Extrapancreatic actions of incretin-based therapies on bone in diabetes mellitus

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I confirm that the word count of this thesis is less than 100,000 words excluding the title page, contents acknowledgements, summary or abstract, abbreviations, footnotes, diagrams, maps, illustrations, tables, appendices, and references or bibliography
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SUMMARY

Diabetes mellitus is correlated with modifications in bone microarchitectural and mechanical strength, leading to increased bone fragility. The incretin hormones, with a classical effect to increase insulin secretion following food ingestion, are now postulated to have important direct effects on bone. As such, glucose-dependent insulinoitropic polypeptide (GIP) has dual actions on bone cells; enhancing bone-forming activity of osteoblasts and suppressing bone resorption by osteoclasts. The sister incretin of GIP, glucagon-like peptide-1 (GLP-1), is also suspected to directly influence bone health in a beneficial manner, although mechanism are less clear at present. The physiological actions of incretins are attenuated by dipeptidyl peptidase (DPP-4) activity and it is speculated that introduction of DPP-4 inhibitor may also positively affect quality of the skeleton. As such, this thesis evaluates the potential beneficial effects of a DPP-4 resistant GIP analogue, namely [D-Ala²]GIP, on osteoblastic-derived, SaOS-2 cells, and also preliminary in vivo studies on the impact of genetic deficiencies of GIPRs and GLP-1Rs on bone mineral density and content. Further studies characterised the beneficial effects of incretin-based therapies on metabolic control, bone microstructure and bone mechanical integrity in animal models of pharmacologically-, genetically- and environmentally-induced diabetes. GIP and related stable analogue increased bone-forming biomarkers in SaOS-2 cells and importantly, [D-Ala²]GIP was shown to be more potent than native GIP. Knockout mouse studies revealed that both GIPR and GLP-1R signaling are important for optimum bone mass. All diabetic mouse models displayed reduced bone mass, altered bone micromorphology and impairment of bone mechanical strength, similar to the human situation, confirming their appropriateness. The incretin-based therapeutics, [D-Ala²]GIP and Liraglutide, in streptozotocin-diabetic significantly increased bone matrix properties, indicating recovery of bone strength at the tissue level. The beneficial effects of administration of [D-Ala²]GIP-oxyntomodulin on bone health in db/db mice were more prominent as the Oxm analogue did not only improve bone strength at tissue level, but also at whole-bone level. These modifications were independent of metabolic status. Twice-daily Exendin-4 therapy improved glycaemic control and increased work required to resist bone fracture in high-fat fed mice. It was also established that Sitagliptin had neutral effects on bone microstructure and mechanical strength in high-fat mice. In
summary, these data demonstrate the negative impact of diabetes mellitus on normal skeleton development and bone quality. Moreover, this thesis highlights the growing potential of incretin-based therapies for ameliorating bone defects and improving the increased fragility fracture risk associated with diabetes.
## ABBREVIATIONS

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<th>Description</th>
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<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>AlkP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AMD</td>
<td>Absorbing mineral density</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>B.Dm</td>
<td>Bone diameter</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMDD</td>
<td>Bone mineral density distribution</td>
</tr>
<tr>
<td>BSE</td>
<td>Back-scattered electron</td>
</tr>
<tr>
<td>BV</td>
<td>Bone volume</td>
</tr>
<tr>
<td>bw</td>
<td>Body weight</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CSMI</td>
<td>Cross-sectional moment of inertia</td>
</tr>
<tr>
<td>Ct.Th</td>
<td>Cortical thickness</td>
</tr>
<tr>
<td>(db/db)</td>
<td>Diabetic (db/db) mice</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy x-ray absorptiometry</td>
</tr>
<tr>
<td>dl</td>
<td>Decilitre</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinoactive polypeptide</td>
</tr>
<tr>
<td>GIPR</td>
<td>Glucose-dependent insulinoactive polypeptide receptor</td>
</tr>
<tr>
<td>GL</td>
<td>Grey level</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
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<tr>
<td>GLP-1R</td>
<td>Glucagon-like peptide-1 receptor</td>
</tr>
<tr>
<td>GPa</td>
<td>Gigapascal</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>keV</td>
<td>Kilo electron volt</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out mice</td>
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</tbody>
</table>
kV Kilovolt
l Litre
Ma.Dm Marrow diameter
MALDI-TOF-MS Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
mg Miligram
microCT X-ray microcomputed tomography
min Minutes
ml Mililitre
mm Milimeter
mmol Milimolar
MMP Matrix metalloproteinase
mN Milinewton
MPa Megapascal
MSC Mesenchymal stem cell
N Newton
nm Nanometer
OPG Osteoprotegerin
Oxm Oxyntomodulin
pA Picoampere
pJ Picojoule
PMMA Polymethylmethacrylate
qBEI Quantitative back-scattered electron imaging
qXRI Quantitative x-ray imaging
RANKL Receptor activator on nuclear factor-Kappa B ligand
RIA Radioimmunoassay
ROI Region of interest
rpm Revolutions per minute
RT-PCR Reverse transcriptase-polymerase chain reaction
SEM Standard error of the mean
STZ Streptozotocin
Tb.N Trabecular number
Tb.Sp Trabecular separation
Tb.Th Trabecular thickness

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<table>
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<th>Abbreviation</th>
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<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TV</td>
<td>Trabecular volume</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>Z</td>
<td>Atomic number</td>
</tr>
<tr>
<td>µA</td>
<td>Microampere</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
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DECLARATION

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Chapter 1

General Introduction
1.0 OVERVIEW

Diabetes has become a global issue as it is estimated that more than 387 million people worldwide are affected by this modern disease (International Diabetes Federation 2014b). The alarming number of diabetes cases has listed this disorder as the potential seventh leading cause of death in 2030 (Mathers & Loncar 2006). Common complications that are associated with diabetes include retinopathy (blindness), neuropathy (nerve damage), cardiovascular disease, stroke and nephropathy (kidney failure) (Diabetes UK 2014) and most recently, diabetes is also related to bone loss. Extensive studies on the impact of diabetes on bone have linked the disease to severe impairment of bone mechanical integrity, reduced bone mineral density (BMD) in T1DM and unaffected or even increased BMD in T2DM. Regardless of the inconsistencies in BMD, both types of diabetic patients have increased risk of fractures (Janghorbani et al. 2007, Vestergaard 2007). Early diagnosis and treatment of diabetes is vital to prevent long term complications that could lead to bone fractures and mortality in elderly people. Furthermore, the link between diabetes and bone fragility suggests that if blood glucose management is improved or if signaling pathways contributing to diabetic bone pathologies are corrected, overall bone quality could be enhanced, improving the increased risk of fracture in diabetic patients.

1.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a metabolic disease where production of insulin is insufficient or body cannot effectively utilise insulin produced by pancreas. As a consequence, there will be high blood glucose levels in the body. Diagnosis of diabetes mellitus is made when fasting plasma glucose is ≥ 126 mg/dl (7.0 mmol/l) or plasma glucose level is ≥ 200 mg/dl (11.1 mmol/l) measured 2 hours after 75 g-oral glucose tolerance test (Zemmit et al. 2001). Generally, the disease is classified into 2 major forms; type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM).
1.1.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM), previously referred to as juvenile-onset or insulin-dependent diabetes, is characterised by inability of the body to synthesise insulin due to destruction of insulin-producing pancreatic beta cells. This results in a dependency on exogenous insulin to stabilise blood glucose concentrations following feeding. The pathogenesis of this autoimmune disorder has been extensively studied in rodent models and humans and several key target for autoantibodies and autoreactive cells have been identified; insulin, glutamic acid decarboxylase (GAD), protein tyrosine phosphatase insulinoma antigen (IA-2), zinc transporter 8 (ZnT8) and islet glucose-6-phosphatase catalytic subunit-related protein (IGRP) (Culina et al. 2013, Yoon & Jun 2005, Zhang et al. 2008). Pancreatic beta cells autoantigens together with lymphocytes and macrophages trigger overexpression of CD4$^+$ and beta cell-specific cytotoxic CD8$^+$ T cells (Pinkse et al. 2005) which eventually lead to the destruction of insulin-producing beta cells.

The primary cause of T1DM is autoimmune beta-cell destruction. However, there is also correlation between genetic and several environmental factors in the pathogenesis of T1DM. Genetic components that strongly influence T1DM are reported to be on major histocompatibility complex human leukocyte antigen (HLA) alleles, which are associated with ‘protection from’ or ‘susceptibility towards’ T1DM. More than 90% of T1DM patients have either DR3-DQ2 or DR4-DQ8 haplotypes, while the percentage of normal controls with these haplotypes is less than 40% (Atkinson & Eisenbarth 2001). These genes are known to be important regulators in immune response by introducing peptide antigens to T lymphocytes which stimulate the production of antibodies against beta-cell antigens. Further exposure to environmental triggers such as viruses in susceptible individuals can cause abnormal activation of immune system that will generate multiple autoantibodies to islet cells.

T1DM is most prevalent in children and young people and it accounts for about 10% of diabetes cases worldwide (International Diabetes Federation 2014a). Statistical studies conducted by EURODIAB (EUROpe and DIABytes) from 1989 to 2003 estimated that diabetes cases in children below 15 years old will rise by 70% in 2020.
A more recent study on trends over 20-year incidence projected that the prevalence of Type 1 diabetes has annual increase of 3.4% (Patterson et al. 2012).

1.1.2 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM), previously known as adult-onset or non-insulin-dependent diabetes, develops when beta cells do not make enough insulin or the insulin produced does not work appropriately. T2DM is a progressive disease and starts gradually with insulin insensitivity or impairment of the body’s response to insulin (Stumvoll et al. 2005). Compensatory mechanism in the body respond to insulin resistance by secreting more insulin to maintain normal glucose levels, but eventually pancreatic beta-cells can no longer produce sufficient insulin to maintain normal glucose levels. Over time, worsening insulin resistance and the decline in beta cell function will lead to hyperglycaemia and eventually progress to the diabetic state. The environmental factors that trigger T2DM are pregnancy, ageing and modern lifestyle (high calorie intake, obesity, physical inactivity) (Chen et al. 1988, Hu 2011, Stumvoll et al. 2005). Similar to T1DM, the combination of genetic predisposition and environmental factors contributes to the onset of insulin resistance and its progression to T2DM (Leahy 2005). The key genes associated with genetic predisposition of T2DM are \( TCF7L2,\ PPARG,\ KCNJ11,\ CAPN10,\ HHEXIIDE,\ MC4R,\ FTO\) and \( KCNQ1\) (Tahrani et al. 2011, Valeriya et al. 2007).

T2DM usually appears in middle-aged and older people and accounts for 90% of all diabetes cases (International Diabetes Federation 2014a). T2DM has become an epidemic as this disease progresses slowly and undetected many years before diagnosis is made. With increasing prevalence of obesity and T2DM, a huge burden is imposed on the public health-system (Diabetes UK 2012).
1.1.3 Existing treatment for diabetes

The only therapeutic option for T1DM patients is exogenous insulin replacement therapy. However, T1DM complications are not always prevented by exogenous insulin therapy (Atkinson et al. 2014). Other important gluoregulatory hormone which is co-secreted with insulin by pancreatic beta cells, amylin, is also reduced in T1DM, leading to generation of synthetic amylin analogue, Pramlintide. Amylin replacement with Pramlintide improves glycaemic control by delaying gastric emptying and suppressing postprandial glucagon secretion (Fineman et al. 2002, Nyholm et al. 1999). In a long term clinical study, adjunct therapy with Pramlintide has been shown to provide better glycaemic control in T1DM individuals compared to insulin replacement alone (Ratner et al. 2004). As such, this amylin-related analogue was clinically approved in April 2004 by the US Food and Drug Administration (FDA) to be used as add-on with insulin therapy in T1DM patients (Ryan et al. 2005). Another option for T1DM treatment is islet-cell transplantation, leaving the recipients dependent on lifelong immunosuppression drugs to prevent organ rejection. Most importantly, the limiting factor in this type of treatment is a short supply of organs and until new sources of functional islets are found, the future for organ transplantation in T1DM treatment remains sceptical (McCall & Shapiro 2012).

