12 hr and 24 hrs after overnight fasting in the presence of unsupplemented DMEM/ Hams F-12 without FCS. GLP-1 (7-36) is understood to initiate mitogen- activated protein kinases and extracellular- regulated kinases (MAPK/ ERK pathway) which is mediated by protein kinases (PKA and PKC) (Portha, 2011). Furthermore, these signalling cascades and also serum- glucocorticoid- regulated kinase (SGK-1) are associated with regulation of SCNN1A/ ENaC expression in the collecting duct (Thomas, 2008). I assessed the responses of SGK-1 gene and protein to concentrations of GLP-1 (7-36). I then used the same experimental design to assess the response of ERK1/2 phosphorylation to GLP-1 (7-36). I subsequently assessed the involvement of PKA and PKC on ERK1/2 activation. HCD cells were treated in a time and concentration dependent fashion with various concentrations of GLP-1 (7-36) for 2, 5, 15, 30 60 minutes. Extreme care was taken to avoid excessive movement/ causing stress to the cells during this process. Angiotensin II at a concentration of 10 nM/ L was used as positive control to ERK1/2 activation. In separate experiments, cells were cultured in the presence of PKC and

PKA inhibitors for appropriate incubation periods prior to treatment with GLP-1 (7-36). For these experiments ERK1/2 inhibitor was used as control to ensure the correct protein bands were being visualised.

7.1.2 RT-PCR

Total RNA was extracted and underwent subsequent cDNA synthesis before samples were prepared for analysis by RT-PCR. Quantitative PCR of *SGK1* and *SCNN1A* were performed on an ABI 7000 system (Applied Biosystems, Carlsbad, California). PCRs were carried out using solutions purchased from Primer Design, UK. Protocol conditions consisted of 50 cycles: enzyme activation for 10 minutes at 95 C; denaturation for 15 seconds at 95 C; fluorogenic data was collected through the SYBR® green channel at 60 C for 60 seconds; melting curve analysis. Pre-validated primers were purchased from Primer Design, UK (**Table 7.1.2.1 Primer sequences)**. As a negative control, preparations lacking RNA or reverse transcriptase were used in place of the cDNA. RNAs were assayed from three independent biological replicates.

Table 7.1.2.1: Primer sequences.

Gene	Forward primer	Reverse Primer	bp
Human SCNN1A	5'-CTGCCAGAGAACTCCTATG-3	5'-TCAATTTTGGGGAAGACAAGATG-3	94
Human SGK-1	5'-CCCGTCGTCCAATCCTCAT-3	5'-CTTCTGCCTTGTGTCTTGCTA-3	104

Pre-validated primers. Patent protected, bp, base pairs

7.1.2.2 Western blotting

In brief, proteins (20-40 µg) were separated by sodium dodecyl sulphate (SDS) polycrylamide gel electrophoresis (10% or 12% resolving gel) at 40mV for 3 hours in electrophoresis buffer containing 25mmol/l Tris, 192mmol/l glycine, and 0.1% (wt/ vol) SDS. Proteins were transferred on to Immobilin P membrane in transfer buffer (25 mmol/l Tris, 192 mmol/l glycine, 20% (vol/ vol) methanol) for 1 hour at 100 volts, 4 ^{o}C. Following protein transfer, membranes were blocked for 1 hour in TBS-T (TBS plus 0.1% tween-20) containing 5% (wt/ vol) bovine serum albumin (BSA) at 4 ^{o}C and subject to four separate 20 minute washes. Membranes were analysed using specific polyclonal antibodies against Human SGK-1 (AbCam), Human α-ENaC (AbCam), phosphorylated P38, ERK 1/2 and total ERK 1/ 2 (AbCam) at dilutions of 1:2000 + 0.5% BSA. After five, 10 minute washes, the membranes were then incubated for 1 hour at ambient temperature with corresponding secondary antibodies (horseradish peroxidase- conjugated) anti-rabbit (diluted to 1:2000) or anti-mouse (diluted to 1:2000) in TBS-T+0.05% BSA. Membranes were then washed for a further five, 15 minute washes in TBS-T followed by three 10 minute washes in TBS. Specific proteins were detected using ECL-Plus chemiluminescence detection system (Amersham Biosciences) and were visualized after 1-5 minutes exposure of membranes to x-ray film. Blots were probed with an appropriate antibody to control for loading inefficiencies for subsequent densitometry analysis of protein expression levels (AbCam).

7.2 Data analysis

Auto radiographs were quantified using densitometry (Scion Corp. 2008). Statistical analysis of the data was performed using one-way ANOVA test with Dunnett's post test. Data are expressed as arithmetic mean ± SEM. *N* denotes number of experiments and $P < 0.05$ denotes statistical significance.

7.3 Results

7.3.1 Expression of SGK-1, and SCNN1A in HCD cells

Studies confirmed the presence of *SGK-1* and *SCNN1A* mRNA and protein in HCD cells. Optimisation of PCR and subsequent analysis of RNA preparations of HCD cells revealed the presence of *SGK-1* and *SCNN1A* mRNA. To confirm that mRNA was appropriately translated, protein expression was quantified using western blot analysis, revealing bands at approximately 75 kDa and 50 kDa, for *SCNN1A* and *SGK-1* respectively.

7.3.2 Up-regulation SCNN1A expression after treatment with GLP-1(7-36)

Serum- and glucocorticoid- regulated kinase (SGK-1) is an important regulator of Na^+ retention by intensifying the stability and expression of SCNN1A/ ENaC. To test my hypothesis that GLP-1(7-36) down-regulates *SGK-1* and *SCNN1A* mRNA and protein levels, HCD cells were treated with 0.1 nM/ L, 1 nM/ L, 10 nM/ L and 100 nM/ L GLP-1(7-36) amide for 2 hrs, 4hrs, 12 hrs and 24 hrs. Cells grown under these conditions exhibited a modest decrease in *SGK-1* mRNA expression which decreased at 4 hours (2-fold compared to control (no supplement), $P = 0.03$) at a concentration of 1 nM GLP-1 (7-36) (**Figure 7.3.2.2 Concentration- dependent effects of GLP-1 (7-36) on *SGK-1* mRNA expression in HCD cells**). Contrary to my hypothesis HCD cells treated with 10nM GLP-1(7-36) enhanced *SCNN1A*

mRNA expression 4-fold by 24 hours (*P*= 0.015) (**Figure 7.3.2.3- Concentration- dependent (a) and time- dependent (b) effects of GLP-1 (7-36) on *SCNN1A* mRNA expression**). Western blotting also showed modest increases of SGK-1 ($P < 0.05$ versus control) with corresponding increases in α-ENaC ($P < 0.05$) protein expression after 24 hrs stimulation with 10 nM GLP-1 (**Figure 7.3.2.4- Effect of GLP-1 (7-36) on SGK1 protein expression; Figure 7.3.2.5 - Effect of GLP-1 (7-36) on α-ENaC protein expression**).

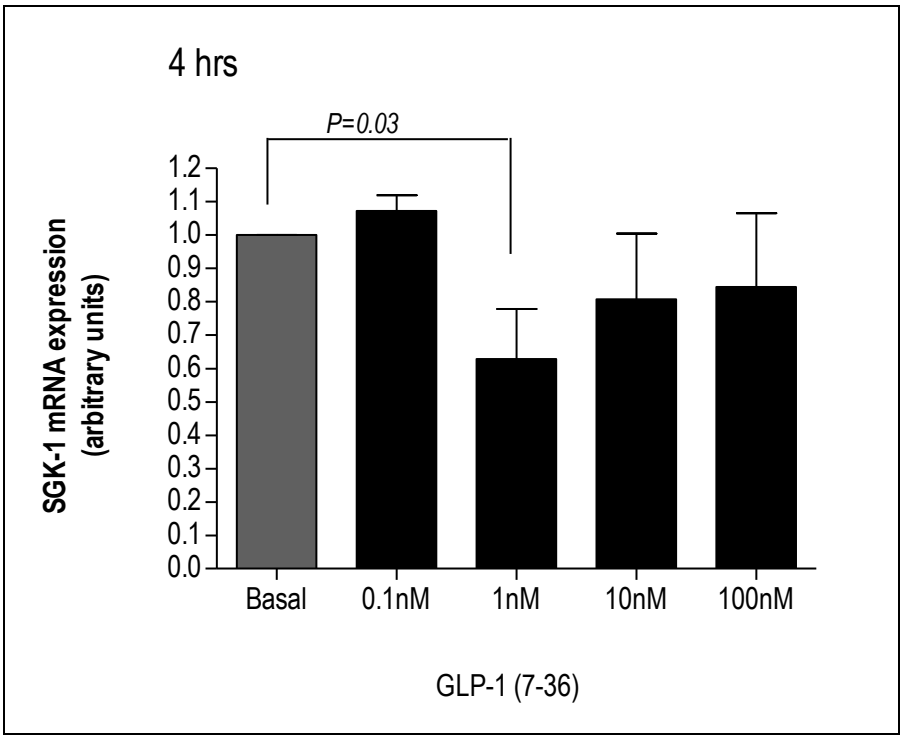
7.3.3 α-ENaC signalling pathways

To elucidate further the cellular signalling pathways involved in the increased response to α-ENaC after treatment with GLP-1, I examined the effect of GLP-1 treatment on ERK 1/2 phosphorylation in HCD cells. SGK-1 is understood to phosphorylate ERK1/2; this latter also being activated by PKC (Soundararajan, 2009). Phosphorylation of ERK 1/2 was demonstrated to increase significantly upon stimulation with 10 nM GLP-1 ($P < 0.01$). At the highest concentration of GLP-1 (7-36) (100 nM), ERK1/2 phosphorylation was diminished to basal levels. ERK1/2 phosphorylation peaked at 5 minutes, gradually returning to baseline values by 30 minutes (**Figure 7.3.3.1- Time- dependent (2-60 minutes) effects of GLP-1 (7-36) on ERK 1/2 phosphorylation**). This effect was approximately two-thirds the intensity of angiotensin II- stimulated ERK1/2 response. Treatment with protein kinase inhibitors revealed a 2- fold decrease in ERK1/2 compared to control (no supplement) ($P < 0.01$) after treatment with PKC inhibitor. Treatment with ERK1/2 inhibitor totally abolished this effect which confirmed specificity of antibodies for ERK/12 (**Figure 7.3.3.2- Effects of 10nM GLP-1 on ERK 1/2**

protein expression in HCD cells at 5 minutes after incubating in specific inhibitors for PKA and PKC). Treatment of protein kinase inhibitors did not clearly show inhibition of α-ENaC protein expression. The α-ENaC protein was not visible in these last experiments suggestive that the cell-line had changed at later passages and had ceased producing this protein.

Figure 7.3.2.2 - Concentration- dependent effects of GLP-1 (7-36) on *SGK-1* mRNA expression in HCD cells at **a**) 4 h and **b**) 24 h were assessed by RT-PCR, compared with basal (no supplement). Ubiquitously expressed GapDH was used as control. Data are expressed as relative difference of median of basal. A minimum of three independent experiments were performed and each experiment was carried out in triplicates. Results are analysed using repeated measures ANOVA and Dunnett's multiple comparisons test. Data is expressed as means ± S.E.M.

a) Effect of GLP-1(7-36) on SGK-1 mRNA expression at 4 hrs.



b) Effect of GLP-1(7-36) on SGK-1 mRNA expression at 24 hrs.

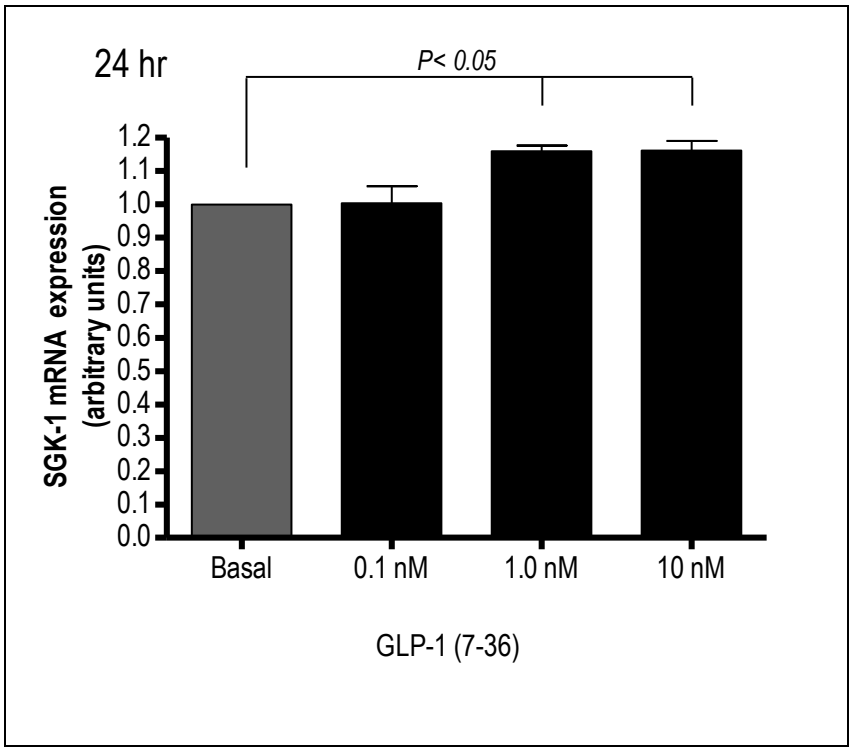
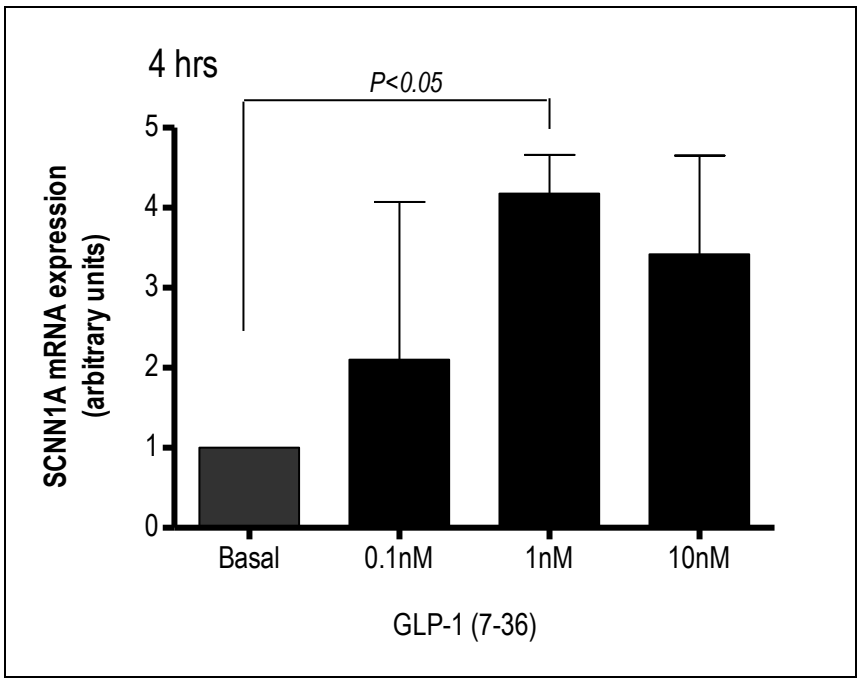