Obesity is normally associated with impaired glucose tolerance and increased risk of developing T2DM. This is due to increased adipocyte accumulation of non-esterified fatty acids, hormones, inflammatory cytokines and other molecules that are involved in the development of insulin resistance (Kahn et al. 2006). Essentially, insulin resistance is improved through intensive lifestyle intervention by monitoring nutrient intake and regular physical activity (Tahrani et al. 2011). However, lifestyle changes are difficult to maintain and not sufficient to keep blood glucose levels normal. Consequently, oral or injectable antidiabetic drugs are often required to manage T2DM (International Diabetes Federation 2014a). Currently available T2DM treatments are summarised in Table 1.1.
Table 1.1 Summary of available treatment for T2DM

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound(s)</th>
<th>Physiological action(s)</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biguanides</td>
<td>Metformin</td>
<td>Suppresses hepatic glucose production and increases insulin sensitivity in muscle</td>
<td>Gastrointestinal side effects such as diarrhoea, abdominal cramping, Risk of lactic acidosis, Vitamin B₁₂ deficiency</td>
</tr>
<tr>
<td>Sulfonylureas</td>
<td>Glibenclamide / glyburide</td>
<td>Binds to sulphonylurea receptors causes $K_{\text{ATP}}$ channels on beta-cell plasma membranes to close, influx of $\text{Ca}^{2+}$ and stimulation of insulin release</td>
<td>Hypoglycaemia, Weight gain</td>
</tr>
<tr>
<td>Meglitinides</td>
<td>Repaglinide, Nateglinide</td>
<td>Binds to sulphonylurea receptors, but at different binding sites to sulphonylurea causes $K_{\text{ATP}}$ channels on beta-cell plasma membranes to close, influx of $\text{Ca}^{2+}$ and stimulation of insulin release</td>
<td>Hypoglycaemia, Weight gain</td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>Pioglitazone, Rosiglitazone</td>
<td>Agonise and activate nuclear transcription factor peroxisome-proliferator-activated-receptor-$\gamma$ (PPAR-$\gamma$) in adipose tissue, increase adipogenesis, reduce release of fatty acids and increase insulin sensitivity in muscle and liver</td>
<td>Weight gain, Oedema / heart failure, Bone fractures, Rosiglitazone increases LDL-C</td>
</tr>
<tr>
<td>Dipeptidyl peptidase-4 (DPP-)</td>
<td>Sitagliptin, Vildagliptin</td>
<td>Inhibits DPP-4 action causes an increase in</td>
<td>Angio-oedema, Association with</td>
</tr>
</tbody>
</table>
Metformin, a biguanide, is the first-line oral therapy for T2DM treatment at present. Glycaemic control by this biguanide is achieved through improving hepatic and peripheral sensitivity to insulin, enhancing insulin-stimulated glucose uptake by skeletal muscle and suppressing hepatic gluconeogenesis (Krentz & Bailey 2005, Wiernsperger & Bailey 1999). Metformin remains the first choice as glucose levels are controlled without increasing body weight or increasing risk of hypoglycaemia in T2DM patients (Hermann et al. 1994). However, it still has side effects such as diarrhoea and risk of lactic acidosis (Inzucchi et al. 2012). Oral metformin does not have direct effect on stimulating pancreatic beta-cell insulin release and when metformin monotherapy is inadequate to maintain normal blood glucose levels, the drug is frequently combined with a sulfonylurea (Inzucchi 2002, Krentz & Bailey 2005).

Sulfonylureas, in contrast to metformin, act by directly stimulating insulin secretion from pancreatic islet beta cells. Sulfonylureas bind to the sulphonylurea receptor-1 (SUR-1), a drug-binding subunit of the ATP-sensitive potassium channel on beta cells, causing the potassium channels to close (Ashcroft 1996). The closure of
potassium channels initiates a series of events; plasma membrane depolarisation, voltage-dependent Ca\textsuperscript{2+} channels are opened, increase Ca\textsuperscript{2+} influx and insulin secreted by exocytosis. Normal glucose concentration for glucose-mediated insulin release is around 5 mmol/l, but in the presence of sulfonylureas, this occurs even at low glucose concentration. Therefore, there is a potential consequence of hypoglycaemia with these agents (Krentz & Bailey 2005). However, as T2DM progresses, which is reflected by beta-cell failure, there is an inadequacy of sulfonylureas to lower blood glucose concentrations. Early introduction of exogenous insulin to sulphonylurea-treated patients has been shown to significantly improve glycaemic control, without causing hypoglycaemia or promoting weight gain (Wright et al. 2002).

Meglinitides bind to non-sulfonylurea sites on SUR-1 and similar to sulfonylureas, their binding on SUR-1 closes the ATP-regulated potassium channels and activates cascades of events which lead to insulin secretion (Pfeiffer 2008). The meglitinides are structurally unrelated to sulfonylureas, where a member of meglinitides, repaglinide is derived from benzoic acid, whilst nateglinide is a phenylalanine derivative (Chachin et al. 2003, Fuhlendorff et al. 1998). Nateglinide has low binding affinity for the K\textsubscript{ATP} channels and rapidly dissociates, while the binding affinity of repaglinide is in between the high-affinity sulphonylureas (glibenclamide and glimepiride) and nateglinide (Hu et al. 2000), indicating shorter duration of actions of meglinitides compared to sulfonylureas. The side effects of these drugs include weight gain and hypoglycemia (Black et al. 2009).

Thiazolidinediones (TZDs) activate peroxisome proliferator-activated receptor \(\gamma\) (PPAR-\(\gamma\)), which closely associated with adipogenesis (Yki-Jarvinen 2004). Activation of PPAR-\(\gamma\) also increases the uptake and storage of free fatty acid (Bogacka et al. 2004). In addition, these insulin-sensitizing drugs regulate glucose levels by improving insulin sensitivity in skeletal muscle and reducing hepatic glucose production (Natali & Ferrannini 2006). A recent study has also shown that a member of the TZDs, Pioglitazone reduces the risk of developing T2DM by 72% in adult with impaired glucose tolerance (DeFronzo et al. 2011). Several adverse effects of TZDs include increased body weight, oedema and elevated risk of bone fractures (Kahn et al. 2006, Schwartz et al. 2006, Yki-Jarvinen 2004).
Sodium–glucose-cotransporter-2 (SGLT2) inhibitors target the activity of SGLT2 by reducing renal glucose reabsorption and increasing urinary glucose excretion, thus preventing increased plasma glucose levels (Chao & Henry 2010). Due to high expression of SGLT2 in kidney, its inhibition plays an important role in controlling reabsorption of glucose in diabetes as SGLT2, facilitated by glucose transporter type 2 (GLUT2), has high capacity (90%) to transport glucose back into blood circulation (Chao & Henry 2010). This insulin-independent activity of SGLT2 inhibitors is compensated by SGLT1, which reabsorbs glucose at slow rate and therefore, minimises the risk of hypoglycaemia (Tahrani et al. 2011). Oral highly-selective SGLT2 inhibitor, Dapagliflozin is associated with mild risk of genital and urinary tract infections (List et al. 2009, Wilding et al. 2009).

Newly-introduced classes of antihyperglycaemia drugs for T2DM are GLP-1R agonists and DPP-4 inhibitors (Nauck et al. 2009). Similar to native GLP-1, the binding of injectable GLP-1 mimetics to GLP-1R stimulates glucose-dependent pancreatic insulin release, suppresses glucagon secretion, delays gastric emptying and reduces appetite (Drucker et al. 2010). The main advantage of GLP-1-based drugs is they promote weight loss, but the stable analogues also have adverse effects such as nausea and vomiting (Lovshin & Drucker 2009). On the other hand, the oral dipeptidyl peptidase 4 (DPP-4) inhibitors stabilise circulating concentrations of endogenous GLP-1 and GIP from DPP-4 activity, thus prolonging bioactivity of the incretin hormones postprandially (Drucker & Nauck 2006). Although DPP-4 inhibitors mediate regulation of insulin and glucagon secretion through GLP-1, the suppression of DPP-4 activity has neutral effect on body weight (Lovshin & Drucker 2009). Additionally, the risk of hypoglycaemia is minimised with incretin-based therapies as their glucoregulatory activities only take place when there are abnormally high glucose concentrations in the body (Stonehouse et al. 2012).
1.2 BONE

Bone is a dynamic organ made up of highly organised structures and consists of three major components; fibrous protein collagen, calcium phosphate-based hydroxyapatite $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ and water (Weiner & Wagner 1998). The two vital roles of bone are to control mechanical function of the body and as storage of calcium and phosphate. Recently, bone has been reported to be involved in regulation of energy metabolism through osteocalcin and insulin interaction (Ducy 2011). Osteocalcin, a hormone secreted by osteoblasts, has been identified as a positive regulator of insulin secretion, insulin resistance and energy expenditure (Lee et al. 2007). Remarkably, insulin signalling in osteoblasts is a positive regulator of osteocalcin production and activation through its ability to indirectly stimulate osteoclastic bone resorption (Ferron et al. 2010). Hence, interaction of osteocalcin and insulin has become part of a complex signalling network between bone and pancreas, the organ that associated with glucose homeostasis.

Bone is continuously remodeled by sequential activity of osteoclasts and osteoblasts. Bone-resorbing cells, osteoclasts break down old bone, followed by formation of new bone by osteoblasts (Figure 1.1) and this coordinated process is controlled by various paracrine and autocrine factors (Matsuo & Irie 2008).
Bone remodeling is coordinated by the activity of osteoclasts and osteoblasts through bone resorption and formation, respectively. A damage (microcrack) is detected by lining cells and signals (local factors) are released to recruit cells from blood and marrow into remodeling compartment. Osteoclastogenesis occurs and the multinucleated osteoclasts resorb bone matrix and the microcrack. This is followed by osteoblastic bone formation, where resorbed lacunae are refilled to the original level by osteoblasts. Osteoblasts, bone-forming cells; osteoclasts, bone-resorbing cells; osteoid, the unmineralized organic portion of bone matrix secreted by osteoblasts; osteocytes, the descendant of the matrix-producing osteoblast, which will form mature bone. Adapted from (Seeman & Delmas 2006).