Figure 7.3.2.3- Concentration- dependent (a) (incubated for 24 hours), and time- dependent (b) effects of GLP-1 (7-36) on *SCNN1A* mRNA expression in HCD cells were assessed by RT-PCR, compared with basal (no supplement). Ubiquitously expressed GapDH was used as control. Data are expressed as relative difference of median of basal. A minimum of three independent experiments were performed and each experiment was carried out in triplicates. Results are analysed using repeated measures ANOVA and Dunnett's multiple comparisons test. Data is expressed as means ± S.E.M.

a) Effects of various concentrations of GLP-1 (7-36) on SCNN1A (ENaC) mRNA expression in HCD cells



b) Effects of 10 nM GLP-1 (7-36) on SCNN1A (ENaC) mRNA expression in HCD cells

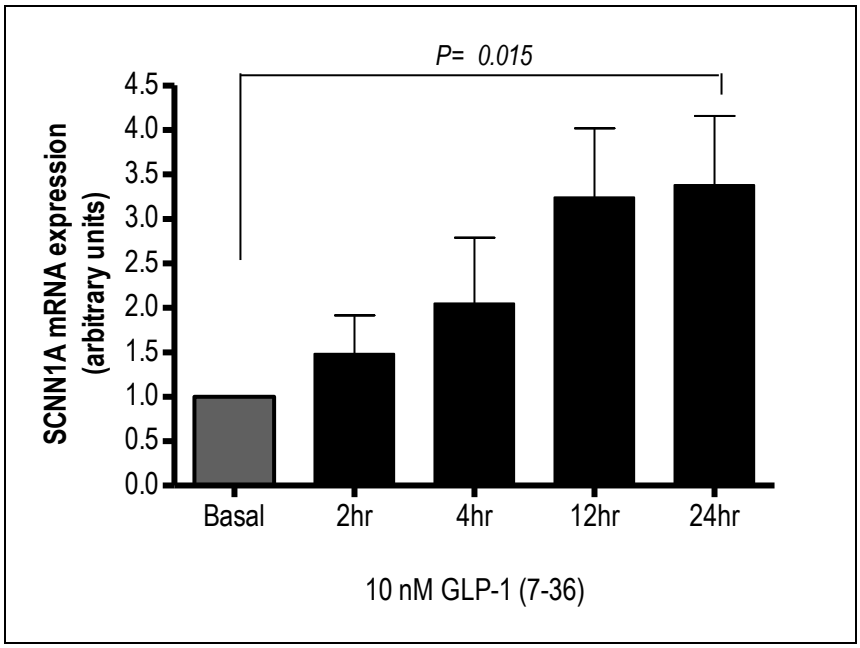


Figure 7.3.2.4- Effect of GLP-1 (7-36) on SGK1 protein expression. SGK-1 levels relative to GapDH is significantly increased in HCD cells compared to basal (no supplement) after 24 hrs incubation with 10 nM GLP-1 using western blotting. Three independent experiments were performed and each experiment was carried out in triplicate. Group comparison by repeated measures ANOVA with *post hoc* Dunnett's multiple comparisons test.

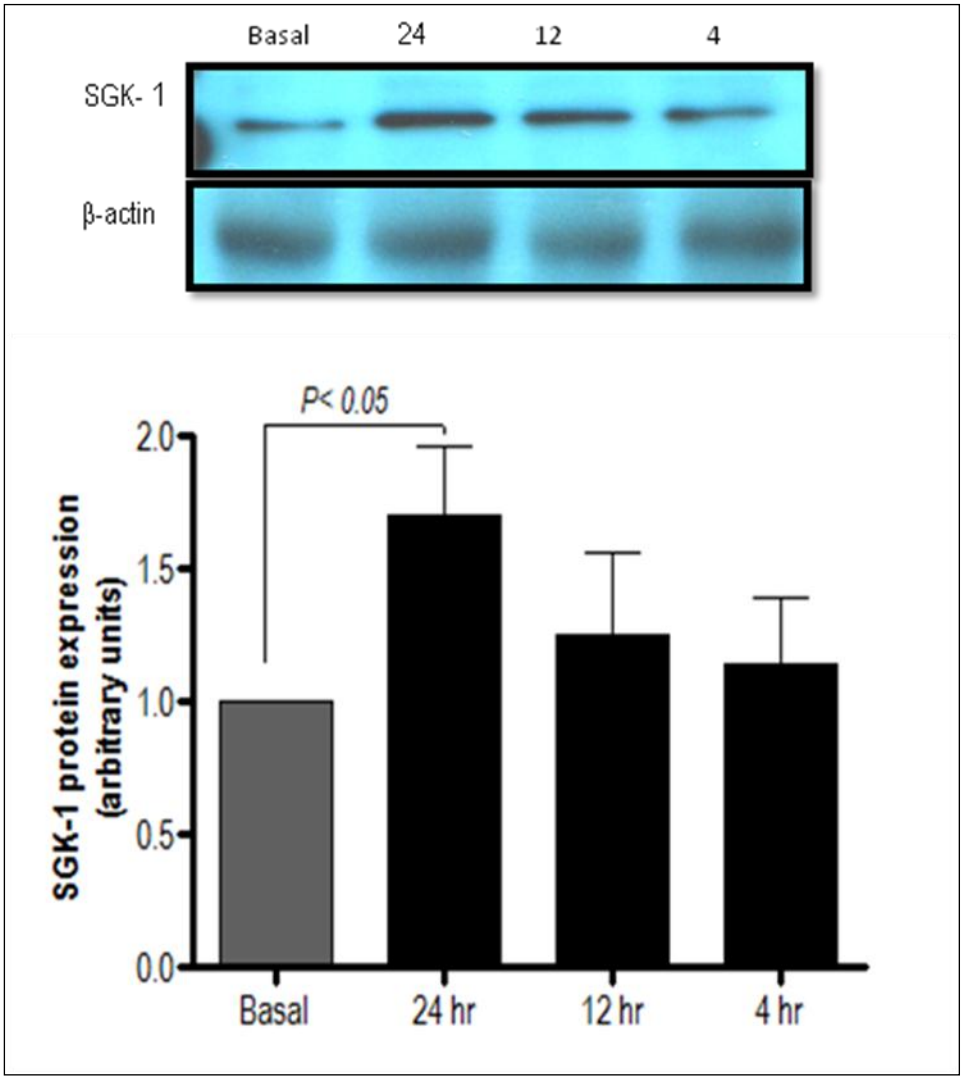


Figure 7.3.2.5 - Effect of GLP-1 (7-36) on α-ENaC protein expression. α-ENaC levels relative to GapDH is significantly increased in HCD cells compared to basal (no supplement) after 24 hrs incubation with 10 nM GLP-1 using western blotting. A minimum of three independent experiments were performed and each experiment was carried out in triplicate. Comparison by paired t-test was performed with 95% confidence interval.

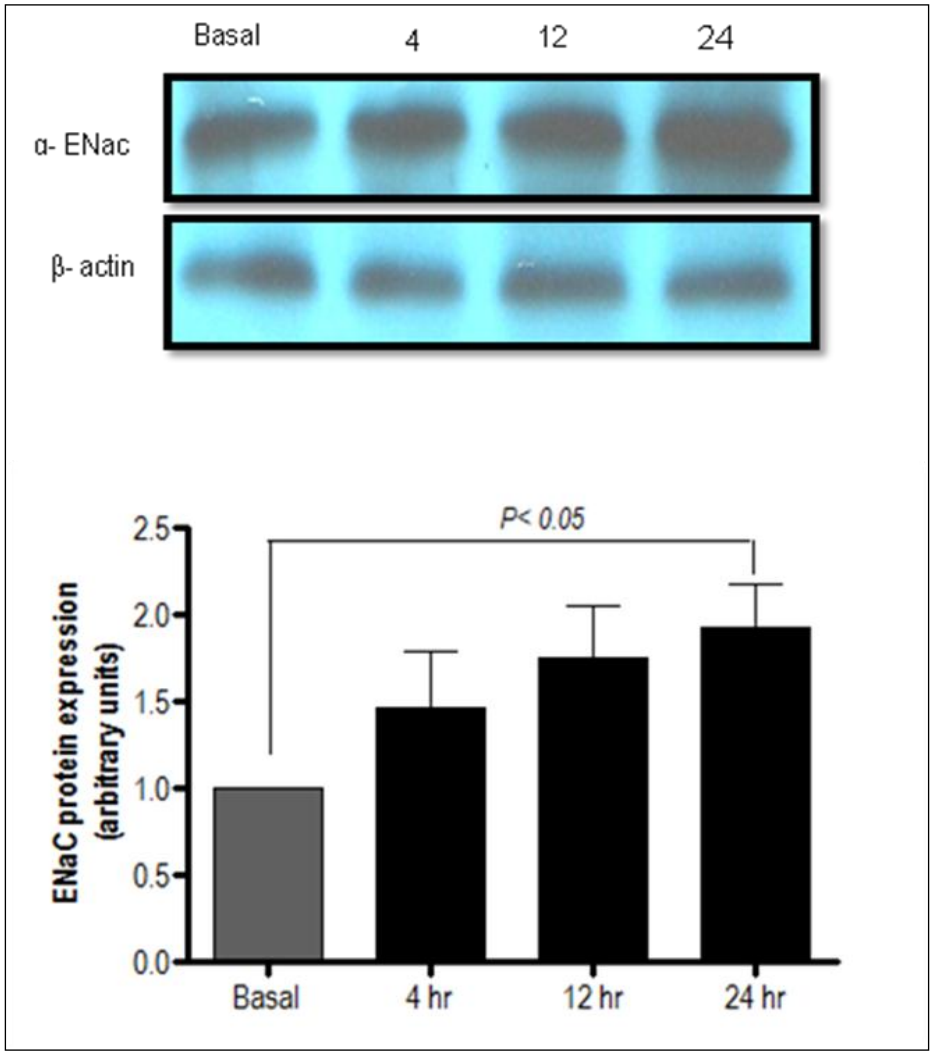


Figure 7.3.3.1- Time- dependent (2-60 minutes) effects of GLP-1 (7-36) on ERK 1/2 phosphorylation in HCD cells were assessed by Western blotting compared with basal (no supplement). Data are expressed as relative to median of basal. A minimum of three independent experiments were performed and each experiment was carried out in triplicate. Group comparison by repeated measures ANOVA and post hoc Dunnett's multiple comparisons test. Results show 10 nM GLP-1 to enhance phosphorylation of ERK1/2, which peaked at 5 minutes, gradually returning to baseline values by 30 minutes.

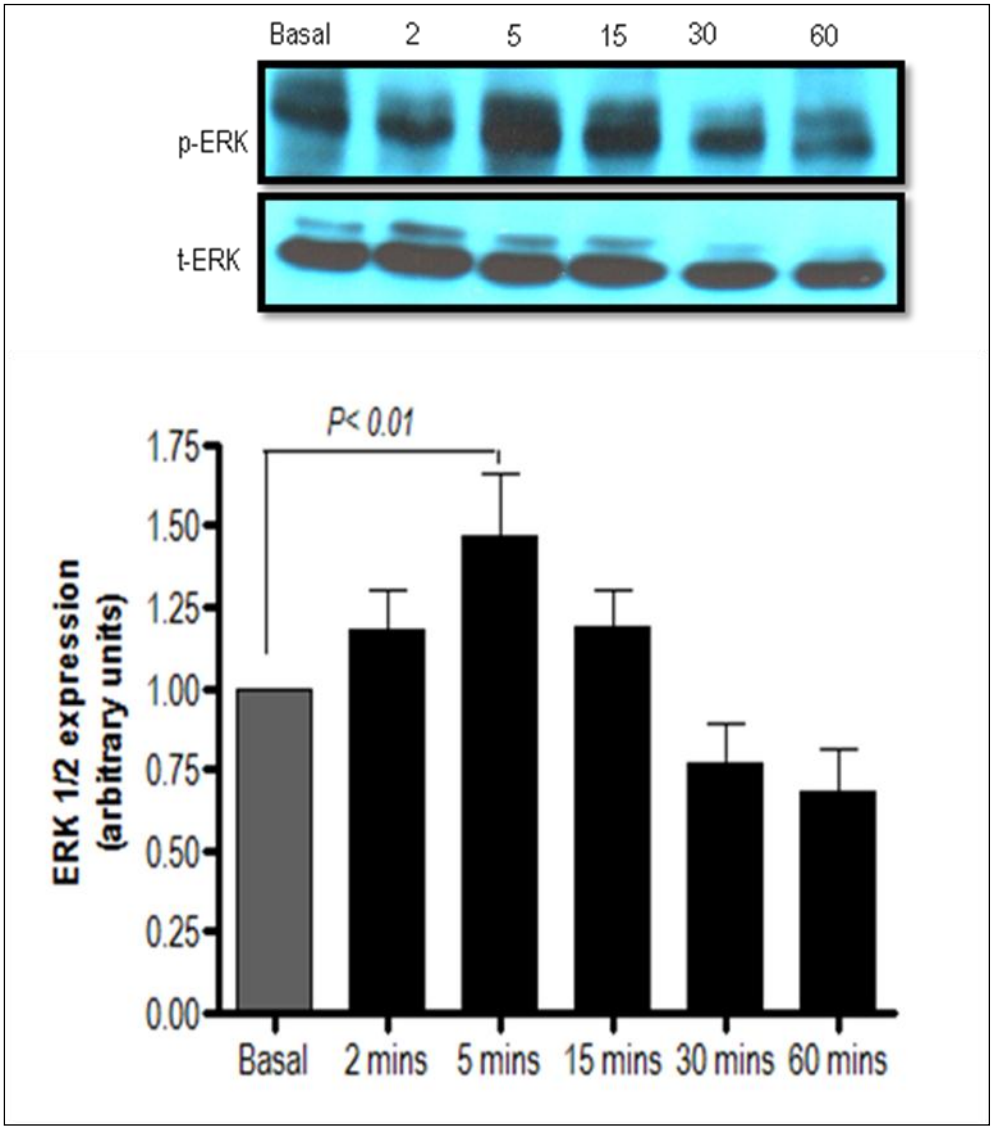
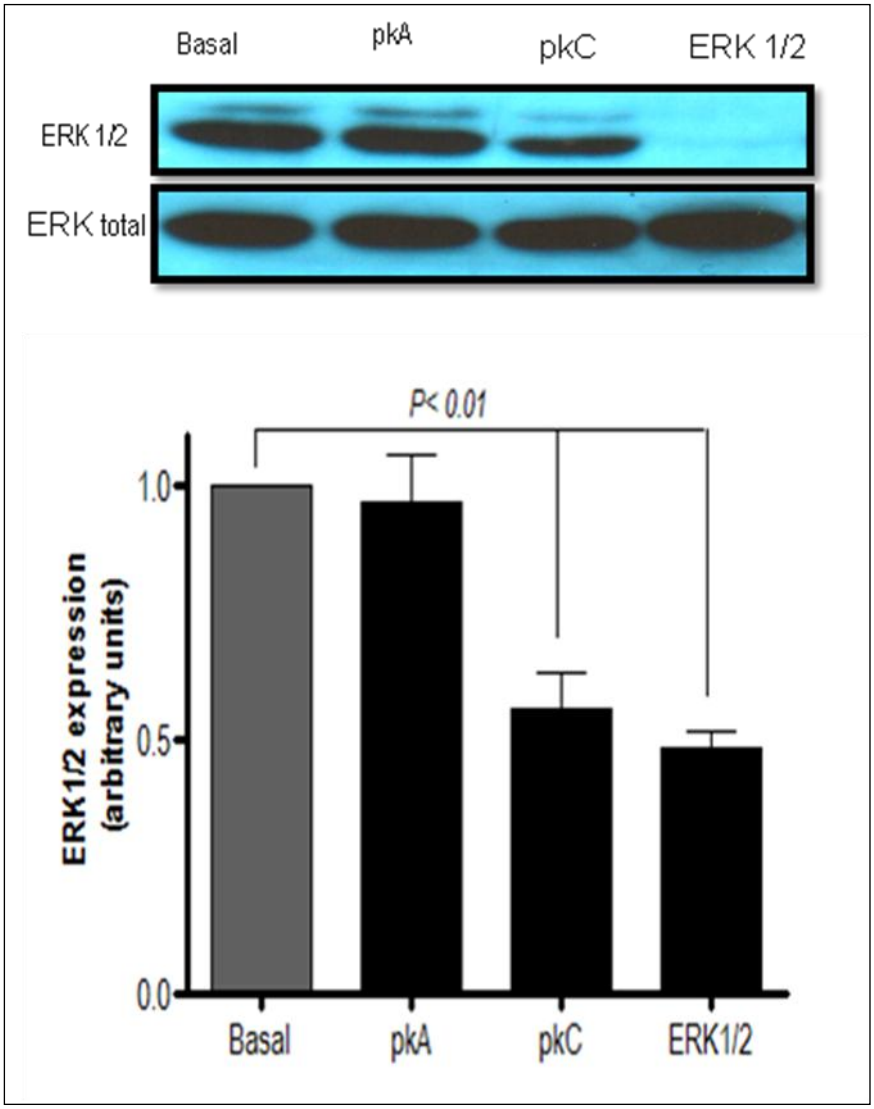


Figure 7.3.3.2- Effects of 10nM GLP-1 on ERK 1/2 protein expression in HCD cells at 5 minutes after incubating in specific inhibitors for PKA and PKC were assessed by Western blotting compared with basal (no supplement). Data are expressed as relative to median of basal. Four independent experiments were performed and each experiment was carried out in triplicate. Group comparison by repeated measures ANOVA and post hoc Dunnett's multiple comparisons test. Results show a significant decrease in ERK 1/2 expression after co-incubation with 10 nM GLP-1 and PKC inhibitor for 5 minutes. ERK 1/2 inhibitor was used as control.



7.4 Discussion

There are many hormonal regulators of ENaC including the most documented hormone, aldosterone, and also angiotensin II, components of the renin angiotensin aldosterone system (RAAS). It is also well understood that stimulation of P13-K promotes the activity of ENaC through upregulation of SGK-1 (Thomas, 2007; 2008). Conversely, activation of ERK1/2 has been shown to suppress ENaC activity and is dependent on activation of PKC although some researchers suggest that activation of ERK1/2 stimulates channel activity (Thomas, 2008; Krug, 2002).

This is the first study to explore the effects of GLP- 1 on α-ENaC expression in the human kidney. The α- ENaC subunit is understood to be involved in transporting Na^+ through the channel, whereas β and γ- subunits have a regulatory role (Hamm, *et al*. 2011). Despite a clear indication from animal and human studies that GLP-1 displays actions in the kidney, no studies to date have focussed on potential GLP-1 actions in distal portions of the nephron, namely the collecting duct where I show GLP-1R to be present and functional. I am able to show GLP-1 (7-36) amide to up-regulate SGK-1 and α-ENaC mRNA expression but GLP-1's action on protein expression was less clear. GLP-1, at a concentration of 1 nM was shown to modestly down-regulate *SGK-1* mRNA at 4 hrs possibly as a consequence of down-regulation by SGK-1 protein. SGK-1 and α-ENaC protein expression were modestly enhanced after stimulation with 10 nM GLP-1 for 24 hrs but surprisingly up-regulation of α-ENaC was not replicated in the experiments using PKC inhibitors as the α-ENaC protein could not be visualised. A

possible reason for this was that morphological changes had occurred within the cells and that α-ENaC protein had stopped being produced by the cells due to the effects of multiple passages. Nevertheless, cell passages did not exceed the recommended maximum of 30 passages. Analysis by western blot showed an increased phosphorylation of ERK1/2 which was attenuated by the presence of PKC inhibitors. Therefore the effect seen in HCD cells after GLP-1 administration may be a result of crosstalk between ERK1/2 and P13-K signalling pathways (Thomas 2008) (**Figure 7.4.1 Possible GLP-1 signalling of ENaC in the collecting duct**). Highest concentrations (100 nM GLP-1 (7-36)) displayed little or no effect.

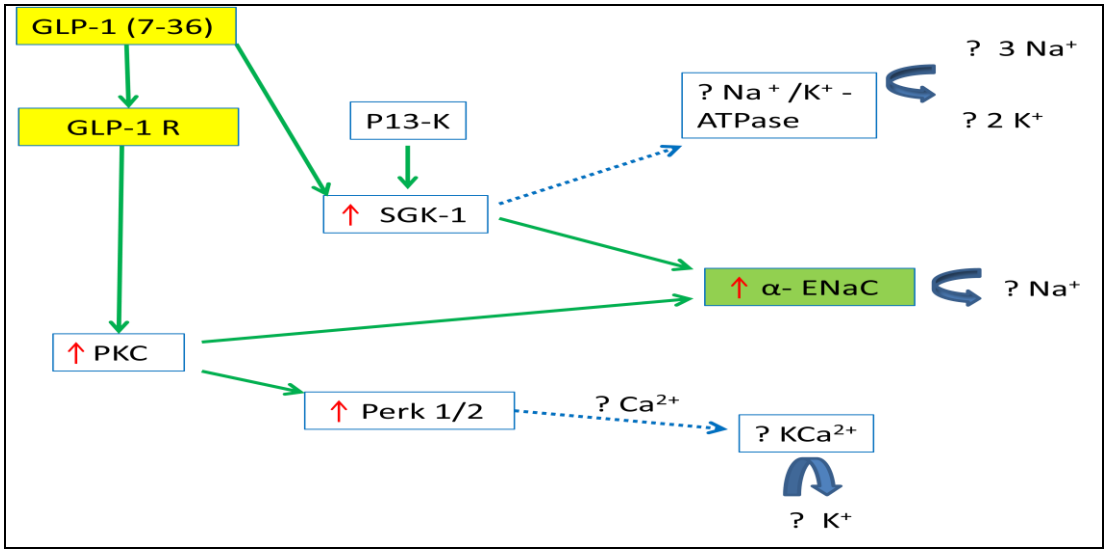


Figure 7.4.1 Possible GLP-1 signalling of ENaC in the collecting duct. A schematic representation of the proposed signalling components of GLP-1R activation (in HCD cells) which may lead to sodium reabsorption in the distal nephron of the human kidney. Where no evidence exists, hypothesis is indicated by a dashed line and "?".

GLP-1 for human GLP-1R is proposed to have an IC50, 0.37 nM (Green, 2004). Furthermore, DPPIV is reported to be present in the human proximal tubule but not in the collecting duct which is interesting as one might not expect GLP-1 metabolites to reach the collecting duct *in vivo* as native GLP-1 should be fully degraded via the liver and peripheral tissues (muscle, connective and adipose) and the remainder by the renal proximal tubule. Furthermore, intact GLP-1 has not been detected in urine (Kugler *et al*, 1985; Malm- Erjefalt, 2010). GLP-1 agonists exenatide and liraglutide are resistant to DPPIV, that is to say, enzymatic degradation by DPPIV (and also by neutral endopeptidases, NEPs) is dramatically slowed (Malm- Erjefalt, 2010). Interestingly, liraglutide is similar to native GLP-1 in that it is eventually cleaved by DPPIV into the truncated GLP-1(9-36) amide (Malm- Erjefalt, 2010). Exenatide is eliminated solely by glomerular filtration and proximal tubular degradation whereas liraglutide does not undergo glomerular filtration due to high binding to serum albumin (Malm- Erjefalt, 2010).

It might have been interesting to explore the effects of other GLP-1 metabolites, such as GLP-1(9-36) amide. Although once believed to be inactive, there is emerging evidence to suggest that the truncated GLP-1 (9-36) amide display effects independent of GLP-1R or could be an inverse agonist to GLP-1R (Thomas & Habener, 2009). In theory, with consideration that DPPIV is concentrated in the tubules, we might expect only small amounts (if any) of intact GLP-1 (7-36) metabolite to reach the collecting duct and that the majority would be excreted by the tubules (and liver and peripheral tissues). We might also speculate that of the minor metabolites to

potentially reach the collecting duct via the tubule, GLP-1(9-36) would be a prime candidate due to cleavage by DPPIV. GLP-1R are present in the collecting duct and GLP-1(7-36) did modestly up-regulate α-ENaC and its corresponding signalling components. These results show a paradox as they suggest that GLP-1 (7-36) might cause an increase in sodium reabsorption in the collecting duct via GLP-1R mediated events which would ultimately lead to an increase in blood pressure. This is contrary to animal and human reports which show GLP-1 to lower blood pressure and increase natriuresis via the tubules. It might be pertinent to explore the effects of other metabolites which might display opposing or allosteric roles.

To conclude, it is not clear how the paradoxical results of this study might be reflected *in vivo* as there is currently a paucity of data into the actions of GLP-1 in the collecting duct. Furthermore, functional studies on ENaC-mediated sodium transport have not yet been performed. A possible limitation to my studies is that I show supraphysiological doses of GLP-1(7-36) to initiate activation of PKC family of serine/threonine protein kinases to phosphorylate α-ENaC and hence it could only be loosely speculated that (in theory) this could result in rapid responses of ENaC which might increase sodium reabsorption in the distal nephron ultimately leading to increased blood pressure (Thomas, 2008). The relationship that this reabsorption has with subsequent studies in man that have shown a sodium losing (natriuresis) needs to be explained. Much research needs to be conducted to establish that a fully functional system is present in the human collecting duct cell lines and that sodium reabsorption is increased by GLP-1 in this kidney segment. Experiments could have been greatly improved by

confirming absence of DPPIV (and NEP) in the HCD cells and by performing sodium transport studies. It might be loosely speculated that if sodium reabsorption does occur at the collecting duct, that overall net increases of reabsorbed sodium would not exceed sodium loss via the proximal tubules and therefore the overall effect of GLP-1 would be to reduce blood pressure. Interestingly, GLP-1 infusions have been shown to increase both sodium and potassium excretion in rats but these experiments did not confirm GLP-1R to be present in distal portions of the kidney in this species (Craijoinas *et al*, 2010; Moreno *et al,* 2002). Nevertheless, this research indirectly supports my findings and suggests that sodium may be being reabsorbed in the distal tubule and collecting duct in exchange for potassium.

8.0 Final discussion and future directions

The main action of GLP-1 is to regulate ingested glucose. GLP-1 is secreted from the L-cells of the intestine in response to ingested nutrients and it enters the blood stream to direct insulin secretion and to regulate growth and apoptosis of pancreatic cells, regulate the uptake of glucagon secretion as well as other metabolic processes (Ceriello *et al*, 2008).

The action of GLP-1 agonists on blood pressure reduction in T2DM patients, as described in **Chapter 4**, is undoubtedly at least in-part due to the weight reducing effects of these agents which is supported by scientific investigation into GLP-1 actions on adipocyte dynamics (Ruiz-Grande, 1992). Recent literature also suggests GLP-1 to mediate autonomic control of glycaemia and satiety by increasing vagal and sympathetic tone and activation of hypothalamic nuclei (Hayes, 2012; Griffion *et al,* 2011; Cabou *et al,* 2008). GLP-1R is located in cardiomyocytes, vascular smooth muscle, endocardium and coronary endothelium and recently discussions with Novo Nordisk researchers have informed that GLP-1 receptor is also located on the sino-atrial node. It is therefore likely that effects of GLP-1 agonists on body weight, blood pressure and heart rate are primarily a consequence of autonomic response to these agents. Location of GLP-1R on sino-atrial tissue possibly explains the chronotropic effects seen in animals and T2DM patients administered GLP-1 (Griffioen *et al*, 2010; Barragan *et al*, 1999; Gardiner, 2006). Furthermore, Novo Nordisk heart rate data indicates that patients with a lower baseline heart rate and hence an assumed increase in vagal tone might be susceptible to greater heart rate increases. Therefore, the use of such agents for glucose control and weight reduction must be

considered carefully as an increase in heart rate might be a deleterious side effect for susceptible individuals.

Undertaking the meta-analysis highlighted the requirement for more standardised reporting of clinical trial results as well as specific concerns as to the need for long-term studies into the cardiovascular safety of GLP-1 agonists. It was surprising that such little emphasis had been placed on investigating the chronotropic effects of GLP-1 agonists in trial participants, despite indication of this effect from supportive scientific evidence. Furthermore, gathering numerical data for the relevant outcomes from trial reports was difficult and although clinical trial organisers were very willing to assist us in supplying the missing data, it was a lengthy process. Regardless of there being strict (CONSORT) guidelines for reporting randomised trials, information was often missing from trial reports including the name of trial registry (National Clinical Trial number) which sometimes resulted in difficulty when comparing trial reports to the corresponding protocols (Brand, 2009; Schulz *et al*, 2010).

In **Chapter 5** I demonstrated that in murine models closely resembling human obesity and diabetes, renal GLP-1R is inhibited and this is possibly a consequence of chronic hyperglycaemia. I was also the first to show that GLP-1R is present in human collecting duct cells. Glucose was found to directly influence GLP-1R expression in immortalised cells of the human proximal tubule and collecting duct (**Chapter 6**). The presence of GLP-1R in renal tissue indicates that a direct receptor-mediated action might occur which is influenced by ambient glucose concentrations. It is possible that GLP-1R directed regulation of glucose is occurring in the kidney which needs

further investigation. Experiments in HCD and HKC8 cells could have been greatly improved by extending the glucose incubation times to 48 hours to more closely mimic chronic hyperglycaemia. However, it is important to consider how GLP-1R expression may differ *in vivo* compared to isolated cell lines. Central (brain) GLP-1R is also strictly dependent on glucose and is closely linked to the development of hyperinsulinemia through feedback of the gut-brain axis during the absorptive state (Cabou *et al,* 2008; Hayes *et al,* 2012). Therefore glucose dependent brain GLP-1R signalling could directly link abnormal metabolic function and blood pressure through deregulation of central and autonomic control of glucose homeostasis, satiety and gastric emptying. The influence of chronic hyperglycaemia on GLP-1R expression in peripheral tissues might directly influence overall glucose control via gut-brain regulatory feedback mechanisms. Therefore hypothalamic factors in the BL/6 and BLK/s mice might contribute to the blunted renal GLP-1R expression. Similarly disrupted insulin signalling from the pancreas in MycER^{TAM} mice might relay to hypothalamic nuclei and to peripheral organs via neuronal afferents to disrupt GLP-1R signalling. This might be another reason why GLP-1R expression was increased in isolated cell lines but blunted *in vivo* where autonomic regulatory neurons would influence GLP-1 receptor expression.