Throughout life, material and structural properties of bone are modulated to adapt to environmental and mechanical changes. Under disease conditions which alter molecular processes in bone, an inability to respond to these changes could lead to fragility and may initiate fracture. Early diagnosis or assessments of bone quality can prevent further bone loss and reduce the risk of bone fragility fracture. Bone quality and remodeling can be assessed at different scale (Table 1.2) by numerous methods such as radiography, histomorphometry and biomechanical test. Sections 1.2.1-1.2.4 and 2.5-2.6 explain in greater detail the techniques and tools that are employed for bone assessment in animal studies.
Table 1.2 Physical scale and characteristics of bone that may affect overall bone quality

<table>
<thead>
<tr>
<th>Scale (m)</th>
<th>Bone characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&gt;10^{-3}$</td>
<td>Whole bone morphology (size and shape)</td>
</tr>
<tr>
<td></td>
<td>Bone density spatial distribution</td>
</tr>
<tr>
<td>$10^{-6}-10^{-3}$</td>
<td>Microarchitecture</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
</tr>
<tr>
<td></td>
<td>Cortical shell thickness</td>
</tr>
<tr>
<td></td>
<td>Lacunar number/morphology</td>
</tr>
<tr>
<td></td>
<td>Remodeling cavity number, size, and distribution</td>
</tr>
<tr>
<td>$10^{-9}-10^{-6}$</td>
<td>Mineral and collagen</td>
</tr>
<tr>
<td></td>
<td>distribution/alignment</td>
</tr>
<tr>
<td></td>
<td>Microdamage type, amount, and distribution</td>
</tr>
<tr>
<td>$&lt;10^{-9}$</td>
<td>Collagen structure and cross-linking</td>
</tr>
<tr>
<td></td>
<td>Mineral type and crystal alignment</td>
</tr>
<tr>
<td></td>
<td>Collagen–mineral interfaces</td>
</tr>
</tbody>
</table>

Several important characteristics of bone that can influence whole bone mechanical properties and bone quality. Adapted from (Hernandez & Keaveny 2006)

1.2.1 Assessment of bone mass

Bone health is primarily determined by bone mass and routine practice to investigate this parameter involves a radiological technique. The most commonly used noninvasive tool to predict bone mass is dual-energy x-ray absorptiometry (DEXA). From DEXA scanning, bone mineral density (BMD), bone mineral content (BMC), fat mass and lean mass can be determined. BMC represents calcium content in bone which absorbs more x-ray radiation than soft tissue and protein, whilst BMD is a measure of average mineral content per unit area or volume of a particular bone segment (Cummings et al. 2002). Bone mineral content can also be assessed with x-
ray microradiograph imaging (qXRI) and quantitatively estimated by using ImageJ analysis (Bassett et al. 2012). Increased BMD is commonly related to low risk of fracture and vice versa. However, this is not always the case as in diabetes mellitus, T1DM and T2DM have contradictory effects on BMD but both incur an increased risk of fracture. Individuals with T1DM appeared to have reduced BMD, which is in agreement with increased skeletal fracture risk, whilst modest increased or unchanged BMD in T2DM patients is surprisingly unable to protect them from bone fragility fractures (Vestergaard 2007). The discrepancies between BMD and fracture risk indicate that BMD alone does not reflect a true measure of bone quality and other characteristics of bone, such as microarchitecture, biomechanical strength and material composition of bone matrix also play a significant role in overall bone integrity (Hernandez & Keaveny 2006, Seeman & Delmas 2006).

1.2.2 Bone microarchitecture

Microscopic architecture and organisation of bone can be explored histomorphometrically and the methods employed include bone histomorphometry or high-resolution tools such as microCT. In bone histomorphometry, embedding, sectioning and staining of bone needs to be carried out prior to analysis. Using different labeling or staining protocols that are specific for molecular activities in bone such as resorption, formation and mineralisation, researchers can specifically view particular bone cell activities (Erben & Glosmann 2012). For example, fluorochrome labeling with alizarin red S is used to determine calcium deposition for bone mineralisation, whereas sirius red demonstrates the presence of collagen (Orriss et al. 2012). In contrast to histomorphometrical techniques, development of microcomputed tomography (microCT) allows for a nondestructive method of bone evaluation. There are three major steps in examining bone by microCT. Firstly, scanning of full bone which will produce X-ray projection images; secondly, reconstruction of 3D images of bone from a stack of projection images; and finally, analysis of the bone (Van't Hof 2012). Importantly, measurement of bone volume and other trabecular variables such as connectivity and frequency of thickness can be obtained from microCT (Chappard et al. 2011).
1.2.3 Biomechanical test

In assessing alterations of bone quality that can lead to skeletal fragility, the main goal is to prevent clinical bone fracture, which principally is a mechanical event (Hernandez & Keaveny 2006). As such, evaluation of the two major contributors in biomechanical of bone, stiffness and strength are essential. Stiffness is defined by the ability of bone to resist deformation during loading, which is determined by elastic modulus, while strength is translated from bone hardness. Bone stiffness and hardness can be examined in rodents by numerous biomechanical techniques including three-point bending test and nanoindentation. The biomechanical concept of the three-point bending test in bone is the relationship between load applied at the midshaft of long bone and displacement of bone until failure, which produces a load-displacement curve similar to well-established stress-strain curves (Turner 2006). From the strain-stress curve for bone, different bone variables can be obtained such as ultimate strength and stiffness.

However, alterations of intrinsic material properties of bone can not be determined with three point bending. In order to provide better understanding of mechanical behaviour of bone, combination of three-point bending test and nanoindentation is employed. Nanoindentation was introduced in 1992 by Oliver and Pharr and it has been widely used to determine hardness and elastic modulus of materials based on an unloading curve produced during indentation (Oliver & Pharr 1992). One of the advantages of this technique is that it can measure mechanical properties of materials at smaller scales (below microns) and therefore, allows higher accuracy of material properties in matrix. Furthermore, the nanoindentation technique is independent of bone size and porosity, which can significantly influence bone hardness and stiffness (Rho et al 1997).

1.2.4 Bone material properties

As stated earlier, bone is mainly composed of collagen and minerals. Thus, any modification in matrix composition or characteristic that has negative impact on bone remodelling could contribute to bone fragility. Scanning electron microscope was first shown to be able to capture trabecular bone images in 1971 (Whitehouse et
al. 1971). Later, it was found that in backscattered electron mode, the density of mineralisation in bone was apparent and therefore could be used to determine bone mineral density distribution (BMDD) (Chappard et al. 2011). In this technique, backscattered electrons (BSEs) in the scanning electron microscope are reflected by atoms in the sample where high atomic-number atoms will have stronger interaction with the electrons and appear brighter in grey level image of the scanning electron microscope. The BSE signal is calibrated and standardised with carbon and aluminum as reference materials for grey level values, while hydroxyapatite is used for the measurement of calcium concentrations (Roschger et al. 1998). Alternatively, the characteristics of bone matrix can be investigated with Fourier transformed infrared (FTIR) microscopy. This technique allows examination of mineral to matrix ratio which reflects the degree of mineralisation of bone matrix, mineral maturity and crystallinity and finally collagen maturity, which is the relative ratio of pyridinium trivalent (mature collagen) to dehydrodihydroxylysinoonorleucine divalent (new collagen) (Paschalis 2012).

1.3 EFFECTS OF DIABETES ON THE SKELETON

Bone loss can be characterised by low bone mass and damage to microstructural properties of bone tissue, which leads to bone fragility and increased risk of fracture. As such, the link between diabetes and bone impairments, along with observations of bone loss in diabetic animals and fracture risk in diabetic patients, requires further consideration.

1.3.1 Hyperglycaemia and AGEs

Bone loss has long been identified as a complication of diabetes. However, pathophysiological aspects that lead to diabetic bone abnormalities are not well understood, as multiple factors are involved in this disorder. A common condition seen in T1DM and T2DM is elevation of blood glucose levels and therefore, hyperglycaemia is thought to contribute to the pathogenesis of bone disorder in diabetes. Hyperglycaemia will initiate formation of advanced glycation end-products (AGEs), where important bone-related proteins such as collagen type 1 are non-
enzymatically glycosylated, leading to alteration of their structure and bioactivity (Brownlee 2001, Saito & Marumo 2010).

AGEs also negatively affect bone remodeling by disrupting functions of osteoblasts and osteoclasts. AGE-mediated attenuation of bone formation through inhibition of proliferation and differentiation, is seen in osteoblastic-derived rat UMR 106 cells (McCarthy et al. 1997). Similarly, suppression of differentiation to osteoblasts by AGEs is shown in human mesenchymal stem cells (Kume et al. 2005). In addition, apoptosis of osteoblasts is significantly stimulated by the presence of AGEs (Alikhani et al. 2007). Furthermore, it is found that AGE-modified proteins enhance bone resorptive activity as seen by an increase in the number of resorption pits in osteoclastic-derived cells (Miyata et al. 1997), forming a positive correlation of osteoclastic bone resorption activities and concentration of AGEs (Dong et al. 2011). Correspondingly, these studies indicate that osteoclasts response on AGEs could lead to further bone loss and reduced bone mechanical competency.

1.3.2 Insulin

In diabetes, blood glucose is not the only metabolic parameter that is altered as insulin levels are also negatively affected. There is mounting evidence to support a positive effect of insulin on bone (Cornish et al. 1996, Fulzele & Clemens 2012, Thrailkill et al. 2005a). Consistently, severe impairments of bone quality have been seen in chemically- and genetically-induced animal models of T1DM mice (Fowlkes et al. 2008, Thrailkill et al. 2005b). On the other hand, T2DM is associated with insulin resistance, excessive weight gain and increased numbers and size of adipocytes. Interestingly, adipocytes and osteoblasts are derived from the same precursor, mesenchymal stem cells (MSCs) (Figure 1.2). Previous studies have shown that numbers of mature osteoblasts are reduced when MSCs were stimulated towards adipogenesis (Lecka-Czernik et al. 2002). Conversely, suppression of adipogenesis was accompanied by a significant induction of osteogenesis in culture cells obtained from PPARγ-deficient mice (Akune et al. 2004). Therefore, bone loss in T2DM could be caused by less bone formation as more progenitor cells are differentiated in favour of adipogenesis rather than osteoblasts. However, as reviewed by McCabe, bone abnormalities as a result of marrow adiposity is a
controversial issue and more studies are required to further elucidate the connection between bone, adipocytes and key factors involved in the differentiation and maturation of MSCs (McCabe 2007).

Figure 1.2 Bone marrow lineage and key determinants of cell maturation

Maturation of mesenchymal stem cells involves numerous key factors such as CEBP (CCAAT/enhancer-binding protein), IL-6 (interleukin-6), MCSF (macrophage colony stimulating factor), OPG (osteoprotegerin), PPARγ (peroxisome proliferator-activated receptor γ), RANK (receptor activator of nuclear factor-kappaB), RANKL (receptor activator of nuclear factor kappa-B ligand), RUNX2 (runt-related transcription factor 2), DLX5 and MSX1 (transcription factors). Adapted from (Rosen & Bouxsein 2006).

1.3.3 Growth factors and cytokines

There are also contributions of endocrine factors in diabetic bone loss such as insulin-like growth factor-1 (IGF-1) and amylin. The positive relationship of IGF-1 and BMD has been reported previously (Rosen 2004) and therefore, it is not surprising to find pronounced reduction of circulating IGF-1 in diabetic patients (Jehle et al. 1998, Sandhu et al. 2002, Teppala & Shankar 2010). On the other hand, inactivation of amylin, a peptide co-secreted with insulin by pancreatic beta-cells, is
associated with low bone mass and increased osteoclastic activity (Dacquin et al. 2004). Furthermore, increases in bone strength and bone formation biomarker, osteocalcin have been demonstrated in T1DM diabetic rats treated with amylin agonist (Horcajada-molteni et al. 2001), indicating the importance of amylin in bone regulation.