In T2DM sodium clearance is inhibited at least in part due to glucose-sodium co-transport in the brush border of the proximal tubule and the effects of hyperinsulinemia in the distal nephron (Gerich, 2010). Therefore, a symptom of chronic hyperglycaemia is glycosuria and the imminent fate of the diabetic patient is a homeostatic response to retain sodium which over time is

suggested to contribute to co-morbid hypertension which is prevalent in type 2 diabetes. The question of why GLP-1 agonists display more beneficial effects on blood pressure remains to be fully researched. More complex mechanisms appear to be involved which in some subjects (about 1 in 3) the pronounced weight loss cannot be the full explanation for all those patients who experience a reduced systolic blood pressure on these agents.

Hyperglycaemia is suggested to increase sodium loss and diuresis by the effect of glycosuria. This phenomenon resets the kidneys to sodium retention and anti-natriuresis as a secondary effect due to enhanced glucose-sodium co-transport in the renal proximal tubule and hyperinsulinemia which has been shown to cause sodium retention in the distal nephron. There is a paucity of mechanistic studies into the action of GLP-1 on sodium transport molecules in the kidney and research thus far been fairly inconclusive. Schlatter and later Crajoinas reported GLP-1 to inhibit sodium transport in isolated cells of the porcine proximal tubule via cAMP dependent PKA signalling pathways (Schlatter, 2006; Crajoinas, 2010). Schlatter was also not able to show GLP-1 to display a direct effect on sodium-glucose transporters but did demonstrate incubation with GLP-1 to result in partial inhibition of NHE3 but he failed to show this with exenatide. This is interesting as it indicates that there might be differing mechanisms to which these two drugs lower blood pressure although exenatide has been shown to increase natriuresis in rodents (Hirata, 2009). Nevertheless, while there is disparity in animals both liraglutide (sharing 97% homology with GLP-1 and similar enzymatic degradation to the native peptide) and exenatide display comparable effects on blood pressure reduction in human

trials. Furthermore, exenatide (Exendin-4) has been shown to increase natriuresis in animals independent of an increase in glomerular filtration rate (Hirata *et al,* 2009). The potential species-specific differences regarding cell system used in Schlatter's study mean it might be pertinent to repeat these experiments in cells of the human proximal tubule (HKC8) cells. Other potential species differences were that GLP-1R was not influenced by glucose or GLP-1 contrary to my own results that show GLP-1R to be directly influenced by glucose in both human proximal tubule and collecting duct cells. It is interesting to note that Schlatter, although using DPPIV inhibitor for his cellular work did not consider the potential for GLP-1 or exenatide to be degraded by neutral endopeptidases (NEP) which have been reported to reside on renal proximal tubule epithelium (Kubaik-Wlekly & Niemir, 2009) so the effects seen in his porcine renal cell system may have been the consequence of GLP-1 or exenatide degradation to minor metabolites by NEP. It is suggested that up to 50% native GLP-1 is degraded by NEP, although this is controversial (Grieve, 2009).

I have shown that α-ENaC mRNA and associated mediators are up-regulated by GLP-1 which is possibly through direct GLP-1R- mediated action. The disparity as to the concentration of GLP-1 to produce such effects is not clear with concentrations of GLP-1(7-36) close to the IC50 value resulting in down-regulation of SGK-1 mRNA expression but higher concentrations of 10 nM eliciting an up-regulation of SGK-1 and α-ENaC mRNA and their corresponding proteins at 24 hours and also of phosphorylated ERK1/2. Neither DPPIV nor NEP is reported to be present in the collecting duct but I was not able to confirm the absence of DPPIV or

NEPs in the human collecting duct cell line utilised in this study without further research. Research in animals has shown that different responses are observed at either physiological or supraphysiological concentrations of GLP-1 and other analogues although this does appear to be highly dependent on route of administration (Bojanowska & Stepniak). Further exploration into dose effects might allow a more solid explanation. I showed GLP-1(7-36) amide to modestly up-regulate α-ENaC and that this may be mediated by P13-K and PKC dependent SGK-1 and ERK1/2 signalling pathways, respectively. The finding from this cell model suggest that GLP-1 might increase sodium reabsorption via the collecting duct which is paradoxical to the natriuresis and blood pressure reduction described in animals and humans. However, the incongruity between my results in cell lines and the existing literature could be the result of a number of factors. For instance, a concert of effects would occur *in vivo* which would not be replicated in a cell system. It is also possible that GLP-1 displays differing regional effects on renal sodium transport, and that it inhibits sodium reabsorption in the tubules, but modestly increases reabsorption by the collecting duct where sodium balance is finely controlled resulting in a greater overall natriuretic than anti-natriuretic effect. Furthermore, the concentration of DPPIV in the tubules might mean that GLP-1 is fully degraded in this segment and intact GLP-1 (7-36) fails to reach the collecting duct *in vivo.* Functional GLP-1R is however present in human collecting duct cells which might suggest that a proportion of metabolites do reach the collecting duct. It remains unknown whether some active GLP-1(7-36) travels to the collecting duct or if the majority could be GLP-1(9-36) or other

minor metabolites. Therefore, it might be speculated that if sodium reabsorption *is* occurring via the collecting duct, this effect might be limited to isolated collecting duct cell lines.

To take this thesis further and to bring into context the cardiovascular action of GLP-1 or liraglutide, the effects of administration of GLP-1 (9-36) to MycER mice could be explored within insulin responsive tissues such as the kidney in the absence of endogenous insulin production. Furthermore, mechanistic studies could be repeated in proximal tubule and collecting duct cells to explore the action of GLP-1(9-36) or other metabolites on sodium transport. Clinical trials in man using long-acting GLP-1 agonists raise the possibility that GLP-1 metabolites reach the collecting duct and thus initiate sodium reabsorption via GLP-1R-mediated up-regulation of ENaC. This action might explain why the longer acting GLP-1 agonists are reported to have a reduced effect on lowering blood pressure. Once weekly exenatide preparations are currently undergoing phase 3 trials and one might predict an extended accumulation in the kidney with these agents. Long acting release formulations for liraglutide, which shares a similar metabolite profile to GLP-1, are also under development and early reports suggest they increase heart rate but reduce blood pressure less. Interestingly data pooled by meta-analysis from clinical trials into exenatide LAR preparations showed a significantly lower mean difference in blood pressure reduction than shorter acting formulations compared to active control. This could be reflected in the potential for increased sodium reabsorption via the collecting duct by these agents and requires more detailed studies to explore this hypothesis. Further research into the actions of these formulations and the

corresponding metabolites on sodium reabsorption via ENaC would be beneficial.

8.1 Conclusion

As the principal action of GLP-1 is to regulate glucose, it is probable that improvement of overall metabolic control could also play a role in the improved blood pressure seen in patients undergoing GLP-1 therapy through control of metabolic and cardiovascular homeostasis by GLP-1 produced in the brain. Brain GLP-1 is glucose dependent which minimises the risk of hypoglycaemia by blunting insulin secretion when insulin levels return to normal (Cabou *et al,* 2008). Brain GLP-1 has been linked specifically to insulin sensitivity and cardiovascular control. GLP-1 is secreted from enteroendocrine cells which controls pancreatic enteroendocrine secretion (stimulation of insulin and inhibition of glucagon) and is also synthesized in the caudal regions of the nucleus of the solitary tract (Cabou *et al,* 2008). GLP-1 released into the hypothalamus controls food intake, blood pressure and heart rate (Yamamoto *et al,* 2003; Barragan *et al,* 1999; Hayes *et al,* 2012). Central administration of exendin-4 has been demonstrated to stimulate intravenous glucose insulin secretion and conversely, centrally administered antagonist exendin (9-39) blocks post-prandial glucose induced insulin secretion (Knauf *et al,* 2005). This relay between the gut-brain axis leads to hyperinsulinemia as a consequence of increases in brain GLP-1 which also causes a redistribution of blood flow to mesenteric organs by modulation of heart rate and blood pressure through changes in autonomic control (Cabou *et al,* 2008). It is becoming clear that neuronal circuits in the

central nervous system play a crucial role in metabolic and cardiovascular homeostasis through increasing insulin sensitivity and vascular effects of insulin and the production of nitric oxide in the hypothalamus (Cabou *et al*, 2008). Therefore a central control of glucose metabolism via gut-brain-kidney axis is possibly the primary mechanism by which GLP-1 agonists displays weight and blood pressure reducing effects with neuronal control of insulin secretion and sensitivity being central to such effects. However, modulation of autonomic control mechanisms might result in unfavourable side effects such as increased heart rate in patients receiving GLP-1 agonists. Further research into the long term cardiovascular safety of these agents is required, especially in patients with low baseline heart rate who might be susceptible to such effects.

Appendix

A1.0 Cell culture techniques and solutions for HCD and HKC8 cells

A1.1 Trypsin solution for subculturing HCD and HKC8 cells:

20ml 1x PBS

9ml of 1mM EDTA

1ml of 2.5% trypsin

This mixture was either stored at -20^{0}C, or at 4^{0}C for 10-14 days.

A1.2 Standard culture medium for HCD cells

DMEM/Hams-F12 mix containing:

2% FCS (10ml in 500ml)

20 mM Hepes (supplement with 1ml 1M Hepes)

5ml ITS (sigma I1884; final concs: insulin (5 μg/ml), transferrin (5 μg/ml),

Na_2SeO_3 (5 ng/ml))

5 X10^{-8}M Dexamethasone (2.5μl of 10^{-2}M stock in ethanol, stored at –20)

Store all stocks at -20^{o}C

A1.3 Normal growth medium for HKC8 cells

DMEM/Hams-F12 (containing Hepes glutamax)
5% FCS,
Feed every other day.

A1.4 Medium for serum free culture for experiments for both HCD and HKC-8 cells:

485ml DMEM/Hams-F12 mix

5ml ITS [insulin (5 μg/ml), transferrin (5 μg/ml), Na_2SeO_3 (5 ng/ml)]

62.5 µl T_3 (20 µg/ml) (stock 1mg in 1ml of 1M NaOH then + 49ml of water, store –20^{o}C)

12.5 µl epidermal growth factor (stock, 100 µg/ml in 0.1% BSA, store –80oC)

50 µl hydrocortisone hemisulphate (stock, 50 µg/ml; 1mg in 1ml of EtOH + 19ml H_2O, store –20^{o}C)

5 ml glutamine (100X stock solution = 0.3 mg/ml)

All stock solutions were stored at -20^{o}C

A1.5 Routine Maintenance

Feeding cells:

Feed cells every 1-2 days

Pipette out old medium from flask to waste pot.

Using fresh pipettes and a good aseptic technique, transfer fresh medium (12 -15ml for T-75 flasks; 5ml for T-25 flasks) to the flask.

Place flask in CO_2 incubator which should read 37^{0}C and 5% CO_2.

A2. Sub-culturing cells:

Split when approximately 90% confluent. Time taken to reach this will vary but usually takes 4-7 days if split 1:20.

Normal growth medium is very low in FCS; therefore inactivate trypsin with medium containing a higher FCS concentration. For example, for 75^{2} cm flask, use 3mls of trypsin solution and then deactivate with 10mls of DMEM/F12 + 10% FCS. Spin cells 1100rpm, 5 mins and remove medium. Re-suspend cells in 20mls normal growth medium; add 1ml to new flask (containing approx. 12 mL medium) for stocks.

Freezing cells (Freeze in FCS with 10% DMSO)

Trypsinise cells and spin at 1100 rpm, 5 mins RT. Resuspend pellet in FCS with 10%.

From 1 confluent 75cm^2 flask add 2-3ml freezing solution and divide into 2 cryovials.

Freeze down slowly at –70^{o}C for a few hours or O/N (put vials inside polystyrene packing) and then liquid N_2.

When reviving cells, bring to RT quickly.

A2.0 Western blotting solutions

A2.1 Sodium Dodecyl Sulphate (SDS) (4%)

10 ml 20% SDS solution
50 ml dH2O
Solution stored at room temperature (RT)

A2.2 Western blotting Loading buffer

625 µl Tris-HCl (pH 6.8) 125 mM
500 µl SDS 4%
1 ml Glycerol
200 µl Dithiothreitol (DTT)
125 µl Bromophenol Blue
250 µl Distilled H2O

A2.3 10% western blotting gel:

4 ml Resolving gel
3 ml Resolving buffer
5 ml dH_2O
10 µl TEMED
100 µl 10% APS

A2.4 12% western blotting gel

5 ml Resolving gel
3 ml Resolving buffer
4 ml dH_2O
10 µl TEMED
100 µl 10% APS

A2.5 Stacking gel

0.65 ml Resolving gel
1.25 mL Stacking gel
3.05 mL dH_2O
50 µl APS
5 µl TEMED

A2.6 Tris-buffered Saline-Tween (TBS-T) (10X): 0.5M Tris Base, 9% NaCl, pH 7.6

61 g Trizma base
90 g NaCl
1 L dH2O
Mix thoroughly to dissolve and adjust pH with HCL. Store at RT.

A2.7 TBS-T (1X*)*

TBS-T (10X) diluted (1:10) with dH2O

A2.8 Blocking Solution for PVDF membranes

10 g bovine serum albumin
200 ml TBS 0.5% TBS (Tween 20 (0.1% (v/v), Sigma UK)

A2.9 Blocking with immunizing peptide

Blocking buffer consisting of TBST plus 3% BSA
Antibody
Blocking (immunizing peptide)

A3.0 Transfer buffer

100 ml Tris glycine
200 ml methanol
700 ml dH2O

A3.1 Running buffer

10 X Running buffer
100 ml in 900 ml dH2O

A4.0 PCR

A4.1 Gel agarose

0.5 g agarose
50 ml 1 x TBE
1.5 µl ethidium bromide

References

Abate N & Chanalia M (2001) Ethnicity and type 2 diabetes. Focus on Asian Indians. *Journal of Diabetes and its Complications*. **15**: 320-327

Abu-hamdah R, Rabiee A, Meneilly GS, Shannon RP, Anderson DK, Elahi D.(2009) The extra pancreatic effects of GLP-1 and related peptides*. J. Clin Endocrinol. Metab.* **94** (6): 1843-1852

Ali L, Khan AKA, Hasan Z, Mosihuzzaman M, Nahar N, Nasreen T, Nur-E-Alam M, Rokeya (1995). Characterization of the hypoglycaemic effects of *Trigonella Foenum Graecum* seed. *Planta medicine* **61**: 358-60

Almind, K., Biorbaek, C., Vestergaard, H., Hansen, T., Echwald, S.M., Pederson O (1993) Amino acid polymorphisms of insulin receptor substrate-1 in non-insulin dependent diabetes mellitus. *Lancet* **342**:828-32

Amori RE, Lau J, Pittas AG, (2007). Efficacy and safety of incretin therapy in type 2 diabetes: Systematic review and meta-analysis. *JAMA* **298** (2): 194-206.

Anselmino M, Öhrvik J, Rydén L (2010). Resting heart rate in patients with stable coronary artery disease and diabetes: a report from the euro heart survey on diabetes and the heart. *Eur Heart J* **31**:3040-3045.

Apovian CM, Bergenstal RM, Cuddihy RM, Qu Y, Lenox S, Lewis MS et al (2010). Effects of exenatide combined with lifestyle modification in patients with type 2 diabetes. *Am. J. Med*. **123**:468-77.

Arnette D, Gibson TB, Lawrence MC, January B, Khoo S, McGlynn K, Vanderbilt CA, Cobb MH (2003) Regulation of ERK1 and ERK2 by glucose and peptide hormones in pancreatic β-cells. *J. Biol. Chem*. **278**:32517–32525.