Conflicting data has been published on the participation of adipokines (e.g leptin and adiponectin) and cytokines (e.g osteoprotegerin) in bone regulation under diabetic conditions. For instance, leptin-deficient obese diabetic (ob/ob) mice exhibit severe bone impairments, and improvement in bone mass was seen with administration of leptin (Steppan et al. 2000). Similarly, leptin treatment increases bone mechanical properties in normal mice (Cornish et al. 2002), indicating the anabolic effect of leptin on bone. However, in clinical human studies, unchanged or increased serum leptin concentrations are observed in diabetic individuals (Alexopoulos et al. 2006, Luna et al. 1999). Bone-related protein, osteoprotegerin (OPG) is known to have positive effect on bone by inhibiting excessive osteoclastogenesis and in line with that, genetically modified mice lacking OPG gene have been shown to have elevated osteoclast numbers and activity (Nakamura et al. 2003). In the contrary, serum OPG levels are significantly increased in T1DM children (Galluzzi et al. 2005), suggesting a negative correlation of OPG and bone. These contradictions could be due to comparisons made on different models (mouse vs human) and the limited data available in this area. As such, further investigations are needed to explore the possible effects of adipokines and cytokines in diabetic bone abnormalities.

1.3.4 Bone impairments in animal models of diabetes

One particular model of animals extensively used in the laboratory is mouse. The high degree of similarity between mouse and human genetics (more than 95% of human genomes conserved in the mouse) has increased the value of mouse models for research on human disorders (The Jackson Laboratory 2015). Detrimental effects of diabetes on bone quality have also been vastly investigated in rodent models of diabetes. Diabetes can develop in animals either spontaneously- or through genetic modifications or induction of diabetes based on experimental protocols. Thrailkill and colleagues showed that trabecular and cortical bone were severely compromised
in spontaneous nonobese diabetic (NOD) mice as assessed by microCT and three-point bending tests (Thrailkill et al. 2005b). The modifications in microstructure and bone strength contributed to an impairment of bone formation as seen by reductions of bone formation biomarkers and formation of new bone. In addition to trabecular bone loss, a more recent study reported increases in bone marrow adiposity and adipocyte-related proteins, whilst osteoblast-specific osteocalcin was reduced in NOD mice (Botolin & McCabe 2007).

Bone abnormalities have also been investigated in several frequently used genetically-induced rodent models of diabetes such as obese Zucker (fa/fa) rats, obese diabetic (ob/ob) mice and diabetic (db/db) mice. These diabetic models exhibit gross weight gain, hyperglycaemia and hyperinsulinemia due to impairments in leptin signaling (Rees & Alcolado 2005). Homozygous mutation of the leptin gene (ob gene) in ob/ob mice or defective leptin receptor in db/db mice and obese Zucker rats severely affects bone microstructure and bone strength. Obese diabetic (ob/ob) mice have shorter femoral bone compared to normal mice and densitometry and histomorphometry analysis reveal reduced bone mass, cortical thickness, and trabecular bone volume in the diabetic mice (Hamrick et al. 2004, Matsunuma et al. 2004, Steppan et al. 2000). Similar findings are observed in Zucker rats (Tamasi et al. 2003). Additionally, reductions in whole-femur BMD and BMC as well as whole-bone biomechanical properties were reported in db/db mice (Ealey et al. 2006). Further studies by Williams and colleagues found not only alterations of mechanical properties at the organ-level, but also changes in material properties of bone matrix in leptin receptor-deficient db/db mice (Williams et al. 2011).

Streptozotocin (STZ) is a glucosamine-nitrosourea analogue and when introduced into the body, it is taken up by pancreatic beta cells, causing cell death. Due to the toxicity of STZ to insulin-producing islets beta cells, this compound has been widely used, either as single high dose or multiple low doses, to induce hyperglycaemia and insulin deficiency in normal rodents (Lu et al. 1998). Defective bone formation is consistently seen in STZ-induced diabetic rodents (Coe et al. 2013, Fowlkes et al. 2008, Motyl & McCabe 2009). Additionally, multiple low-dose STZ-injected mice demonstrate increased bone marrow adiposity which is accompanied by increased expression of adipocyte biomarkers and reductions in markers for bone formation
In terms of the biomechanical consequences of diabetes, diabetic bones require less load and energy absorption to reach failure (Reddy et al. 2001), emphasising a reduction in biomechanical integrity.

Other models of T2DM can be obtained through high-fat dietary feeding. The intake of high-fat diet, which mimics Westernised diet, can cause metabolic disturbances and lead to obesity, insulin resistance and hyperinsulinemia (Zhang et al. 2009). With regards to bone, conflicting results are reported on the bone mass of high-fat dietary-induced diabetic mice (Halade et al. 2010, Ionova-Martin et al. 2010, Patsch et al. 2011), which possibly due to different type of high-fat diet used in the studies. In agreement with reduced bone mass in high-fat fed mice, Halade et al. found that bone marrow adipocytes were significantly increased (Halade et al. 2010), whilst Patsch et al. reported a reduction in bone volume as assessed by microCT (Patsch et al. 2011). Studies by Ionova-Martin et al. observed significant reductions in parameters of microstructural and biomechanical strength, despite increased bone quantity and mass in high-fat diet group (Ionova-Martin et al. 2010). In general, the studies agree that high fat feeding has adverse effects on overall bone quality.

1.3.5 Bone mineral density and fracture risk in human diabetes

Recent meta-analysis of discrepancies in BMD and fracture risk conclude that regardless of BMD (decreased, unchanged, elevated), DM patients are associated with increased risk of fractures (Janghorbani et al. 2007, Vestergaard 2007). However, the relative fracture risk is higher in T1DM than T2DM. The majority of studies in T1DM consistently report the reduction of BMD in T1DM patients (Rakel et al. 2008) and in line with that, T1DM individuals are more susceptible to bone fragility fracture (Vestergaard 2007). Cross-sectional studies of the relationship between BMD and increased fracture risk are mostly related to the lower spine, and although there are limited reports on T1DM-associated bone fragility at other sites (Hampson et al. 1998, Miazgowski & Czekalski 1998), most of these studies demonstrate a pattern towards increased fracture risk (Vestergaard 2007). T2DM individuals have normal-to-increased BMD when measured at femoral neck, vertebral, distal tibia, humerus and radius (De Liefde et al. 2005, Petit et al. 2010, Schwartz et al. 2000) and persistently high BMD is seen even after correction for
body composition compared to age-matched healthy subjects. Despite elevated BMD, T2DM patients still have increased bone fragility fracture (Vestergaard 2007).

Several factors limit the usefulness of these studies such as duration and severity of diabetes, assessment of different types of bone markers and different methods for measurement of bone density (Epstein & LeRoith 2008). Furthermore, in view of risk of fractures, BMD should not be the only aspect that is considered (Moseley 2012) as diabetes-related extraskeletal factors, such as neuropathy and retinopathy could contribute to the elevated rate of falling as a result of increased postural instability and impaired eye-sight (Schwartz et al. 2002). As a consequence of the first fall, diabetic patients may exhibit reduced bone competency and therefore, may be prone to further bone fracture.

1.4 INCRETIN EFFECT

The incretin effect is described as the difference in insulin secretion after glucose ingestion or intravenous glucose infusion. The term was first introduced when the oral administration of glucose was observed to induce elevated insulin release compared with the same amount of glucose given intravenously (Nauck et al 1986). The incretin effect is considered as an important event in controlling blood glucose homeostasis as insulin response from this effect is estimated to account for 50% to 70% of total insulin release (Nauck et al. 2011). In response to food ingestion, incretin hormones are released into circulating blood and enhance glucose-mediated insulin secretion from pancreatic beta-cells. The two gut hormones that have been identified to control the enteroinsular axis are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Baggio & Drucker 2007). Although other gastrointestinal hormones can stimulate insulin release and lower blood glucose concentrations, only GIP and GLP-1 are physiologically relevant (McIntosh et al. 2009).
1.5 GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP)

GIP was isolated from crude extracts of porcine small intestine and initially known as gastric inhibitory polypeptide for its ability to inhibit the secretion of gastric acid in dogs (Pederson et al. 1975). Further studies with purified GIP demonstrated the stimulatory effect of GIP on insulin release in humans and animals. The incretin effect of GIP was considered more important as the insulinotropic action was seen at physiological levels compared to its inhibitory action in gastric acid release which only occurred at pharmacological levels. Therefore, it is renamed as glucose-dependent insulinotropic polypeptide (Baggio & Drucker 2007).

1.5.1 Synthesis, secretion and signaling

GIP is a 42-amino acid peptide synthesised within K-cells, which are abundant in the upper duodenum and proximal jejunum of small intestine. The human GIP gene comprises of 6 exons (Figure 1.3A) with most of the sequences for GIP in exon 3. The GIP sequence is highly conserved for human and shares more than 90% similarity in amino acids sequence with mouse, rat, porcine and bovine GIP (Baggio & Drucker 2007). Bioactive GIP is processed from ProGIP, a prohormone precursor of 153 amino acids (as shown in Figure 1.3) and the enzyme responsible for the post-translational processing is prohormone convertase (PC) 1/3. PC1/3-mediated cleavage of the ProGIP sequence occurs at single arginine residues, leaving biologically active GIP(1-42) along with N-terminal and C-terminal signal peptides (Ugleholdt et al. 2006).

Basal GIP concentrations for healthy humans is around 0.01 nmol/l and the peak value of GIP level reached within 60 min after nutrient intake, increases by 10 to 15-fold (Visboll et al. 2001). GIP binds to the GIP receptor (GIPR), a member of the seven-transmembrane domain-spanning receptor which belongs to G-protein coupled receptor (GPCR) family (Usdin et al. 1993).
1.5.2 Pancreatic actions

The main role of GIP is as incretin hormone that regulates glucose homeostasis. Upon nutrient intake, GIP is secreted by K-cells, binds to its receptors on islet beta-cells of pancreas and subsequently enhances glucose-mediated insulin secretion. Activation of GIPR signaling increases cAMP levels and inhibits $K_{ATP}$ channels. Inhibition of ATP-sensitive potassium channels leads to influx of $Ca^{2+}$ and stimulation of insulin secretion by exocytosis (Ding & Gromada 1997). Binding of GIP to its G-protein couple receptor also activates cAMP-mediated phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) and MAP kinase signaling pathways which are closely associated with promotion of proliferation, differentiation and inhibition of apoptosis in beta cells (Ehses et al. 2003, Trumper et al. 2001, Trumper et al. 2002).

1.5.3 Extrapancreatic actions

The primary physiological action of GIP is in pancreas. However, the widespread expression of GIPRs in other tissues such as in adrenal cortex, brain, heart, lung, pituitary, adipose and bone suggests wider extrapancreatic actions of GIP in the body (Figure 1.4) (Baggio & Drucker 2007). GIP is suggested to participate in fat metabolism from the observations that obese humans (Creutzfeldt et al. 1978, Salera
et al. 1982) and ob/ob mice (Flatt et al. 1983) had increased concentrations of plasma GIP. In agreement, there is evidence of functional GIPRs in primary adipocytes and the adipose cell line 3T3-L1 (Rupert et al. 1998). Additionally, a more recent study in isolated adipocytes demonstrated that GIP dose-dependently stimulates lipogenesis (Omar et al. 2013).

In addition to adipose tissue, extrapancreatic effects of GIP have also been shown in the brain. GIPR mRNA and its specific binding sites are detected in several regions of the brain such as cerebral cortex and hippocampus, including old factorybulb (Kaplan & Viagna 1994, Usdin et al. 1993), indicating a direct role of GIP in regulating brain function. Administration of exogenous GIP in rats and incubation of GIP with cultured adult hippocampal progenitor cells has been demonstrated to stimulate the proliferation of hippocampal progenitor cells (Nyberg et al. 2007). In agreement, the same group of researchers reported that genetic deficiency of the GIPR reduced the number of new proliferating cells by 50%. GIP-overexpressing transgenic mice display better memory recognition than wild type mice (Ding et al. 2006) and consistent with this finding, administration of a stable GIP analogue for 28 days improved cognitive function in high-fat fed diabetic mice with cognitive dysfunction (Porter et al. 2011). Collectively, these observations show that GIP positively influences brain function.