Aronow WS, Ahn C, Mercando AD, Epstein S (1996). Association of average heart rate on 24-hour ambulatory electrocardiograms with incidence of new coronary events at 48-month follow-up in 1311 patients (mean age 81 years) with heart disease and sinus rhythm. *Am J Cardiol*. **78**; 1175–1176.

Ban K, Kim K-H, Cho C-K, Sauve´ M ,Diamandis EP, Backx PH, Drucker DJ,Husain M (2010) Glucagon-Like Peptide (GLP)-1(9-36) Amide-Mediated Cytoprotection Is Blocked by Exendin(9-39) Yet Does Not Require the Known GLP-1 Receptor*. Endocrinology*, **151**(4):1520–1531

Ban K, Noyan-Ashraf MH, Hoefer J, Bolz S-S, Drucker DJ, Husain M (2008). Cardioprotective and Vasodilatory Actions of Glucagon-Like Peptide 1 Receptor Are Mediated Through Both Glucagon-Like Peptide 1 Receptor - Dependent and Independent Pathways. *Circulation*; **117**:2340-2350

Barnett A, Brice R, Hall G, Holt R, Kanumilli N, Mulnier H (2010) Nice technology appraisal on liraglutide: Interpretation and practical implications. *Diabetes Primary Care* **12** (6): 1-11

Barnett AH, Burger J, Johns D, et al (2007) Tolerability and efficacy of exenatide and titrated insulin glargine in adult patients with type 2 diabetes previously uncontrolled with metformin or a sulphonylurea: a multinational, randomised, open label two period crossover non-inferiority trial. *Clinical Therapeutics* Nov; **29**(11):2333-48

Barragán JM, Eng J, Rodríguez R, Blázquez E (1999) Neural contribution to the effect of glucagon-like peptide-1-(7-36) amide on arterial blood pressure in rats. *Am J Physiol.* Nov; **277**(5 Pt 1):E784-91

Basten-Dionne PO, Valenti L, Kon N, Gu W, Buteau J (2011). Glucagon-like peptide 1 inhibits the sirtuin deacetylase SirT1 to stimulate pancreatic {beta}-cell mass expansion. *Diabetes* **60** (12): 3217-22

Beinborn M, Worrall CI, McBride EW, Kopin AS (2005) A human glucagon-like peptide-1 receptor polymorphism results in reduced agonist responsiveness*. Regul Pept*. Aug 15; **130**(1-2):1-6.

Bens M, Chassin C (2006), Vandewalle A. Regulation of NaCl transport in the renal collecting duct: lessons from cultured cells. *Pflugers Arch*. **453**(2):133-46

Bergenstal R, Lewin A, Bailey T, Chang D, Gylvin T, Roberts V (2009). Efficacy and safety of biphasic insulin aspart 70/30 versus exenatide in subjects with type 2 diabetes failing to achieve glycemic control with metformin and a sulfonylurea. *Curr Med Res Opin* **25**(1):65-75.

Bergenstal RM, Wysham C, MacConell L, Malloy J, Walsh B, Yan P (2010), Efficacy and safety of exenatide once weekly versus sitagliptin or pioglitazone as an adjunct to metformin for treatment of type 2 diabetes (DURATION-2): a randomised trial. *Lancet* **376**:431-9.

Bharucha AE, Charkoudian N, Andrews CN et al. (2008). Effects of glucagon-like peptide-1, yohimbine, and nitrergic modulation on sympathetic and parasympathetic activity in humans. *Am J Physiol Regul Integr Comp Physiol.* 295(3):R874–R880.

Blackburn, G.L., Kanders B.S. (1994), *Obesity: pathophysiology, psychology and treatment*.Chapman & Hall: Great Britain. pp: 81-101

Blevins T, Pullman J, Malloy J, Yan P, Taylor K, Schuteis C et al (2011). DURATION 5: Exenatide once weekly resulted in greater improvements in glycemic control compared with exenatide twice daily in patients with type 2 diabetes. *J. Clin. Endocrinol. Metab.* **96**:1301-10.

Blonde L, Klein EJ, Han J,et al (2006) Interim analysis of the effects of exenatide treatment on A1C, weight and cardiovascular risk factors over 82 weeks in 314 overweight patients with type 2 diabetes. *Diab. Obes. Metab.* **8**:436-447.

Blonde L, Russell-Jones D. The safety and efficacy of liraglutide with or without oral antidiabetic drug therapy in type 2 diabetes: an overview of the LEAD 1-5 studies. *Diabetes Obes Metab* 2009;**11**(Suppl 3):26-34.

Bode B (2012). An overview of the pharmacokinetics, efficacy and safety of liraglutide. *Diab. Res. Clin. Pract*. **97**:27-42.

Bonny O and Hummler E (2000) Dysfunction of epithelial sodium transport: From human to mouse. *Kidn. Intern.* **57**: 1313-18.

Bojanowska E, Stempniak B. (2003) Effects of glucagon-like peptide-1 (7-36) amide on neurohypophysial hormone secretion induced by acute hyperosmotic challenge. *Neuropeptides* Feb;**37**(1):45-50.

Briaud I, Lingohr MK, Dickson LM, Wrede CE, Rhodes CJ (2003). Differential activation mechanisms of Erk-1/2 and p70(S6K) by glucose in pancreatic β-cells. *Diabetes* **52**:974–983.

Brűning JC, Winnay J, Bonner-Weir S, Taylor SI, Accili D, Kahn CR (1997). Development of a novel polygenic model of NIDDM in mice heterozygous for *IR* and *IRS1* null alleles. *Cell* **88**: 561-572

Bubein JK, (2010). Epithelial Na^+ channel (ENaC), hormones and hypertension. *J. Biol. Chem.* **285** (31): 23527-31.

Bullock BP, Heller RS, Habener JF (1996).Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor.*Endocrinology* **137**: 2968-78

Bunck MC, Cornér A, Eliasson B, et al. (2010). One year treatment with exenatide vs. insulin glargine: effects on post-prandial glycemia, lipid profiles and oxidative stress. *Atherosclerosis* Sept; **212** (1): 223-229.

Bunck MC, Cornér A, Eliasson B, Heine RJ, Shaginian RM, Taskinen MR, et al (2011). Effects of exenatide on measures of beta-cell function after 3 years in metformin-treated patients with type 2 diabetes. *Diabetes Care* **34**(9):2041-7.

Bunck MC, Diamant M, Corner A, Eliasson B, Malloy JL, Shaginian RM, et al (2009). One-year treatment with exenatide improves beta-cell function, compared with insulin glargine, in metformin-treated type 2 diabetic patients: a randomized, controlled trial. *Diabetes Care* **32**(5)762-768.

Buse JB, Bergenstal RM, Glass LC, Heilmann CR, Lewis MS, Kwan AYM et al (2011). Use of twice daily exenatide in basal insulin-treated patients with type 2 diabetes. A randomized controlled trial. *Ann. Int. Med.***154**:103-112.

Buse JB, Henry RR, Han J, Kim DD, Fineman MS, Baron AD.(2004) Effects of exenatide (Exendin-4) on glycaemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulphonylurea*. Diabetes Care* **Nov 27** (11): 2628-35

Buse JB, Klonoff DC, Nielson L, Guan X, Bowlus CL, Holcombe JH, Maggs DG, Wintle ME (2007) Metabolic effects of two years of Exenatide treatment on diabetes, obesity, and hepatic biomarkers in patients with type 2 diabetes: An interim analysis of data from the open label, uncontrolled extension of three double blind, placebo controlled trials. *Clinical Therapeutics* **29**; (1), 139-153

Buse JB, Rosenstock J, Sesti G, et al. (2009) Liraglutide once a day versus exenatide twice a day for type 2 diabetes: a 26- week randomised, parallel-group, multinational, open label trial (LEAD-6)*. Lancet* **374**; 39-47.

Buse JB, Sesti G, Schmidt WE, Montanya E, Chang C-T, Xu Y, et al (2010). Switching to once-daily liraglutide from twice-daily exenatide further improves glycemic control in patients with type 2 diabetes using oral agents. *Diabetes Care* **33**(6):1300-1303.

Cabou C, Campistron G, Marsollier N, Leloup C, Cruciani-Gugliemacci C, Penicaud L, Drucker DJ, Magnan C, Burcelin R (2008) Brain GLP-1 regulates arterial blood flow, heart rate and insulin sensitivity. *Diabetes.* **57**: 2577-87

Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J. D, Rossier, B. C. (1994) Amiloride- sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature* **367**, 463–467.

Carattino MD, Hughey RP, Kleyman TR (2008). Proteolytic processing of the epithelial sodium channel gamma subunit has a dominant role in channel activation. *J.Biol.Chem*. **283**:25290–25295.

Carattino MD, Sheng S, Bruns JB, Pilewski JM, Hughey RP, Kleyman TR (2006). The epithelial Na+ channel is inhibited by a peptide derived from proteolytic processing of its alpha subunit. *J.Biol.Chem*. **281**:18901–18907.

Carraro-Lacroix LR, Malnic G, Girardi AC (2009) Regulation of Na+/H+ exchanger NHE3 by glucagon-like peptide 1 receptor agonist exendin-4 in renal proximal tubule cells. *Am J Physiol Renal Physiol.* **8**: 231-7

Castellino P, DeFronzo RA (1990). Glucose metabolism and the kidney. *Semin. Nephrol*; **10**: 458-63

Ceriello A, Colagiuri S, Gerich J, Tuomilehto J (2008); Guideline Development Group Guideline for management of postmeal glucose. *Nutr. Metab. Cardiovasc. Dis*. **18**(4):S17-33.

Cheong YH, Kim MK, Son MH, Kaang BK (2011) Glucose exposure pattern determines glucagon-like peptide 1 receptor expression and signaling through endoplasmic reticulum stress in rat insulinoma cells. *Biochem. Biophys. Res. Comm*. **414**: 220–225

Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND et al. (1996). Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* **84**; 491-95

Chen H, Simar D, Morris MJ (2009). Hypothalamic neuroendocrine circuitry is programmed by maternal obesity: interaction with postnatal nutritional environment. *PloS One.* **4:** e6259.

Cheng Q, Leung PS (2011). An update on the islet renin–angiotensin system. *Peptides*. **32**: 1087–1095.

Chin, M. Isono, K. Isshiki, S. Araki, T. Sugimoto, B. Guo, H. Sato, M. Haneda, A. Kashiwagi, D. Koya (2005), Estrogen and raloxifene, a selective estrogen receptormodulator, ameliorate renal damage in db/db mice, *Am. J. Pathol.* **166** 1629–1636.

Chiquette E, Ramirez G, DeFronzo R. (2004) A meta-analysis comparing the effect of thiazalidinediones on cardiovascular risk factors. *Arch. Intern. Med.* **164** (25): 2097-2104.

Christensen BM, Perrier R, Wang Q, Mercier Zuber A, Maillard M, Mordasini D et al. (2010) Sodium and potassium balance depends on α- ENaC expression in the connecting tubule. *J. Am. Soc. Nephrol.* **21**: 1942-51.

Clausen TD, Mathiesen E, Ekbom P, Hellmuth E, Mandrup-Poulsen T, Damm P (2005). Poor pregnancy outcome in women with type 2 diabetes. *Diabetes Care.* **28:** 323–8.

Clausen TD, Mathiesen ER, Hansen T, Pederson O, Jensen DM, Lauenborg J et al. (2008). High prevalence of type 2 diabetes and pre-diabetes in adult off spring of women with gestational diabetes mellitus or type 1 diabetes: the role of intrauterine hyperglycemia. *Diabetes Care.* **31:** 340–46.

Clee SM, Attie AD (2007). The Genetic Landscape of Type 2 Diabetes in Mice. *Endocrine Reviews* **28**(1):48–83.

Cochrane handbook for systematic reviews. Version 5: available online at www.cochrane- handbook.org (June 2011)

Coleman DL (1992). The influence of genetic background on the expression of mutations at the diabetes (db) locus in the mouse. VI: Hepatic malic enzyme activity is associated with diabetes severity. *Metabolism* **41**:1134–1136

Coleman DL, Hummel KP (1973) The influence of genetic background on the expression of the obese (Ob) gene in the mouse. *Diabetologia* **9**:287–293.

Crajoinas RO, Oricchio FT, Pessoa TD, Pacheco BP, Lessa LM, Malnic G, Girardi AC. (2011). Mechanisms mediating the diuretic and natriuretic actions of the incretin hormone glucagon-like peptide-1. *Am J Physiol Renal Physiol.* Aug;**301**(2): F355-63.

Crowley SD, Gurley SB, Oliverio MI, Pazmino AK, Griffiths R, Flannery PJ, Spurney RF, Kim HS, Smithies O, Le TH and Coffman TM (2005) Distinct roles for the kidney and systemic tissues in blood pressure regulation by the renin-angiotensin system, *J. Clin. Invest.***115**; 1092–1099

Cundy T, Gamble G, Townend K, Henley PG, MacPherson P, Roberts AB (2000). Perinatal mortality in Type 2 diabetes mellitus. *Diabet Med.* **17:** 33–39.

Dall'agnoll R, Lino Von Poser G, (2000) The use of complex polysaccharides in the management of metabolic diseases: the case of *Salanum Lycocarpum* fruits. *Journal of Ethnopharmacology*. **71**; 337-341

Davies MJ, Donnelly R, Barnett AH, Jones S, Nicolay C, Kilcoyne A. (2009). Exenatide compared with long-acting insulin to achieve glycaemic control with minimal weight gain in patients with type 2 diabetes: results of helping evaluate exenatide in patients with diabetes compared with long-acting insulin (HEELA) study. *Diabetes Obes Metab.* Dec; **11**(12):1153-64

Day C & Bailey C (1988) Hypoglycemic agents from traditional plant treatments for diabetes. *International Industrial Biotechnology* **8**; (3):5-8.

Deacon CF, Knudsen LB, Madsen K, Wiberg FC, Jacobsen O, Holst JJ (1998). Dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity. *Diabetologia.* **41**: 271-278.

De Glisezinski, F. Crampes, I. Harant, M. Berlan, J. Hejnova, D. Langin, D. Rivière, and V. Stich Endurance training changes in lipolytic responsiveness of obese adipose tissue *American Journal of Physiology Endocrinology & Metabolism* **275**; E951-E956, 1998.

DECODA Study Group (2003) Age- and Sex- Specific Prevalence of diabetes and impaired glucose regulation in 11 Asian cohorts. *Diabetes care*. **26**; 1770-1780

DeFronzo RA, Cooke CR, Andres R, Faloona GR, Davis PJ (1975). The effect of insulin on renal handling of sodium, potassium, calcium, and phosphate in man. *J Clin Invest*. **55**: 845–855.

DeFronzo RA, Davidson JA, Del Prato S (2012). The role of the kidneys in glucose homeostasis: A new path toward normalizing Glycaemia. *Diabetes Obes. Metab.* Sept 28. **14** (1): 5-14.

DeFronzo RA, Ratner RE, Han J, Kim DD, Fineman MS, Baron AD, et al (2005). Effects of exenatide (exendin-4) on glycemic control and weight over 30 weeks in metformin-treated patients with type 2 diabetes. *Diabetes Care*. **28**(5):1092-1100.