With regards to bone, functional GIPRs were revealed on bone-forming cells, the osteoblasts and bone-resorbing cells, the osteoclasts (Bollag et al 2000, Zhong et al 2007). GIPRs were also expressed on osteocytes (Bollag et al 2001) and cultured bone marrow stromal cells (BMSCs) (Ding et al. 2008). The expression and potential osteoprotective effects of GIP on bone will be further discussed in Section 1.8.
The effects of GIP are mediated by direct interaction with GIPRs on specific tissues. Taken from (Baggio & Drucker 2007).

1.5.4 DPP-4 and GIP metabolism

GIP is secreted immediately after food intake. However, the endogenous peptide has a short half-life due to the action of the ubiquitous enzyme dipeptidyl peptidase-4 (DPP-4). Active GIP in rodents is reported to have a half-life of less than 2 min (Kieffer et al. 1995) while in humans, GIP has a half-life of around 5-7 min (Deacon et al. 2000). DPP-4 is a specialized serine protease that cleaves penultimate residues from the N-terminal of bioactive proteins that contain alanine or proline residues at position 2 (Mentlein et al. 1993). Thus, DPP-4 inactivates biologically active GIP by cleaving at its first two N-terminal amino acids (Tyr\(^1\)-Ala\(^2\)), leaving the N-terminally truncated metabolite, GIP(3-42) (Kieffer et al. 1995). DPP-4-facilitated cleavage of active GIP(1-42) eliminates insulinotropic activity, but receptor affinity is only
reduced by fourfold (Deacon et al. 2006). Inhibition of DPP-4 action by specific DPP-4 inhibitor has previously been shown to reduce degradation of exogenous GIP, resulting in increased levels of intact GIP in plasma (Deacon et al. 2001, Deacon et al. 2002). In addition to rapid degradation by DPP-4, GIP is also subjected to renal extraction through kidney filtration (Meier et al. 2004). In order to overcome the inactivation of GIP by DPP-4, structurally-modified DPP-4 resistant GIP molecules are developed. Furthermore, promoting the adherence of long-acting GIP agonists to serum proteins will delay renal clearance and improve the bioactivity of GIP.

1.5.5 Structurally-modified stable GIP agonists

In order to harness the insulinotropic properties of GIP, substantial numbers of DPP-4 resistant GIP molecules have been developed. One of the pharmacological approaches is through modifying the N-terminal amino acid residues of GIP to prevent DPP-4-mediated degradation, hence prolonging bioactivity of circulating GIP (Irwin & Flatt 2009). Several Tyr1-substituted GIP molecules that have been generated and tested include N-acetyl, N-Fmoc, N-gluticol, N-pyroglutamyl and N-palmitate groups. These analogues have been shown to be resistant to DPP-4 and have enhanced bioactivity compared to native GIP in vitro (O’Harte et al. 2000, Gault et al. 2002, O’Harte et al. 2002). In addition, synthesis and characterisation of novel Ala2-modified GIP analogues has also been carried out and these include [Abu2]GIP, [Gly2]GIP, [Sar2]GIP, [Ser2]GIP and [D-Ala2]GIP. However, only [Gly2]GIP, [Ser2]GIP and [D-Ala2]GIP have notable biological activity and antihyperglycaemic properties compared to native GIP (Hinke et al. 2002, Gault et al. 2003a).

Although N-terminally-protected GIP analogues are resistant to DPP-4-mediated inactivation, these molecules are still susceptible to in vivo renal filtration. Therefore, in order to prolong the pharmacodynamic profile of GIP, a C-16 palmitate fatty-acid (PAL) chain is attached covalently to the Lys16 or Lys37 residue of the GIP peptide. The fatty-acid GIP derivatives that have been developed to date are GIP(Lys37PAL), GIP(Lys16PAL), N-AcGIP(Lys37PAL), N-AcGIP(Lys16PAL), N-pGluGIP(Lys37PAL) and N-pGluGIP(Lys16PAL) (Irwin et al. 2005a, Irwin et al. 2005b, Irwin et al. 2006a). The attachment of C-16 fatty acid protects the GIP-
modified molecules from being degraded by DPP-4, whilst biological and insulinotropic activities are significantly enhanced compared to native GIP peptide (Irwin & Flatt 2009). In comparison of efficacy of different long-acting GIP analogues, a previous study has shown that the biological activity of stable fatty-acid GIP derivatives is similar to stable non-fatty acid GIP molecule (Irwin et al. 2006b), indicating an equal effectiveness of the DPP-4 resistant molecules.

1.6 GLUCAGON-LIKE PEPTIDE-1 (GLP-1)

Glucagon-like peptide-1 (GLP-1), along with its sister incretin, GIP, are responsible for incretin effect. In 1983, a study with GIP-lacking rat gut extracts found that 50% incretin activity was preserved in these extracts (Ebert et al. 1983). It was then suggested that GIP only partially accounting for the incretin effect and that there are other gut factors which carry similar insulinotropic properties to GIP. Sharing 50% sequence of glucagon, GLP-1 together with GLP-2 were later identified from the sequence of hamster preproglucagon (Bell et al. 1983). Subsequent work on their potential insulinotropic action revealed the ability of GLP-1 to stimulate insulin secretion from the isolated perfused rat pancreas (Mojsov et al. 1987) and later was shown to be one of hormones that accounts for incretin effect.

1.6.1 Synthesis, secretion and signaling

GLP-1 is one of the products of the proglucagon gene which has 6 exons and the entire GLP-1 coding sequence is within exon 4 (Figure 1.5A). The proglucagon gene is expressed not only in pancreatic α-cells but also in L-cells of the intestine and central nervous system. Posttranslational processing of proglucagon will generate different peptides, depending on where the gene is expressed. While in pancreas, proglucagon is cleaved by prohormone convertase PC2 to produce glucagon (GLUC), glicentin-related polypeptide (GRPP), intervening peptide-1 (IP-1) and major proglucagon fragment (MPGF) (Rouille et al. 1997a). As a major product of proglucagon in alpha-cells, glucagon is known to play its role by counteracting insulin action to maintain normal glucose levels. However, the actual roles of other post-translational products remain largely unknown.
Structures of (A) the proglucagon gene, (B) mRNA, and (C) protein. (D) Posttranslational processing of proglucagon in the pancreas generates Glicentin-related polypeptide (GRPP), glucagon (GLUC), intervening peptide-1 (IP-1), and major proglucagon fragment (MPGF), whereas the products in the intestine and brain are glicentin, oxyntomodulin (OXM), intervening peptide-2 (IP-2), and GLP-1 and GLP-2. Adapted from (Baggio & Drucker 2007).

In the intestine and central nervous system, the 180-amino acid proglucagon peptide liberates oxyntomodulin, glicentin, GLP-1, intervening peptide-2 (IP-2) and GLP-2 (Figure 1.5D). GLP-1 is the major product of proglucagon gene in the intestine and it is one of the important glucoregulatory hormones that control glucose homeostasis. In addition to GLP-1, oxyntomodulin is also released postprandially and the peptide is believed to exert its effects by binding to both GLP-1 and glucagon receptors (Pocai 2012). Activation of glucagon receptor increases blood glucose concentration, whilst simultaneous GLP-1R activation counteracts this effect, thus balancing glucose metabolism in the body. Oxyntomodulin is recently speculated to have therapeutic potential for diabetes and obesity for its dual actions in improving glucose homeostasis and lowering body weight (Pocai 2014). Similar to GIP, processing of proglucagon precursor to GLP-1 is dependent on prohormone convertase PC1/3. Proglucagon is initially cleaved to glicentin and the major
proglucagon fragment (MPGF). Subsequent cleavage of MPGF at the monobasic site Arg\textsuperscript{77} and the dibasic site Arg\textsuperscript{109}-Arg\textsuperscript{110} releases GLP-1 (Rouille et al. 1997b).

Fasting plasma concentrations of biologically active GLP-1 in humans is normally in a range of 5-10 pmol/L and increases approximately 2- to 3-fold following food intake (Vilsboll et al. 2001). The secretion of GLP-1 follows biphasic pattern where early phase takes 10-15 minutes and the subsequent later phase takes much longer, around 30-60 min (Herrmann et al. 1995). Upon release, GLP-1 binds to its heterotrimeric receptor, a member of G-protein-coupled receptor (GPCR) superfamily (Mayo et al. 2003) and activates adenylate cyclase. The activation of adenylyl cyclase is followed by a rise in cAMP levels and direct inhibition of \(K\textsubscript{ATP}\) channels which leads to elevation of intracellular Ca\textsuperscript{2+} concentration, and subsequently, stimulation of insulin release (Gromada et al. 1998).

### 1.6.2 Pancreatic actions

The primary physiological action of GLP-1 in the pancreas is to stimulate insulin release from islet beta-cells in a glucose dependent manner (Mojsov et al. 1987). Two different signaling pathways are involved in GLP-1-mediated cAMP-activated insulin release, either through PKA or Epac2 or cAMP-independent phosphatidylinositol-3 kinase (PI-3K)/protein kinase C (PKC) signaling pathways (Baggio & Drucker 2007). In addition, activation of GLP-1R signaling enhances transcription of the insulin gene and biosynthesis of insulin, thus preventing exhaustion of insulin stores (Drucker et al. 1987, Wang et al. 1997). GLP-1-mediated insulin gene transcription and synthesis occur through pancreas duodenum homeobox 1 (PDX-1), an essential transcription factor for pancreatic and beta cell function by upregulating PDX-1 expression and binding to the insulin gene (Wang et al. 1999). GLP-1 also strongly suppresses glucagon secretion from pancreatic alpha-cells (Holst et al. 2011) and its inhibitory action is likely mediated through stimulation of the glucagon inhibitor, somatostatin and somatostatin subtype receptor 2 (SSTR2) in pancreatic cells (de Heer et al. 2008).
1.6.3 Extrapancreatic actions

Several other tissues have been shown to express GLP-1Rs, such as the heart, brain, liver, intestinal tract, muscle and adipose tissue (Dunphy et al. 1998, Merchenthaler et al. 1999, Wei & Mojsov 1996). As shown in Figure 1.6, wide distribution of GLP-1Rs throughout the body is an indication that GLP-1 possesses several extrapancreatic actions. Indeed, glucoregulatory activity of GLP-1 does not only take place in pancreas, but also in other tissues by reducing hepatic glucose production in liver and increasing glucose uptake in muscle and adipose tissue (Baggio & Drucker 2007, Larsson et al. 1997). In the brain, activation of GLP-1R signaling enhances neurogenesis, reduces apoptosis and protects neuronal function (Perry et al. 2002, Perry et al. 2003). Moreover, improvements in learning and memory activities were observed in rodents when treated with stable GLP-1 mimetics (During et al. 2003, Porter et al. 2010). Therefore, GLP-1 could be useful in the treatment of neurodegenerative disorders (Holscher 2012).

Protective properties of GLP-1 have also been demonstrated in isolated and intact rat heart (Bose et al. 2005) and GLP-1 was also shown to improve ventricular performance and myocardial glucose uptake in dogs (Nikolaidis et al. 2004). Although there are limited data available, several recent studies postulate that GLP-1 affects bone in a beneficial manner. The potential osteoprotective actions of GLP-1 will be explained in further detail in Section 1.8.
GLP-1 exerts its effects by directly binding to GLP-1Rs on pancreas, stomach, brain and heart. However, GLP-1 acts through indirect mechanisms in liver, fat and muscle. Adapted from (Baggio & Drucker 2007).

1.6.4 DPP-4 and GLP-1 metabolism

There are several forms of GLP-1 secreted in vivo that include GLP-1(1-37), GLP-1(1-36)amide, but only GLP-1(7-37) and GLP-1(7-36)amide are thought to be biologically active (Baggio & Drucker 2007). Both bioactive GLP-1 molecules appear to have similar potency in stimulating insulin secretion (Orskov et al. 1993) with significant amounts of GLP-1(7-36)amide found in circulation system of humans (Orskov et al. 1994). GLP-1 is rapidly secreted into blood circulation following feeding. However, the half-life of intact GLP-1 is short, around 2 min (Kieffer et al. 1995), due to the activity of DPP-4. Native GLP-1 contains alanine at position 2 and therefore is a substrate for DPP-4. The specific cleavage of DPP-4 at N-terminal
dipeptides from GLP-1 produces GLP-1(9-36)amide and GLP-1(9-37) with abolished insulinotropic activities (Mentlein et al. 1993, Kieffer et al. 1995). Similar to GIP, GLP-1 can also be eliminated from circulation by renal filtration (Meier et al. 2004). The short half-life of GLP-1 has prompted generation of stable long-acting forms of GLP-1 (Knudsen et al. 2000).

1.6.5 Structurally-modified stable GLP-1 agonists

In order to harness the highly significant glucose-lowering and insulin-releasing properties of GLP-1, numerous structurally modified GLP-1R agonists have been generated and tested for their therapeutic potential. These GLP-1 analogues possess modification of amino acid residues at N-terminus of the peptide, producing GLP-1R molecules that are resistant to DPP-4 activity (Deacon et al. 1998). Furthermore, biological activity of GLP-1 can be prolonged by delaying renal filtration and this can be achieved through fatty acid derivatisation (Green & Flatt 2007).

1.6.6 Exendin-4

Exendin-4 (Exenatide / Byetta) is a naturally occurring 39 amino acid peptide isolated from the saliva of the lizard Heloderma suspectum (Eng et al. 1992). Exendin-4 exhibits roughly 53% similarity in structure to mammalian GLP-1 and it is resistant to DPP-IV action due to the presence of a glycine residue at position 2 in place of alanine (Drucker & Nauck 2006). As a result, Exendin-4 remains in blood circulation longer than native GLP-1 (Parkes et al. 2001). Exendin-4 mimics GLP-1 actions by enhancing insulin secretion in a glucose-dependent manner, reduces glucagon release, decreases food intake and slows gastric emptying (Nielsen et al. 2004).

Exenatide is the synthetic form of exendin-4 and it was the first GLP-1 mimetic approved by US Food and Drug Administration (FDA) in 2005 as an alternative for the treatment of T2DM (Drucker & Nauck 2006). Exenatide is administered twice daily at a dose of 5 - 10 µg and postprandial and fasting plasma glucose is markedly reduced in patients with T2DM receiving subcutaneous Exenatide (Koltermann et al. 2003). In addition, improvement in glucose levels was observed when Exenatide was
used as an adjunctive therapy in diabetic patients who had poor glycaemic control with metformin and/or sulfonylurea combinations (DeFronzo et al. 2005, Fineman et al. 2003, Kendall et al. 2005).

1.6.7 Liraglutide

Liraglutide (NN2211, Victoza) is an effective GLP-1 analogue and has high sequence homology (approx. 97%) to native GLP-1. Liraglutide differs from native GLP-1 sequence at lysine 26 and 34, where lysine 26 has an addition of C-16 free fatty acid via a γ-glutamic acid spacer while at position 34, lysine is substituted by an arginine residue (Knudsen et al. 2000). The additional acyl chain in Liraglutide promotes non-covalent binding of the GLP-1 mimetic to serum protein albumin and prevents renal clearance, thus prolonging its action (Knudsen et al. 2000). Similar to Exenatide, Liraglutide binds to and fully activates GLP-1Rs to glucose-dependently stimulate the release of insulin and subsequent reduction in blood glucose concentrations (Lovshin & Drucker 2009).

The half-life of Liraglutide is about 13 h and therefore, it is suitable for once-daily subcutaneous administration in humans (Agerso et al. 2002, Degn et al. 2004, Knudsen et al. 2000). In 2009, approval was obtained from the European Medicines Agency to clinically use Liraglutide in T2DM treatment, and this was followed by the US Food and Drug Administration in early 2010 (Peterson & Pollom 2010). Several clinical studies of tolerability and efficacy of Liraglutide have been conducted and it was demonstrated that once-daily subcutaneous administration of Liraglutide at a dose of 0.6-1.8 mg was well tolerated (Neumiller & Campbell 2009). Additionally, Liraglutide can also be used as monotherapy or in combination with other glucose-lowering agents, such as metformin and/or a sulphonylurea, in poorly controlled T2DM patients (Neumiller & Campbell 2009).

1.6.8 Newly-approved/developed GLP-1 therapeutics

A structurally-modified form of the Exenatide molecule, Lixisenatide is one of the latest drugs approved by the European Medicines Agency in early 2013 for the management of T2DM. This molecule is a synthetic version of Exendin-4 and it
differs from Exendin-4 by having deletion of one proline residue and an addition of six lysine molecules at the C-terminus (Barnett 2011). Once-daily administration of Lixisenatide monotherapy or as adjunct therapy with metformin or sulfonylureas has been shown to significantly improve postprandial blood glucose levels in inadequately controlled T2DM individuals (Fonseca et al. 2012, Horowits et al. 2013). Similar to Exenatide and Liraglutide, monotherapy of Lixisenatide has common gastrointestinal adverse effects such as vomiting and nausea with low numbers of treatment-associated hypoglycaemic problems reported (Christensen et al. 2011).

Two once-weekly GLP-1 derivatives that have been recently approved are Exenatide extended-release and Albiglutide. Exenatide long-acting release (LAR) (Bydureon) contains exenatide in poly(lactic-co-glycolic) microspheres (Tharakan et al. 2011) which is gradually released by diffusion of the microspheres. The long-acting release property of the once-weekly Exenatide derivative provides a continuous exposure to Exenatide and elicits sustained improvements in glycaemic control and body weight (Bergenstal et al. 2010, Buse et al. 2010). Exenatide LAR was authorized for marketing in January 2012 by FDA and is taken at a dose of 2 mg / week (Lund et al. 2014, Neumiller 2012). On the other hand, Albiglutide consists of two molecules of GLP-1 fused to human serum albumin and this molecule is resistant to DPP-4 degradation through substitution of alanine for glycine at position 2 of the GLP-1 peptides (Lund et al. 2011). In an evaluation test for efficacy with three different dosing regimes, pronounced improved glycaemic control and modest weight loss were demonstrated with weekly administration of Albiglutide (Rosenstock et al. 2009), making this sustained-release GLP-1R agonist suitable for less frequent dosing as compared to existing T2DM treatment. In April 2014, Albiglutide (Tanzeum) was approved to be used once weekly by FDA at a dose of 30-50 mg (GlaxoSmithKline 2015).

Taspoglutide has α-aminoisobutyric acid residues replacing Ala8 and Gly35 in human GLP-1(7-36)amide (Dong et at. 2010). The molecule exhibits enhanced stability, thus higher potency in vivo compared to native GLP-1 (Sebokova et al. 2010). Although a clinical study comparing once-weekly administration of Taposglutide to twice-daily Exenatide demonstrated significantly improved glycemic control in
T2DM patients, the use of this long-acting agonist has limitations due to its side effects (Rosenstock et al. 2013).

1.7 DPP-4 INHIBITORS

Another attractive therapeutic strategy for diabetes is through DPP-4 inhibition (Holst & Deacon 1998). As mentioned, DPP-4 inactivates native GLP-1(7-36)amide and GIP(1-42) by cleaving the alanine residue at position 2 of these peptides, leaving the truncated forms GLP-1(9-36)amide and GIP(3-42) (Baggio & Drucker 2007). Hence, inhibition of DPP-4 activity by DPP-4 inhibitors prevents the degradation of biologically active incretins and prolongs insulinotropic action of endogenous GLP-1 and GIP (Green et al. 2006). Several DPP-4 inhibitors are already clinically available, namely Sitagliptin, Vildagliptin, Saxagliptin and Linagliptin (Duez et al. 2012).

Vildagliptin (Galvus), Sitagliptin (Januvia), Saxagliptin (Onglyza) and Linagliptin (Tradjenta) are orally available and highly selective inhibitors of DPP-4. Once-daily administration of Sitagliptin for a period time of 24 weeks reduced HbA1c concentrations and postmeal plasma glucose levels of type 2 diabetic patients whose glucose tolerance was inadequately controlled by diet and exercise (Aschner et al. 2006). Additionally, Sitagliptin used as an adjunct to ongoing metformin or thiazolidinedione therapy improved glycaemic control in type 2 diabetic subjects (Charbonnel et al. 2006, Rosenstock et al. 2006).

Vildagliptin was introduced in the UK in 2008, a year later than Sitagliptin, and is is administered twice daily at a dose of 50 mg orally. Vildagliptin monotherapy or in conjunction with metformin or insulin therapy significantly reduced HbA1c in type 2 diabetic patients (Bosi et al. 2007, Fonseca et al. 2007, Pratley et al. 2006). Vildagliptin improves glycaemic control in pre-diabetic or impaired glucose tolerance patients by increasing postprandial plasma level of biologically active GLP-1 and GIP and pancreatic beta-cell sensitivity to glucose, as well as suppressing glucagon secretion (Rosenstock et al. 2008). Saxagliptin is the third DPP-4 inhibitor approved for clinical use in 2009 and used as an add-on therapy at a dose of 5 mg with metformin or thiazolidinedione therapy (DeFronzo et al. 2009, Hollander et al. 2012).
The introduction of Linagliptin was in 2011, and similar to other DPP-4 inhibitors, glycaemic control is improved with Linagliptin monotherapy or in combination with other oral glucose-lowering agent (Duez et al. 2012, Gomis et al. 2012).

DPP-4 inhibitors offer several advantages over existing diabetes therapies. Firstly, enhancement of insulin secretion and suppression of glucagon release follow a glucose-dependent manner, thus there less risk of hypoglycaemia (Herman et al. 2005). Furthermore, no weight gain was observed in type 2 diabetes patients treated with DPP-4 inhibitors (Barnett 2006, Karasik et al. 2008, Rosenstock et al. 2008). Lastly, long-term inhibition of DPP-4 has beneficial effects on islet beta-cell mass and function as seen in diabetic mice (Mu et al. 2006, Pospisilik et al. 2003, Reimer et al. 2002).

1.8 FUNCTION OF INCRETINS ON BONE

Bone formation and breakdown are closely related to nutrient intake and it has been shown that with meal ingestion, biomarkers for bone resorption are reduced (Henrikson et al. 2003), suggesting a link between gut and bone regulation. GIP and GLP-1 are secreted in response to food ingestion and since gastrointestinal hormone levels change upon food intake, it is suspected that incretins may play a role in bone turnover. GIP has direct action on bone as GIPR mRNA and protein are present in normal bone cells (osteoblast and osteoclast) and progenitor bone cells (Bollag et al. 2000, Bollag et al. 2001, Ding et al. 2008, Zhong et al. 2007).