Defronzo RA, Triplitt C, Qu Y, Lewis MS, Maggs D, Glass LC (2010), Effects of exenatide plus rosiglitazone on beta-cell function and insulin sensitivity in subjects with type 2 diabetes on metformin. *Diabetes Care*. **33**(5):951-7.

Derosa G, Maffioli P, Salvadeo SA, Ferrari I. Ragonisi F, Querci IG et al. (2010). Exenatide vs. Glimepiride in patients with diabetes. *Diabetes Technol. Therap*. **12**(3):233-40.

Derosa G, Putignano P, Bossi AC, Bonaventura A, Querci F, Franzetti IG, et al (2001). Exenatide or glimepiride added to metformin on metabolic control and on insulin resistance in type 2 diabetic patients. *Eur J Pharmacol* ; **666** (1-3)251-256.

Diamant M, Gaal L, Stranks S, Northrup J, Cao D, Taylor K et al. (2010). Once weekly exenatide compared with insulin glargine titratedto target in patients with Type 2 Diabetes (Duration 3): An open-label randomised trial. *Lancet.* Jun 26 **375** (9733); 2234-43

Dillon JS, Tanizawa Y, Wheeler MB (1993) Cloning and functional expression of the human glucagon-like peptide-1 (GLP-1) receptor. *Endocrinology* **133**; 1907–1910

Doyle ME, Egan JM (2007) Mechanisms of GLP-1 in the pancreas. *Pharmacol. Ther.* **113** (3): 546-93

Drucker DJ (2006) The biology of incretin hormones. *Cell Metab.* **3**: 154

Drucker D, Buse JB, Taylor K, Kendall DM, Trautman M, Zhuang D et al (2008). Exenatide once- weekly versus twice daily for the treatment of type 2 diabetes: a randomized open- label, non- inferiority study. *Lancet*. **372**:1240-50.

Dushay J, Gao C, Gopalakrishnan GS, Crawley M, Mitten EK, Wilker E, Mullington J, Maratos-Flier E (2012), Short-Term Exenatide Treatment Leads to Significant Weight Loss in a Subset of Obese Women Without Diabetes *Diabetes Care*. **35**:4-11

Edwards CM, Edwards AV, Bloom SR (1997) Cardiovascular and pancreatic endocrine responses to glucagon-like peptide-1(7-36) amide in the conscious calf. *Exp Physiol*. Jul **82**(4); 709-16

El-Atat FA, Stas SN, Mcfarlane SI, Sowers JR, (2004). The relationship between hyperinsulinemia, hypertension and progressive renal disease. *J. Am. Soc. Nephrol.***15** (11): 2816–2827.

Elias CF, Kelly JF, Lee CE, Bjorbaek C, Drucker DJ, Kuhar MJ, Saper CB, Elquist JK (2000) Chemical characterization of leptin activated neurons in the rat brain. *J Comp Neurol* **423**:261–268.

Evan GI, Littlewood TD (1993) The role of c-MYC in cell growth. *Curr. Opin. Gen. Dev.* **3**: 44-49.

Fakhoury WKH, Lereun C, Wright D (2010). A meta-analysis of placebo-controlled clinical trials assessing the efficacy and safety of incretin- based medications in patients with type 2 diabetes. *Pharmacol*. **86**:44-57.

Feinglos MN, Saad MF, Pi-Sunyer FX, An B, Santiago O (2005). Effects of liraglutide (NN2211), a long-acting GLP-1 analogue, on glycaemic control and bodyweight in subjects with Type 2 diabetes. *Diabet Med*;**22**(8):1016-1023.

Flint A, Raben A, Astrup A, Holst JJ. Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. *J Clin Invest* 1998;**101**(3):515–520.

Follman D, Elliott P, Suh I, Cutler, J. (1992) Variance imputation for overviews of clinical trials with continuous response. *J. Clin. Epidemiol.* **45** (7): 769-773

Forbes S, Moonan M, Robinson S, Anyaoku V, Patterson M, Murphy KG, Ghatei MA, Bloom SR, Johnston DG (2005) Impaired circulating glucagon-like peptide-1 in response to oral glucose in women with previous gestational diabetes. *Clin Endocrinol*. **62**; 51-55

Fornesca V, Falahati A, Zychma, M, Madsbad S, Plutzky J, (2009). A meta-analysis of six clinical trials demonstrate that the once-daily GLP-1 analogue, Liraglutide reduces systolic blood pressure. Presented at EASD, 2009

Forti A, Garcia EG, Yu MB, Jimenez MC, Brodows RG, Oliveira JH (2008).Efficacy and safety of exenatide administered before the two largest daily meals of Latin American patients with Type 2 Diabetes (Safety and efficacy of Exenatide taken before lunch and before dinner compared with before breakfast and before dinner in patients with Type 2 Diabetes using oral antidiabetic therapy- Trial 10749).*Current Medical Research and Opinion* **24** (9): 2437-2447

Franks PW, Bhattacharayya S, Luan J, Montague C, Brennand J, Challis B et al. (2003) Association between physical activity and blood pressure is modified by variants in G-protein coupled receptor- 10. *Hypertension*. **43**: 224-28.

Froguel P & Boutin P (2001) Genetics of human obesity. *Best Practice & Research Clinical Endocrinology & Metabolism.* **15** (3); 391-401

Gaillard D, Puchelle ME, Hinnrasky J, Coscoy S, Hofman P, Matthay MA, Puchelle E, Barbry P (2000) Early expression of β- and γ-subunits of epithelial sodium channel during human airway development. *Am J Physiol Lung Cell Mol Physiol* **278**:L177-L184.

Gallwitz B, Bohmer M, Segiet T, Molle A, Milek K, Becker B, et al. (2011). Exenatide twice daily versus premixed insulin aspart 70/30 in metformin-treated patients with type 2 diabetes: a randomized 26-week study on glycemic control and hypoglycaemia. *Diabetes Care* **34**(3):604-606.

Gao Y, Yoon KH, Chuang L-M, Mohan V, Ning G, Shah S, et al (2009). Efficacy and safety of exenatide in patients of Asian descent with type 2 diabetes inadequately controlled with metformin or metformin and a sulphonylurea. *Diabetes Res Clin Pract*. **83**(1):69-76.

Garber A, Henry R, Ratner R, et al. (2009) for the LEAD-3 (Mono) Study Group. Liraglutide versus glimepiride monotherapy for type 2 diabetes (LEAD-3 Mono): a randomised, 52-week, phase III, double-blind, parallel-treatment trial. *Lancet.* **373**; 473-81.

Gardiner SM, March JE, Kemp PA, Bennett T (2008) Autonomic nervous system dependent and independent effects of exendin-4 infusion in conscious rats. *British Journal of Pharmacology* **154**; 60-70

Gerich JE (2010). Role of the kidney in normal glucose homeostasis and in the hyperglycaemia of diabetes mellitus: therapeutic implications. *Diabetic Med.* Feb; **27** (2): 136-42

Gill A, Hoogwerf BJ, Burger J, Bruce S, MacConell L, Yan P, et al (2010). Effect of exenatide on heart rate and blood pressure in subjects with type 2 diabetes mellitus: a double-blind, placebo-controlled, randomized pilot study. *Cardiovasc Diabetol.* **9**:6

Goke, R. and J. M. (1988) Conlon Receptors for glucagon-like peptide-1(7-36) amide on rat insulinoma-derived cells. *J Endocrinol*. **116**(3); 357-362.

Goldstone AP, Mercer JG, Gunn I, Moar KM, Edwards CMB, Rossi M, Howard JK, Rasheed S, Turton MD, Small C, Heath MM, O'Shea D, Steere J, Meeran K, Ghatei MA, Hoggard N (1997), Bloom SR: Leptin interacts with glucagon-like peptide-1 neurons to reduce food intake and body weight in rodents. *FEBS Lett* **415**:134–138

Goldstone AP, Morgan I, Mercer JG, Morgan DG, Moar KM, Ghatei MA, Bloom SR (2000). Effect of leptin on hypothalamic GLP-1 peptide and brain-stem preproglucagon mRNA. *Biochem Biophys Res Commun* **269**:331–335.

Gomez E, Pritchard C, Herbert TP (2002) cAMP-dependentprotein kinase and Ca^2 influx through L-type voltage-gated calcium channels mediate Raf-independent activation of extracellular regulated kinase in response to glucagon-like peptide-1 in pancreatic β-cells. *J Biol Chem* **277**:48146–48151.

Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL, (1995) Insulin receptor phosphorylation, Insulin receptor substrate-1 phosphorylation, and phosphatidylinositol-3 kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J. Clin. Invest.* **95**; 2195-2204

Goodyear, L. J., Kahn, B. B. (1998), Exercise, glucose transport and insulin sensitivity. *Annual Review of Medicine* **49**; 235-61

Graziano MP, Hey PL, Borkowski D, Chicci GC, and Strader CD (1993) Cloning and functional expression of a human glucagon-like peptide-1 receptor. *Biochem. Biophys. Res. Commun.* **196**; 141–146

Greischel A, Binder R, Baierl J (2010). The Dipeptidyl Peptidase-4 Inhibitor Linagliptin Exhibits Time- and Dose-Dependent Localization in Kidney, Liver, and Intestine after Intravenous Dosing: Results from High Resolution Autoradiography in Rats. *Drug Met. Disp.* **38** (9): 1443-48

Gribble FM, Williams L, Simpson AK, Reimann F (2003) A novel glucose-sensing mechanism contributing to glucagon-like peptide-1 secretion from the GLUTag cell line. *Diabetes* **52**:1147–1154

Griffioen K J, Wan R, Okun E, et al. (2010). GLP-1 receptor stimulation depresses heart rate variability and inhibits neurotransmission to cardiac vagal neurons. *Cardiovasc. Res.* Aug **14**; 1-7

Griffiths, P.L. & M.E. Bentley. (2001). The nutrition transition is underway in India.*The Journal of Nutrition* **131**; 2692-2700.

Gustavson SM, Chen D, Somayaji V, Hudson K, Baltrukonis DJ, Singh J, et al (2011). Effects of a long-acting GLP-1 mimetic (PF-04603629) on pulse rate and diastolic blood pressure in patients with type 2 diabetes mellitus. *Diabetes, Obesity and Metabolism* **13**: 1056–1058.

Gutzwiller JP, Hruz P, Huber AR, Hamel C, Zehnder C, Drewe J, Gutmann H, Stanga Z, Vogel D, Beglinger C. (2006) Glucagon-like peptide-1 is involved in sodium and water homeostasis in humans. *Digestion* **73**(2-3):142-50.

Gutzwiller JP, Tschopp S, Bock A, Zehnder CE, Huber AR, Kreyenbuehl M, Gutmann H, Drewe J, Henzen C, Goeke B, Beglinger C. (2004) Glucagon-

like peptide 1 induces natriuresis in healthy subjects and in insulin-resistant obese men. *J Clin Endocrinol Metab* **89**: 3055–3061.

Nelson- Hansson JH Williams C, Suzuki H, Schild L, Shimkets R, Lu Y,Canessa C, Iwasaki T, Rossier B & Lifton RP (1995) Hypertension caused by a truncated epithelial sodium channel gamma subunit: genetic heterogeneity of Liddle syndrome. *Nature Genetics* **11** 76–82.

Hamm, LL, Zhuang Feng Z, Hering-Smith, MS KS (2010). Regulation of sodium transport by ENaC in the kidney. *Curr. Opin. Nephrol. Hypertens.* **19** (1): 98-105.

Harrington EA. Fanidi A, Evan GI (1994). Oncogenes and cell death. *Curr. Opin. Gen. Dev.* **4**: 120-9.

Harris M, Firsov D, Vuagniaux G, Jackson Stutts M, Rossier BC (2007). A Novel eutrophil Elastase Inhibitor Prevents Elastase Activation and Surface Cleavage of the Epithelial Sodium Channel Expressed in *Xenopus laevis* Oocytes. *J. Biol. Chem.* **282**: (1): 58–64

Harris M, Garcia-Caballero A, Stutts MJ, Firsov D, Rossier BC (2008). Preferential assembly of epithelial sodium channel (ENaC) subunits in Xenopus oocytes: role of furin-mediated endogenous proteolysis. *Biol.Chem* **283**:7455–7463.

Harris RBS, Mitchell TD, Yan X, Simpson JS, And Redmann SM (2001). Metabolic responses to leptin in obese *db*/*db* mice are strain dependent. *Am J Physiol Regulatory Integrative Comp Physiol.* **281**: R115–R132.

Hayes MR (2012). Neronal and intracellular signalling pathways mediating Glucagon-Like Peptide-1 energy balance and glycaemic effects. *Physiol. Behaviour.* **106**: 413-6.

Heine RJ, Van Gaal LF, Johns D, Mihm MJ, Widel MH, Brodows RG. Exenatide versus insulin glargine in patients with suboptimally controlled type 2 diabetes (2005).Effect of AC2993 (synthetic Exendin-4) compared with insulin glargine in patients with type 2 diabetes also using combination therapy with sulfonylurea and metformin. *Annuals of Internal Medicine.* **143**: 559-569

Higgins JPT, Green S (editors) Cochrane handbook for systematic reviews of interventions version 5.1.0 (updated, March 2011). The Cochrane Collaboration 2011. Available from www.cochrane-handbook.org (accessed 18th April 2012)

Hills CE, Bland R, Bennett J, Ronco PM & Squires PE (2006) High glucose up-regulates ENaC and SGK1 expression in HCD-cells. *Cellular Physiol. Biochem*. **18** 337–346.

Hills CE, Squires PE, Bland R (2008). Serum and glucocorticoid regulated kinase and disturbed renal sodium transport in diabetes. *J Endocrinol.* Dec. **199**(3):343-9.

Hirata K, Kume S, Araki S, Sakaguchi M, Chin-Kanasaki M, Isshiki K, Sugimoto T, Nishiyama A, Koya D, Haneda M, Kashiwagi A, Uzu T (2009) Exendin-4 has an anti-hypertensive effect in salt-sensitive mice model.*Biochem Biophys Res Commun*. Feb 27 **380**(1); 44-9.

Holst JJ, Gromada J. (2004) Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. *Am. J. Physiol. Endocrinol. Metab.* **287**(2); E199-206.

Horita S, Seki G, Yamada H, Suzuki M, Koike K, Fujita T (2011). Insulin Resistance, obesity, Hypertension, and Renal Sodium Transport. *Intern. J Hyperten*; 2011: 1-8.

Hozawa A, Ohkubo T, Kikuya M, Ugajin T, Yamaguchi J, Asayama K, et al. (2004) Prognostic value of home heart rate for cardiovascular mortality in the general population: the Ohasama study. *Am J Hypertens.* **17**;1005–10

Hughey RP, Mueller GM, Bruns JB, Kinlough CL, Poland PA, Harkleroad KL, Carattino MD, Kleyman TR (2003). Maturation of the epithelial Na+ channel involves proteolytic processing of the alpha and gamma-subunits. *J.Biol.Chem*. **278**:37073–37082.

Huml M, Kobr J, Siala K, Vararovska J, Pomahacova R, Karlikova M, Sykora J (2011). Gut peptide hormones and pediatric type 1 diabetes mellitus. *Physiology Research.* **60**; 647-58

Hummel KP, Coleman DL, and Lane PW (1972). The influence of genetic background on expression of mutations at the diabetes locus in the mouse. I C57BL-KsJ and C57BL-6J strains*. Biochem. Genetics* **7**: 1–13.

Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A, Boucher R & Rossier BC (1996) Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nature Genetics* **12**: 325–328.

Ji HL, Su XF, Kedar S, Li J, Barbry P, Smith PR, Matalon S & Benos DJ (2006). Delta-subunit confers novel biophysical features to αβγ- human epithelial sodium channel (ENaC) via a physical interaction. *J. Biol. Chem.* **281**: 8233–8241.

Jouven, X., Empana, J.P., Schwartz, P.J., Desnos, M., Courbon, D., Ducimetiere, P. (2005) Heart-rate profile during exercise as a predictor of sudden death. *N. Engl.J. Med.* **352**: 1951–1958

Kadowaki T, Namba M, Yamamura A, Sowa H, Wolka AM, Brodows RG (2009). Exenatide exhibits dose-dependent effects on glycemic control over

12 weeks in Japanese patients with suboptimally controlled type 2 diabetes. *Endocr J*. **56**(3):415-424.

Kellenberger S & Schild L (2002) Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. *Physiol. Reviews* **82**: 735–767.

Kendall DM, Riddle MC, Rosenstock J, et al. (2005) Effects of exenatide (Exendin-4) on glycaemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulphonylurea. *Diabetes Care* **28**: 1083-91

Kim D, MacConell L, Zhuang D, Kothare PA, Trautmann M, Fineman M, et al (2007). Effects of once-weekly dosing of a long-acting release formulation of exenatide on glucose control and body weight in subjects with type 2 diabetes. *Diabetes Care* **30**(6):1487-93.

King H, Aubert RE, Herman WH (1998) Global burden of diabetes, 1995-2025: Prevalence and numerical projections. *Diabetes Care* **21**: 1414-1431

Klonoff DC, Buse JB, Nielsen LL, Guan X, Bowlus CL, Holcombe JH, (2008). Exenatide effects on diabetes, obesity, cardiovascular risk factors and hepatic biomarkers in patients with type 2 diabetes treated for at least 3 years. *Curr Med Res Opin* **24** (1):275-86.

Körner M, Stöckli M, Waser B, Reubi JC (2007) GLP-1 receptor expression in human tumors and human normal tissues: potential for in vivo targeting. *J Nucl Med.* **48** (5):736-43

Kreymann B, Williams G, Ghatei MA, Bloom SR (1987) Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet* **2** (8571): 1300-4.

Krug AW, Claudia Schuster C, Gassner B, Freudinger R, Mildenberger S, Troppmair J, Gekle M (2002). Human Epidermal Growth Factor Receptor-1 Expression Renders Chinese Hamster Ovary Cells Sensitive to Alternative Aldosterone Signaling. *J. Biol. Chem.* **277** (48): 45892–45897

Kriz & Bankir (1988). The Renal Commission of the International Union of Physiological Sciences (IUPS): A standard nomenclature for structures of the kidney. *Kidn. International*, **33**: 1-7.

Kucher V, Boiko N, Pochynyuk O, Stockland JD (2011). Voltage-dependent gating undergoes loss of ENaC function in speudohyperaldosteronism type 1. *Biophys J.* April 20. **100** (8): 1930-9.

Kuhn M (2004) Molecular Physiology of natriuretic peptide signalling *Basic Res Cardiol* **99**:76-82

Laakso M, Malkki M, Kekäläinen P, Kuusisto J, Deeb SS, (1994) Insulin receptor substrate-1 variants in non-insulin dependent diabetes. *J. Clin. Invest.* **94**: 1141-6.

Lang F, Gorlach A, Vallon V (2009). Targeting SGK-1 in diabetes. *Exp. Opion. Ther. Targets* **13** (11): 1303-11.

Larsen PJ, Tang-Christensen M, Jessop DS (1997). Central administration of glucagon-like peptide-1 activates hypothalamic neuroendocrine neurons in the rat. *Endocrinology*.**138**:4445–55.

Larsson H, Holst JJ, Ahrén B (1997). Glucagon-like peptide-1 reduces hepatic glucose production indirectly through insulin and glucagon in humans. *Acta Physiol Scand* **160**: 413–422.

Leroith D (ed.) (1999), *Advances in Molecular and Cellular Endocrinology (volume 3),* JAI Press Inc: London. pp 104-108

Levine HJ . Rest heart rate and life expectancy. *J. Am. Coll. Cardiol*. 1997 Oct; **30** (4): 1104-6

Littlewood RD, Hancock DC, Danielian PS, Parker MG, Evan G (1995). A modified estrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Research.* **23** (10): 1686- 90.

Liu Y, Nakagawa Y, Wang Y, Li R, Li X, Ohzeki T, Friedman TC (2003). leptin activation of corticosterone production in hepatocytes may contribute to the reversal of obesity and hyperglycemia in leptin-deficient *ob*/*ob* mice. *Diabetes* **52**:1409–1416.

Liutkus J, Guzman JR, Norwood P, et al. (2010). A placebo- controlled trial of exenatide twice-daily added to thiazolidinediones alone or in combination with metformin. *Diabetes Obes. Metab*. **12**: 1058-1065.

MacDonald PE, El-kholy W, Riedel MJ (2002). The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* **51**(Suppl. 3):S434–S442.

Madsbad S, Kielgast U, Asmar M, Deacon CF, Torekov SS, Holst JJ. An overview of once-weekly glucagon-like peptide-1 receptor agonists - available efficacy and safety data and perspectives for the future. Diabetes, *Obesity & Metab*. **13**: 394-407.

Madsbad S, Schmitz O, Ranstam J, Jakobsen G, Matthews DR (2004) International Study Group.Improved glycemic control with no weight increase in patients with type 2 diabetes after once-daily treatment with the long-acting glucagon-like peptide 1 analog liraglutide (NN2211): a 12-week, double-blind, randomized, controlled trial. *Diabetes Care*. Jun **27**(6); 1335-42.

Mannucci E, Rotella CM (2008) Future perspectives on glucogon-like peptide-1, diabetes and cardiovascular risk. *Nutrit. Met. Cardio. Dis*. **18** (9): 639-45.

Mannucci,G (2000) Glucagon-like peptide (GLP)-1 and leptin concentrations in obese patients with Type 2 diabetes mellitus. *Diabetic Medicine* **17**(10); 713-719.

Marre M, Shaw J, Brandle M, et al. (2009) Liraglutide once-daily human GLP-1 analogue, added to a sulphonylurea over 26 weeks produces greater improvements in glycaemic and weight control compared with adding rosiglitazone or placebo in subjects with type 2 diabetes (Lead 1-SU). *Diabet. Med.* **26**; 268-278

Martin BC, Warram JH, Krolewski AS, Bergman RN, Soeldner JS, Kahn CR (1992). Role of glucose and insulin resistance in development of type II diabetes mellitus: results of a 25 year follow-up study. *Lancet* **340**: 925-929.

Marunaka Y (1997) Hormonal and osmotic regulation of NaCl transport in renal distal nephron epithelium. *Jpn J Physiol*. Dec **47**(6):499-511

McKeigue, P.M., Shah, B., Marmot, M.G. (1991). Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians. *Lancet* **337**; 382-86.

Meier JJ, Gallwitz B, Askenas M, Vollmer K, Deacon CF, Holst JJ, Schmidt WE, Nauck MA (2005) Secretion of incretin hormones and the insulinotropic effect of gastric inhibitory polypeptide in women with a history of gestational diabetes. *Diabetolgia* **48**; 1872-1881

Meyer C, Dostou JM, Gerich JE (1999). Role of human kidney in glucose counterregulation. Diabetes. May; **48** (5): 943-8

Meyer C, Dostou JM, Welle SL, Gerich JE (2002). Role of human liver, kidney and skeletal muscle in postprandial glucose homeostasis. *Am. J. Physiol. Endocrinol. Metab*. **282**: E419-E427.

MHRA (2011). Safety warnings and messages for medicines. www.mhra.gov.uk/safetyinformation/ (October, 2011)

Mircea Cheta, D. et al (1999), *Preventing Diabetes- Theory, Practice and New Approaches,* John Wiley and Sons LTD: England. pp 102-125

Misra, A., J.S. Wasir & N.K. Vikram. (2005). Waist circumference criteria for the diagnosis of abdominal obesity are not applicable uniformly to all populations and ethnic groups. *Nutrition (Burbank, Los Angeles County, Calif.)* **21**; 969-976.

Mojsov S, Weir GC, Habener JF (1987) Insulinotropin: glucagon-like peptide I (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas.*J Clin Invest*. Feb **79**(2); 616-9

Montrose-Rafizadeh C, Avdonin P, Garant MJ, Rodgers BD, Kole S, Yang H (1999), Levine MA, Schwindinger W, Bernier M.Pancreatic glucagon-like peptide-1 receptor couples to multiple G proteins and activates mitogen-activated protein kinase pathways in Chinese hamster ovary cells. *Endocrinology.* Mar;**140**(3):1132-40

Moreno, C., M. Mistry, et al. (2002) Renal effects of glucagon-like peptide in rats. *Eur. J. Pharmacol.* **434**(3); 163-167.

Moretto TJ, Milton DR, Ridge TD, MacConell LA, Okerson T, Wolka AM, Brodows RG (2008) Efficacy and tolerability of exenatide monotherapy over 24 weeks in antidiabetic drug-naive patients with type 2 diabetes: A randomized, double blind, placebo-controlled parallel-group study. *Clinical therapeutics* **30** (8); 1448-1460

Muscelli E, Mari A, Casolaro A, Camastra S, Seghieri G, Gastaldelli A, Holst JJ, Ferrannini E (2008) Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients. *Diabetes* May;**57**(5):1340-8.

Nakagami, T., Q. Qiao, B. Carstensen, C. Nhr-Hansen, G. Hu & J. Tuomilehto, et al. (2003). Age, body mass index and Type 2 diabetes-associations modified by ethnicity. *Diabetologia* **46**; 1063-1070.

Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, et al. (2009) Medical management of hyperglycemia in type 2 diabetes: A consensus algorithm for the initiation and adjustment of therapy. *Diabetes Care* **32**(1):193–203.

National Institute for Health and Clinical Excellence. Technology Appraisal TA248. Exenatide prolonged-release suspension for injection in combination with oral antidiabetic therapy for the treatment of type 2 diabetes. London, NICE, 2012.

Nauck M A, Duran S, Kim D, Johns D, Northrup J, Festa A, et al (2007). A comparison of twice-daily exenatide and biphasic insulin aspart in patients with type 2 diabetes who were suboptimally controlled with sulfonylurea and metformin: a non-inferiority study. *Diabetologia* **50**(2):259-267.

Nauck M, Frid A, Hermansen K, et al. (2009). Efficacy and safety comparison of Liraglutide, Glimepiride and placebo, all in combination with metformin in type 2 diabetes. *Diabetes Care* **32**: 84-90.

Nelson P, Poon T, Guan X, Schnabel C, Wintle M, Fineman M (2007) The incretin mimetic Exenatide as a monotherapy in patients with Type 2 Diabetes: An Open-Label Study to Examine the Long-Term Effect on

Glucose Control (HbA1c) and Safety and Neves VR, Kiviniemi AM, Hautala AJ, Karjalainen J, Piira O-P, Catai AM, Makikallio TH, Huikuri HV, Tulppo MP (2011) Heart rate dynamics in cardiac patients with and without type 2 diabetes. *Front. Physiol.* **2** (57): 1-7

Nishizawa M, Nakabayashi H, Kawai K, Ito T, Kawakami S, Nakagawa A *et al.* (2000). The hepatic vagal reception of intraportal GLP-1 is via receptor different from the pancreatic GLP-1 receptor. *J Auton Nerv Syst* **80**: 14–21.

Nolan CJ, Damm P, Prantki M (2011). Type 2 diabetes across generations: from pathophysiology to prevention and management. *Lancet.* **378**: 169-81

O'Rahilly, Wareham NT (2003) Association between physical activity and blood pressure modified by variants on G-protein coupled receptor-10. *Hypertension* **43**: 224-28.

Ojima A, Ishibashi Y, Matsui T, Maeda S, Nishino Y, Takeuchi M, Fukamiz K, Yamagishi S (2013) Glucagon-Like Peptide-1 Receptor Agonist Inhibits Asymmetric Dimethylarginine Generation in the Kidney of Streptozotocin-Induced Diabetic Rats by Blocking Advanced Glycation End Product - Induced Protein Arginine Methyltranferase-1 Expression. *Am. J. Pathology*. **182** (1): 132-141

Orskov C, Holst JJ, Poulsen SS and Kirkegaard P (1987) Pancreatic and intestinal processing of proglucagon in man. *Diabetologia* **30**:874-881

Park CW, Kim HW, Ko SH, Lim JH, Ryu GR, Chung HW, Han SW, Shin SJ, Bang BK, Breyer MD, Chang YS (2007). Long term treatment of glucagon-like peptide- 1 analog exendin-4 ameliorates iabeic nephropathy through improving metabolic anomalies in db/db mice. *J. Am. Soc. Nephrol*. **18** (4): 1227-38

Pascal SM, Guiot Y, Pelengaris S, Khan M, Jonas JC (2008). Effects of c-MYC activation on glucose stimulus-secretion coupling events in mouse pancreatic islets. *Am J Physiol Endocrinol Metab.* Jul; **295**(1):E92-102

Perley MJ & Kipnis DM. (1967) Plasma Insulin Responses to Oral and Intravenous Glucose: Studies in Normal and Diabetic SubjectsThe *Journal of Clinical Investigation* **46** (12); 1954-62

Peti-Peterdi J, Warnock DG, Darwin Bell P (2002) Angiotensin II Directly Stimulates ENaC Activity in the Cortical Collecting Duct via AT1 Receptors. *J Am Soc Nephrol* **13**: 1131–1135.

Portha B, Tourrel-Cuzin C, Movassat M (2011). Activation of the GLP-1 Receptor Signalling Pathway: A Relevant Strategy to Repair a Deficient Beta-Cell Mass. **2011**: 1-11

Pratley R E, Nauck M, Bailey T et al. (2010). Liraglutide vs sitagliptin for patients with type 2 diabetes who did not have adequate glycemic control

with metformin: a 26 week, randomised, parallel group, open- label trial. *Lancet* **375**; 1447-56.

Pratley R, Nauck M, Bailey T, Montanya E, Cuddihy R, Filetti S, et al (2011). One year of liraglutide treatment offers sustained and more effective glycaemic control and weight reduction compared with sitagliptin, both in combination with metformin, in patients with type 2 diabetes: a randomised, parallel-group, open-label trial. *Int J Clin Pract* **65**(4):397-407.

Prie D, Friedlander G, Coreau C, Vanderwalle A, Cassingena R, Ronco PM (1995) Role of adenosine on glucagon-induced cAMP in a human cortical collecting duct cell line. *Kid. Int.* **47**: 1310-18

Ramachandran A (2005) Epidemiology of diabetes in India- Three decades of research. *JAPI* **53**; 34-38

Ramachandran, A., C. Snehalatha, A. Kapur, V. Vijay, V. Mohan & A.K. Das, et al. (2001). High prevalence of diabetes and impaired glucose tolerance in India: National Urban Diabetes Survey. *Diabetologia* **44**; 1094-1101.