Similar to the pancreas, the binding of GIP to GIPR on osteoblast has been shown to increase cAMP and Ca^{2+} concentrations and subsequently elevate the expression of bone formation biomarkers, collagen type 1 and alkaline phosphatase activity (Bollag et al. 2000), suggesting positive action of GIP on bone formation. GIP has a role in proliferation of osteoblasts as it was observed to have stimulatory effect on the release of transforming growth factor-β (TGF-β) and 3H-thymidine incorporation in osteoblastic-derived cells (Zhong et al. 2003). Investigation of potential osteoprotective effects of GIP using primary mouse osteoblasts and osteoblastic-like SaOS-2 cells found that pretreatment with GIP significantly reduced the percentage
of apoptotic cells (Tsukiyama et al. 2006). Furthermore, GIP may be involved in the early process of osteogenesis by promoting osteoblastic differentiation of bone marrow stromal cells (Ding et al. 2008). There is limited information on GIP actions on bone-resorbing osteoclast cells. However, GIP has been reported to inhibit osteoclast activity and differentiation in vitro (Zhong et al. 2007), indicating a role of GIP in reducing bone resorption.

Rodent models of GIPR knockout have reduced bone size and lower bone mass as well as a decrease in bone formation biomarkers compared with the wild type (Xie et al. 2005). Bone histomorphometrical analyses in GIPR-deficient mice demonstrated that the reduction in bone mass was due to increased multinucleated osteoclasts, which are responsible for active bone resorption (Tsukiyama et al. 2006). In agreement, bone mass, bone size and biomarkers for bone formation were shown to be increased in GIP-overexpressing transgenic mice, whilst bone resorption biomarkers were decreased (Xie et al. 2007). These observations were in harmony with in vitro data that reported a stimulatory effect of GIP on osteoblasts and inhibitory effect on osteoclasts. In addition, using five different techniques of bone assessment, a recent study found that microstructure and strength of bones in mice lacking GIPRs were compromised and these findings support the beneficial effects of GIP on bone (Mieczkowska et al. 2013). Importantly, it was shown earlier that administration of GIP prevented bone loss in ovariectomized rats (Bollag et al. 2001), while a more recent study found that rats injected with a stable GIP analogue appeared to have increased tissue material properties at the cortical bone level (Mabilleau et al. 2014).

Conflicting data on the expression of GLP-1Rs on bone cells has been published (Bollag et al. 2000, Mabilleau et al. 2013, Nuche-Berenguer et al. 2010b). However, the inconsistencies may result from the use of different cell lines. Nevertheless, GLP-1 is speculated to affect bone turnover in a beneficial manner as GLP-1R-deficient mice have reduced bone mass and bone mineral density, whilst bone resorption biomarkers appeared to be significantly increased (Yamada et al. 2008). The same researchers also found that level of calcitonin was reduced and proposed that GLP-1-mediated bone turnover is mediated through a calcitonin-dependent pathway. Recently, bone quality in mice lacking GLP-1Rs function was further
assessed and it was revealed that these mice had reduced cortical bone strength due to alterations of cortical thickness and bone material properties (Mabileau et al. 2013). Other studies demonstrated that bone formation biomarkers were elevated after 3-day subcutaneous administration of GLP-1 or the GLP-1R agonist, Exendin-4 in streptozotocin-induced and insulin-resistant diabetic rats (Nuche-Berenguer et al. 2009, Nuche-Berenguer et al. 2010a), supporting earlier findings that suggested the positive effects of GLP-1 on bone.
1.9 AIMS OF THESIS

The primary aim of this thesis was to investigate the impact of diabetes on bone and to characterise the effects of long-acting anti-diabetic incretin analogues on metabolic control, bone strength and quality in diabetes.

The specific objectives of this thesis were:

1. To demonstrate the positive effects of native GIP and [D-Ala²]GIP on human osteoblastic SaOS-2 cells.

2. Preliminary assessment of metabolic control, bone mineral density and bone mineral content in mice lacking GIP and/or GLP-1 receptors.

3. To investigate the effects of [D-Ala²]GIP and Liraglutide treatment on bone mass and cortical mechanical strength in chemically-induced insulin-deficient diabetic mice.

4. To assess the effects of the triple-acting [D-Ala²]GIP-Oxyntomodulin agonist on cortical bone strength at whole- and tissue-level in genetically-induced C57 BL/KsJ diabetic (db/db) mice.

5. To determine the effects of Exendin-4 and Sitagliptin administration on bone microarchitecture and quality in high-fat fed diabetic mice.

6. To provide evidence to support the use of incretin-based therapies for bone abnormalities in diabetes.
Chapter 2

General Materials and Methods
2.1 PEPTIDES

2.1.1 Synthesis of peptides

Peptides used in these studies were all purchased from GL Biochem Ltd. (Shanghai, China), whilst DPP-4 inhibitor was purchased from ApexBio Technology (Houston, TX, USA) with >95% purity. The peptides used were native GIP(1-42), GLP-1(7-36)amide, [D-Ala²]GIP(1-42), [D-Ala²]GIP-Oxm, Liraglutide, Exendin-4 and the DPP-4 inhibitor was Sitagliptin phosphate monohydrate.

2.1.2 Identification and characterisation

All peptides were identified and characterised by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS). Peptide solution 1.5 µl was individually dispensed into different wells of a 100-well stainless steel MALDI plate and allowed to dry at room temperature. Matrix solution 1.5 µl; 10 mg/ml α-cyano-4-hydroxycinnamic acid in acetonitrile (Sigma-Aldrich, UK), was added to the dried peptides and the mixture was again left to dry at room temperature. The molecular mass of the peptides were determined using a Voyager-DE BioSpectrometry Workstation (PerSeptive BioSystems, Framingham, MA, USA) and recorded as mass-to-charge (m/z) ratio vs percentage intensity of peak. Table 2.1 lists the peptides used together with their theoretical and experimental mass.

2.2 CELL CULTURE

2.2.1 SaOS-2 cells

The SaOS-2 cell line was obtained from ATCC. Cells were cultured in MEM alpha medium (1x) without phenol red (Gibco, Invitrogen) supplemented with 10% of fetal bovine serum (FBS) (Lonza) and 1% of penicillin/streptomycin (5000 U/ml, 5000 µg/ml) (Gibco, Invitrogen). All cells were maintained in sterile tissue culture flasks in a controlled atmosphere (37°C, 5% CO₂/95% air). When cells were in confluence, they were washed with hanks buffer saline solution (HBSS) and trypsinised. Trypsin
Table 2.1: Identification and characterisation of peptides by MALDI-TOF MS

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MALDI-TOF MS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical mass (Da)</td>
<td>Experimental mass (Da)</td>
<td></td>
</tr>
<tr>
<td>GIP(1-42)</td>
<td>4983.60</td>
<td>4983.36</td>
<td></td>
</tr>
<tr>
<td>(D-Ala²)GIP(1-42)</td>
<td>4983.60</td>
<td>4984.32</td>
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<tr>
<td>GLP-1(7-36)amide</td>
<td>3297.68</td>
<td>3297.04</td>
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<tr>
<td>Liraglutide</td>
<td>3751.30</td>
<td>3752.64</td>
<td></td>
</tr>
<tr>
<td>(D-Ala²)GIP-Oxm</td>
<td>4473.00</td>
<td>4472.29</td>
<td></td>
</tr>
</tbody>
</table>

2.3 ANIMALS

Animal models of incretin receptor knock-out (KO) as well as chemically-, genetically- and environmentally-induced diabetic rodents were employed in these studies. All experiments were non-blind studies and performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.3.1 GIPR KO mice

Female GIPR KO mice (kindly provided by Professor Bernard Thorens from University of Lausanne, Switzerland) were derived from the C57BL/6J background as outlined previously (Preitner et al. 2004). These mice were individually housed in an air-conditioned room at 22 ± 2 with 12 h light : 12 h dark cycle and had free access to standard rodent chow (10% fat, 30% protein, 60% carbohydrate) and drinking water.

2.3.2 GLP-1R KO mice

Male GLP-1R KO mice (obtained from Dr DJ Drucker from University of Toronto, Canada) were derived from the C57BL/6J colony and individually kept in an air-
conditioned room at 22 ± 2 with 12 h light : 12 h dark cycle and had free access to standard rodent chow (10% fat, 30% protein, 60% carbohydrate) and drinking water.

2.3.3 Double incretin receptor (DIR) KO mice

Female DIR KO mice (kindly given by Professor Bernard Thorens from University of Lausanne, Switzerland) were individually caged in an air-conditioned room at 22 ± 2 with 12 h light : 12 h dark cycle and had free access to standard rodent chow (10% fat, 30% protein, 60% carbohydrate) and drinking water. The C57BL/6J genetic background and characteristics of these mice are described previously (Preitner et al. 2004).

2.3.4 NIH Swiss mice

Male NIH Swiss mice (8 weeks, Harlan Ltd., Blackthorn, UK) were housed individually in an air-conditioned room at 22 ± 2 with 12 h light : 12 h dark cycle and had free access to standard rodent chow (10% fat, 30% protein, 60% carbohydrate) and drinking water.

2.3.5 C57 BL/KsJ diabetic (db/db) mice

Male db/db mice (11 weeks, Harlan LTD., Blackthorn, UK) were caged individually in an air-conditioned room at 22 ± 2 with 12 h light : 12 h dark cycle and had free access to standard rodent diet (10% fat, 30% protein, 60% carbohydrate) and drinking water.

2.3.6 High-fat fed NIH Swiss mice

Male NIH Swiss mice (Harlan Ltd., Blackthorn, UK) were housed individually in an air-conditioned room at 22 ± 2 with 12 h light : 12 h dark cycle and had free access to drinking water and high fat diet (45% fat, 20% protein, 35% carbohydrate; Special Diet Service, Essex, U.K.) for 15 weeks prior to start of studies.
2.3.7 Collection of blood samples

Blood samples were obtained from the cut tips of the tail vein of conscious mice and collected into fluoride coated microvette blood tubes (Sarstedt, Germany). The samples were immediately centrifugated using a microcentrifuge (Beckman Instruments, Galway, Ireland) for 3 min at 13 000 g. The separated plasma was aliquoted into 500 μl eppendorf tubes and kept at -20°C prior to analysis.

2.3.8 Intraperitoneal glucose tolerance test

Pre-injection blood collections were taken (0 min) in 18 h fasted mice before intraperitoneal injection of glucose (18 mmol/kg bw). Blood was then collected at 15, 30 and 60 min post-injection and immediately were centrifuged with a microcentrifuge (Beckman Instruments, Galway, Ireland) at 13 000 g for 3 min. Supernatants were then aliquoted into 500 μl eppendorf tubes and stored at -20°C for subsequent plasma glucose and insulin measurements.

2.3.9 Intraperitoneal insulin sensitivity test

Blood glucose levels were initially recorded using an Ascencia glucose meter (Bayer Contour) at 0 min in non-fasted mice. Bovine insulin (25 or 50 U/kg bw in 0.9% saline) was then intraperitoneally administered and blood glucose sampled at 30 and 60 min post-injection.

2.4 IN VIVO BIOCHEMICAL ANALYSIS

2.4.1 Plasma glucose determination

Plasma glucose concentrations were determined by the glucose oxidase method using Analox analyser GM-9 with Analox instruments glucose reagent. Glucose oxidase catalyses the oxidation of glucose to gluconic acid and the rate of oxygen consumption is directly proportional to concentration of glucose in the sample. The analyser was calibrated with 8 or 25 mmol/l glucose standard until it achieved a
stable reading of the standard used and 5 µl of samples was injected into it. The reading was then recorded.