Ramachandran, A., Snehalatha, C, Vijay V, King H (2002) Impact of poverty on the prevalence of diabetes and its complications in urban southern India. *Diabetic medicine.* **19**: 130-135

Ramachandran, A., Snehalatha, C., Baskar ADS, Mary S, Sathish Kumar, CK, Selvam S, Catherine S, Vijay V (2004). Temporal changes in prevalence of diabetes and impaired glucose tolerance associated with lifestyle transition occurring in the rural population in India. *Diabetolgia*. **47**; 860-865

Ratner R, Han J, Nicewarner D, Yushmanova I, Hoogwerf BJ, Shen L (2011). Cardiovascular safety of exenatide BID: an integrated analysis from controlled clinical trials in participants with type 2 diabetes. *Cardiovasc Diabetol*. **10**:22-32.

Ratner RE, Maggs D, Nielsen LL, Stonefouse AH, Poon T, Zhang B, Bicsak TA, Brodows RG, Kim DD.(2007) Long-term effects of exenatide therapy over 82 weeks on glycemic control and weight in over-weight metformin-treated patients with type 2 diabetes mellitus. *Diabetes, Obesity and Metabolism.* **7**(8); 419-428

Reimann F, Gribble FM (2002) Glucose-sensing in glucagon-like peptide-1-secreting cells. *Diabetes* **51**:2757–2763

Richter G, Rüdiger Göke, Burkhard Göke and Rudolf Arnold (1990) Characterization of receptors for glucagon-like peptide-1(7–36) amide on rat lung membranes. *FEBS Letters* **267** (1); 78-80

Rung J, Cauchi S, Albrechtsen A, Shen L, Rocheleau G, Cavalcanti-Proença C (2009) Genetic variant near IRS1 is associated with type 2 diabetes, insulin resistance and hyperinsulinemia. *Nat Genet*. **41**:1110-1115.

Russell-Jones D, Vaag A, Schmitz O, et al. (2009). Liraglutide vs insulin glargine and placebo in combination with metformin and sulfonylurea therapy in type 2 diabetes mellitus (LEAD-5 met+SU): a randomised controlled trial. *Diabetologia* **377**;192.

Ryder REJ, Thong KY, Cull ML, Mills AP, Walton C, Winocour PH; on behalf of the ABCD nationwide exenatide audit contributors. The Association of British Clinical Diabetologists (ABCD) nationwide exenatide audit. *Practical Diabetes Int* 2010; **27**(8): 352–357.

Saad MJA, Araki, Miralpeix M, Rothenberg PL, White ME, Kahn CR (1992). Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. *J. Clin. Invest.* **90**; 1839-49

Sanz C, Vázquez P, Navas MA, Alvarez E, Blázquez E (2008). Leptin but not neuropeptide Y up-regulated glucagon-like peptide 1 receptor expression in GT1-7 cells and rat hypothalamic slices. *Metab. Clin. Exper.* **57**: 40–48.

Schlatter P, Beglinger C, Drewe J, Gutmann H (2007) Glucagon-like peptide 1 receptor expression in primary porcine proximal tubular cells. *Reg Peptides.* Jun 7 **141**(1-3):120-8

Schmidt WE, Siegel EG, Creutzfeldt W (1985). Glucagon-like peptide-1 but not glucagon-like peptide-2 stimulates insulin release from isolated rat pancreatic islets. *Diabetologia*. **28**:704–707.

Scrocchi LA , Hill ME, Saleh J, Perkins B, Drucker DJ (2000). Elimination of Glucagon-Like Peptide 1R signalling does not modify weight gain and islet adaptation in mice with combined disruption of leptin and GLP-1 action. *Diabetes* **49**:1552–1560

Sharma K, McCue P, Dunn SR (2003) Diabetic kidney disease in the *db*/*db* mouse. *Am J Physiol Renal Physiol* **284**: F1138–F1144

Shibuya, K. Kanasaki, M. Isono, H. Sato, M. Omata, T. Sugimoto, S. Araki, K. Isshiki, A. Kashiwagi, M. Haneda, Koya D (2005), N-acetyl-seryl-aspartyl-lysyl-proline prevents renal insufficiency and mesangial matrix expansion in diabetic *db/db* mice, *Diabetes* **54**: 838–845.

Shigeto M, Katsura M, Matsuda M, Ohkuma S, Kaku K (2008) Low, but physiological, concentration of GLP-1 stimulates insulin secretion independent of the cAMP-dependent protein kinase pathway. *J Pharmacol Sci*. Nov **108**(3); 274-9

Simonsen L, Holst JJ, Deacon CF. (2006) Exendin-4, but not glucagon-like peptide-1, is cleared exclusively by glomerular filtration in anaesthetised pigs. *Diabetologia.* Apr **49**(4):706-12

Snehalatha, C., V. Viswanathan & A. Ramachandran. (2003). Cutoff Values for Normal Anthropometric Variables in Asian Indian Adults. *Diabetes Care* **26**; 1380-1384.

Snyder PM (2000) Liddles syndrome mutations disrupt cAMP-mediated translocation of the epithelial sodium channel to the cell surface. *J. Clin. Invest.* **105:** 45-53.

Staessen JA, Wang JG. Thijs L. (2001). Cardiovascular protection and blood pressure reduction: a meta-analysis. *Lancet* **358**; 1305-15.

Stumvoll M, Meyer C, Kreider M, Periello G, Gerich J (1998). Effects of glucagon on renal and hepatic glutamine gluconeogenesis in normal postabsorptive humans. *Metabolism* **47**: 1227-1232.

Stumvoll M, Meyer C, Nadkarni V, Gerich JE (1997). Renal glucose production and utilization. *Diabetolgia* **40**: 749-57

Sun P, Yu P, Wang WH (2011). Angiotensin II stimulates epithelial sodium channels in the cortical collecting duct of the rat kidney. *Am. J. Physiol. Renal Physiol*. F679-87

Syme CA, Zhang L, Bisello A (2006) Caveolin-1 regulates cellular trafficking and function of the glucagon-like Peptide 1 receptor. *Mol Endocrinol.* Dec **20**(12); 3400-11.

Tang-Christensen, Vrang N, Larson PJ (2001) Glucagon like-peptide containing pathways in the regulation of feeding behaviour. *International Journal of Obesity.* **25** (5); 542-547

Tanizawa, Y, Riggs, A. C, Elbein, S. C, Whelan, A, Donis-Keller, H (1994) Permutt, M. A. Human glucagon-like peptide-l receptor sequence repeat polymorphisms in genetic analysis. *Diabetes* **43**:752- 757.

Thomas W, McEneaney V, Harvey BJ (2007). Rapid responses to steroid hormones in the kidney. *Nephron Physiol.* **107**(1):1–9.

Thomas W, McEneaney V, Harvey BT (2008) Aldosterone-induced signalling and cation transport in the distal nephron. *Steroids* **73:** 979-84.

Thomsen K, Holstein-Rathlou, NH, Leyssac, PP, (1981). Comparison of three measures of proximal tubular reabsorption: lithium clearance, occlusion time, and micropuncture. *Am. J. Physiol*. **241**: F348– F355

Thorens B (1992) Expression cloning of a pancreatic β-cell receptor for the gluco-incretin hormone, glucagon-like peptide-1. In: (5th ed.),*Proc. Natl. Acad. Sci. USA* **89**; 8641–8645.

Thorens B, Porret A, Bühler L, Deng SP, Morel P, Widmann C (1993) Cloning and functional expression of the human islet GLP-1 receptor.

Demonstration that exendin-4 is an agonist and exendin-(9-39) an antagonist of the receptor. *Diabetes* Nov **42**(11); 1678-82.

Ura S, Araki E, Kishikawa H, Todaka M, Isami S, Shimoda S, Yoshimura R, Matsuda K, Motoyoshi S, Myamura N, Kahn CR, Shichiri M, (1996). Molecular scanning of the IRS-1 gene in Japanese patients with non-insulin dependent diabetes mellitus: identification of five novel mutations in IRS-1 gene. *Diabetologia* **39**; 600-608.

Uttenthal LO, Blázquez E (1990) Characterization of high-affinity receptors for truncated glucagon-like peptide-1 in rat gastric glands. *FEBS Lett.* Mar 12 **262**(1); 139-41.

Vaag AA, Holst JJ, Vølund A, Beck-Nielsen HB (1996). Gut incretin hormones in identical twins discordant for non-insulin-dependent diabetes mellitus (NIDDM)–evidence for decreased glucagon-like peptide 1 secretion during oral glucose ingestion in NIDDM twins. *Eur J Endocrinol*. **135**:425–432.

Van Eyll B, Göke B, Wilmen A, Göke R (1996) Exchange of W39 by A within the N-terminal extracellular domain of the GLP-1 receptor results in a loss of receptor function. *Peptides* **17**(4); 565-70

Van Eyll B, Lankat-Buttgereit B, Bode HP, Göke R, and Göke B (1994) Signal transduction of the GLP-1 receptor cloned from a human insulinoma. *FEBS Lett.* **348**; 7–13.

Vanis L, Gentilcore D, Raynor CK, et al. (2011) Effects on small intestinal glucose load on blood pressure, splanchnic blood flow, glycemia and GLP-1 release in healthy older subjects. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* Jun **300** (6); R1524-31.

Varanasi A, Chaudhuri A, Dhindsa S, Arora A, Lohano T, Vora M, Dandona P, (2011) Durability of effects of Exenatide on glycaemic control, body weight, systolic blood pressure, CRP and triglyceride concentrations. *Endocrin. Prac.* **17** (2); 192-200

Vilsboll T, Christensen M, Junker AE, Knop FK, Gluud LL (2012). Effects of glucagon-like peptide receptor agonists on weight loss: systematic review and meta-analyses of randomized controlled trials. *BMJ* **344**:1-11.

Vilsboll T, Karup J, Madsbad SS, Volund A, Juul AG, Holst JJ (2003) Incretin secretion in relation to meal size in healthy subjects and people with type 1 and 2 diabetes mellitus. *J. Clin. Endocrin. Metab*. **88** (6); 2706-2713

Vilsboll T, Karup J, Madsbad SS, Volund A, Juul AG, Holst JJ (2002). Incretin secretion in relation to meal size in healthy subjects and people with type 1 and 2 diabetes mellitus. *J. Clin. Endocrin. Metab*. **88** (6):2706-2713

Vilsboll T, Krarup T, Deacon CF, Madsbad S, Holst JJ (2001) Reduced post prandial concentrations of intact biologically active glucagon like-peptide 1 in type 2 diabetic patients. *Diabetes* **50**; 609-613

Waknine Y. FDA requests more data for once-weekly exenatide. *Medscape Medical News,* 2010-10-29

Wang, T. J., M. G (2004). Larson. Impact of Obesity on Plasma Natriuretic Peptide Levels. *Circulation* **109**(5): 594-600.

Wei, Y. and S. Mojsov (1995) Tissue-specific expression of the human receptor for glucagon-like peptide-I: brain, heart and pancreatic forms have the same deduced amino acid sequences.*FEBS Letters* **358**(3); 219-224.

Weinman EJ, Steplock D, Shenolikar S (2001) Acute regulation of NHE3 by protein kinase A requires a multiprotein signal complex. *Kidney Int.* Aug **60** (2); 450-4.

Wild S, Roglic G, Green A, Sicree R, King H (2004) Global Prevalence of Diabetes: Estimates for the Year 2000 and Projections for 2030. *Diabetes care* **27**: 1047-1053

Williams RW, Gu J, Qi S, Lu L (2001). The genetic structure of recombinant inbred mice: high-resolution consensus maps for complex trait analysis. *Genome Biology* **2**(11): 1-18.

Willms B, Werner J, Holst JJ, Orskov C, Creutzfeldt W, Nauck MA (1996). Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients. *J Clin Endocrinol Metab* **81**(1):327-332.

World Health Organization (2006) Definition, diagnosis and classification of diabetes mellitus and its complications: Report of a WHO Consultation. Part 1. Diagnosis 285 and classification of diabetes mellitus". http://www.who.int/diabetes/publications/en/.

Wulffele´ MG, Kooy A, De Zeeuw D, Stehouwer CDA, Gansevoort RT. (2004) The effect of metformin on blood pressure, plasma cholesterol and triglycerides in type 2 diabetes mellitus: a systematic review. *J Intern. Med.* **256**: 1-14

Wysham C, Bergenstal R, Malloy J, Yan P, Walsh B, Malone J, et al (2011). DURATION-2: efficacy and safety of switching from maximum daily sitagliptin or pioglitazone to once-weekly exenatide. *Diabet Med* **28**(6):705-714.

Xu G, Kaneto H, Laybutt DR, Duvivier-Kali VF, Trivedi N, Suzuma K, King GL, Weir GC, Bonner-Weir S (2007).Downregulation of GLP-1 and GIP receptor expression by hyperglycemia: possible contribution to impaired incretin effects in diabetes. *Diabetes* Jun;**56**(6):1551-8

Yabe D, Seino Y (2011). Two incretin hormones GLP-1 and GIP: comparison of their actions in insulin secretion and β-cell preservation. *Prog. Biophys. Mol. Biol.* Jul **28**: 248-56

Yamamoto H, Lee CE, Marcus JN, Williams TD, Overton JM, Lopez ME, Hollenberg AN, Baggio L, Saper CB, Drucker DJ (2002) Elmquist JK.Glucagon-like peptide-1 receptor stimulation increases blood pressure and heart rate and activates autonomic regulatory neurons. *J Clin Invest*. Jul **110**(1); 43-52

Yang W, Chen L, Ji Q, Liu X, Ma J, Tandon N, et al (2011). Liraglutide provides similar glycaemic control as glimepiride (both in combination with metformin) and reduces body weight and systolic blood pressure in Asian population with type 2 diabetes from China, South Korea and India: a 16-week, randomized, double-blind, active control trial. *Diabetes Obes. Metab.* **13**(1):81-88.

Yu M, Moreno C, Hoagland KM, Dahly A, Ditter K, Mistry M, Roman RJ (2003). Antihypertensive effect of glucagon-like peptide 1 in Dahl salt-sensitive rats. *J Hypertens*. Jun 21(6); 1125-35.

Zhang R, Jankord R, Flak JN, Solomon MB, D'Alessio DA, Herman JP (2010). Role of Glucocorticoids in Tuning Hindbrain Stress Integration. *J Neurosci.* **30**(44): 14907–14914.

Zhang R, Packard BA, Tauchi M, D'Alessio DA, Herman JP (2009). Glucocorticoid regulation of preproglucagon transcription and RNA stability during stress. *Proc Natl Acad Sci U S A*. **106**:5913–5918.

Zhang Y, Cook JTE, Hattersley AT (1994) Nonlinkage of the glucagon-like peptide 1 receptor gene with maturity onset diabetes of the young. *Diabetologia* **37**: 721–724.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**: 425-432

Zinman B, Gerich J, Buse J, et al. (2009). Efficacy and safety of the human Glucagon-like peptide-1 analog Liraglutide in combination with Metformin and Thiazolidinedione in Patients with type 2 Diabetes (LEAD-4 Met+TZD). *Diabetes Care* **32**(7): 1224-1230.

Zinman B, Hoogerworf B J, Garcia SD, et al (2007). The effect of adding exenatide to a thiazolidinedione in sub- optimally controlled type 2 diabetes. A randomized trial. *Ann. Intern. Med.* **146**: 477-485

Publications