2.4.2 Plasma insulin determination

Plasma insulin concentrations were determined by a modified dextran-coated charcoal radioimmunoassay (RIA). The methodology for insulin RIA is described below.

Iodinated bovine insulin

Iodogen solution was made by dissolving 100 µg/ml of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril in dichloromethane, followed by dispense of the solvent (100 µl) into Eppendorf tubes. The tubes were left in a fume hood with a stream of helium to allow evaporation of the solvent and leaving a uniform layer of iodogen at the bottom of the tubes. Bovine insulin solution (125 µg/ml) was prepared by diluting a 1 mg/ml solution of bovine insulin (in 10 mM HCl) with 500 mM phosphate buffer. 20 µl of the bovine insulin solution and 5 µl of Na125I (100 mCi/ml stock) was added to the iodogen-coated Eppendorf tubes and left on ice with gentle agitation for 15 min. The iodogen reaction was stopped by removing the reaction mixture into a fresh Eppendorf tube with an addition of 500 µl of 50 mM sodium phosphate buffer. The solution was kept on ice prior to HPLC separation.

HPLC separation was carried out using a VydaC8 analytical column (4.6 x 250 mm) and the mobile phases used were 0.12% (v/w) TFA (in purified H2O) and 0.1% TFA (in 70% acetonitrile-30% purified H2O). The separation programme was set for 67 min and 1 ml fractions were collected by fraction collector (Frac-110, LKB). 5 µl from each fraction was aliquoted into LP3 tubes and radioactivity counts were assessed using a gamma counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter). The fractions with highest counts were kept for antibody-binding tests and then pooled together to be used as the 125I-labelled tracer in the insulin radioimmunoassay.
Modified dextran-coated charcoal radioimmunoassay

Radioimmunoassay stock buffer was prepared by adding an acidic solution of 40 mmol/l sodium dihydrogen orthophosphate to 40 mmol/l of basic disodium hydrogen orthophosphate (containing 0.3% (w/v) sodium chloride and 0.02% (w/v) thimerosal) to achieve a solution with pH 7.4 and stored at 4°C until use. On the day of experimentation, working RIA buffer was made up by dissolving bovine serum albumin (0.5 g/100 ml) in stock RIA buffer pH 7.4. Insulin standards were prepared by serial dilutions (0.039 to 20 ng/ml concentration) from frozen rat insulin stock (40 ng/ml) in working RIA buffer. 180 µl of working RIA buffer was added to 20 µl of unknown plasma samples in LP3 tubes to give a total of 200 µl of unknown sample per tube. Guinea pig insulin antibody was later diluted in working RIA buffer to achieve approximately 40% insulin label binding and 100 µl of the antibody was added to the unknown (duplicate) and standard (triplicate) samples, followed by the addition of 100 µl of ¹²⁵I-labelled insulin (approximately 10,000 cpm/100 µl in working assay buffer) into all samples.

All tubes were incubated at 4°C for 48 hours to allow antibody binding reactions to take place. After which, the separation of free from bound ¹²⁵I-labelled was achieved by adding 1 ml of working dextran-coated charcoal (stock dextran-coated charcoal (5% charcoal solution) diluted with stock RIA buffer in 1:5 dilution) to all tubes (except the total tubes of the standards). All tubes were vortexed and incubated for 20 minutes at 4°C in a cooled centrifugal machine (Beckmann Coulter) and then centrifugated for 20 min at 2500 rpm. The supernatant was discarded and free (unbound) ¹²⁵I-labelled tracer absorbed to black charcoal pellet remained in the tubes. Radioactivity in the tubes was counted using a gamma counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter. The total counts (from total tube) minus radioactivity counts of unbound ¹²⁵I-labelled tracer were inversely proportional to insulin present in the unknown samples and standards. The insulin concentration in the unknown samples was determined from a rat insulin standard curve as shown in Figure 2.1.
2.5 MEASUREMENT OF BODY COMPOSITION, BONE MINERAL DENSITY AND MINERAL CONTENT BY DUAL-ENERGY X-RAY ABSORPTIOMETRY (DEXA) SCANNING

DEXA scanning uses x-ray beams of two different energy levels that pass through the whole body where both energies are absorbed by bones and soft tissues. A radiation detector records reduction in intensity of the x-ray beam and attenuation of high and low energy are calculated. The ratio of attenuation of the two energies allows separation of bone and soft tissues as well as lean and fat mass. Bone mineral density (BMD, g/cm²), bone mineral content (BMC, g), lean mass (g), fat mass (g) and percentage of total fat were measured by a Lunar PIXImus dual-energy X-ray absorptiometry (DEXA) scanner (Inside Outside Sales, Wisconsin, U.S.A.). Mice were anaesthetised with isoflurane and pentobarbitol sodium (10 μl/g bw) and then placed on a specimen tray. The animals were exposed to low energy X-rays and a high-resolution picture (0.18 x 0.18 mm pixel size) is captured of the x-rays hitting a luminescent panel. The whole body was scanned and regions of interest (ROIs), as shown in Figure 2.2, were analysed as described previously (Xie et al. 2005). Quality control was performed on daily intervals before acquiring data and calibration of the DEXA machine was performed using the phantom provided by the manufacturer.
Figure 2.2 Animal images from DEXA scanning and its region of interest (ROI)

Figure 2.2A shows total region, whole body (in green) excluding the head (in red)
Figure 2.2B shows femoral region
Figure 2.2C shows lumbar or lower spine region
Figure 2.2D shows tibial region

2.6 ASSESSMENT ON BONE STRENGTH AND QUALITY

Tibias and femurs were cleaned of tissue and then, extracted femurs were stored in ethanol 70, while excised tibias were kept in a fixative (75% ethanol 95, 10% formaldehyde 37 or 40 and 15% distilled water) for 24 h, followed by in absolute acetone and stored at 4°C until required for microstructural and mechanical strength assessment. Five techniques that were then employed; X-ray microcomputed tomography (microCT), quantitative X-ray imaging (qXRI), three-point bending test,
quantitative back-scattered electron imaging (qBEI) and nanoindentation (Figure 2.3).

**Figure 2.3 General representation of experimental design for excised bones**

![Diagram of experimental design](image)

**2.6.1 Quantitative x-ray imaging (qXRI)**

Bone mineral content at cortical or trabecular bone was evaluated using quantitative x-ray imaging which is based on the absorption of x-rays by mineral content in the bone matrix. The x-ray images of femurs were recorded using a Faxitron MX20 digital radiograph (Edimex, Angers, France) with 4 x magnification at 26 kV. A 1.5-mm thick steel plate and polyester plate were used as standards and aligned together.
with femurs each time x-ray images were captured (Figure 2.4). Images obtained in 16-bit DICOM files were processed by stretching the histogram from grey level 0 (represented by polyester) to grey level 255 (represented by steel) and saved in tiff format. Using 16-color lookup table of ImageJ (National Institute of Health, Bethesda, MD), the frequency of occurrence of grey level at trabecular or cortical bone was calculated and mean grey level (GLmean) and absorbing mineral density (AMD) which represent mineral density were determined as previously described (Bassett et al. 2012, Mabilleau et al. 2015, Mieczkowska et al. 2013). Trabecular bone was measured at the metaphysis (starting just below growth plate and 2 mm down), whilst cortical was measured at the midshaft (3 mm below growth plate until the beginning of crest).

Frequency of occurrence of $i$ grey level ($F_i$) :

$$ F_i = 100 \cdot \left( \frac{N_i}{N_t} \right) $$

$N_i$ = number of pixels with the $i$ grey level  
$N_t$ = total number of pixels

The distribution of frequency represented by grey level was plotted and using the following formula, the mean grey level (GLmean) was obtained :

$$ GL_{\text{mean}} = \sum \frac{F_i \cdot GL_i}{100} $$

$GL_i$ = value of the $i$ grey level
Figure 2.4 Normal and x-ray images from x-ray radiograph

![Image of normal and x-ray images from x-ray radiograph]

**Figure 2.4A** shows normal image of two standards and a femur
**Figure 2.4B** shows an x-ray image of steel (GL = 255), polyester (GL = 0) and bone

### 2.6.2 X-ray microcomputed tomography (µCT)

Bone mass and microstructural morphology of tibia were assessed using a high-resolution Skyscan 1172 microtomograph (Bruker-Skyscan, Kontich, Belgium) which was equipped with an X-ray tube working at 50 kV/100 µA. Bones were individually placed in water-containing Eppendorf tubes to keep the sample hydrated and in order to maintain the specimen at stationary condition in the tube, a damp sponge was positioned at both ends of bone in the tube. The Eppendorf tubes were aligned at the central scanner axis on a carbon bed in the sample’s chamber prior to scanning process in the x-ray chamber. Using a fixed pixel size of 9 µm, rotation step set at 0.3° and exposed with a 0.5 mm aluminium filter, bones were scanned cross-sectionally and distally to produce a series of 2D projection images. This was followed by 3D reconstruction of bones from the projection images.
obtained after removing noise background and interactive thresholding using NRecon software.

CTAn software (release 1.11.4.2, Bruker) was then employed to measure trabecular parameters in the volume of interest (VOI) that was located at tibia metaphysis which is approximately 0.5 mm below growth plate on a height of 2 mm. Variables that were assessed with microCT included; BV/TV (bone volume / trabecular volume, %), Tb.Th (trabecular bone thickness, mm), Tb.N (numbers of trabecular bone, 1/mm) and Tb.Sp (trabecular separation, mm). The measurements were performed according to guidelines on bone microstructure proposed by the American Society for Bone and Mineral Research (Bouxsein et al. 2010).

Assessment was also done on cortical bone thickness (Ct.Th, in µm) and cross-sectional moment of inertia (CSMI, in mm⁴) by measuring the diameter of cortical bone (B.Dm, in mm) and bone marrow (Ma.Dm, in mm) at 3-4 mm below the growth plate using lab-made routine ImageJ software (National Institute of Health, Bethseda, MD), as previously explained (Libouban et al. 2008). The measurements were conducted according to guidelines on bone histomorphometry proposed by the American Society for Bone and Mineral Research (Dempster et al. 2013).

Cortical variables were calculated using the following formula;

\[
Ct.Th = \frac{(B.Dm - Ma.Dm)}{2}
\]

\[
CSMI = \frac{\pi}{64} \cdot \left( (B.Dm)^4 - (B.Dm - Ma.Dm)^4 \right)
\]

Where
B.Dm = the diameter of bone
Ma.Dm = the diameter of bone marrow
Figure 2.5 Setup of microCT

Figure 2.5A shows the microtomography system which consists of sample and x-ray chambers.

Figure 2.5B shows how tubes containing bones aligned on a carbon bed in sample chamber.

Figure 2.5C shows the scout view of bones in x-ray chamber.
2.6.3 Three-point bending test

Mechanical properties of femoral bones were assessed by three-point bending test using an Instron-5942 3-point bending machine (Instron, U.S.A.). Prior to experimental day (24 h), bones were rehydrated in saline at room temperature. Femurs were positioned horizontally with the anterior side facing upward, on top of a pair of rounded grips (10 mm apart) as supports (Figure 2.6B). Using a control panel, vertically-moving crosshead was slowly brought down to the midshaft of the specimen and a pressing force was applied to the femur until the bone breaks. A loading speed of 2 mm min\(^{-1}\) was employed and the load and time taken until bone failure was recorded by the captor. The load-time curve was converted to a force-displacement curve (Figure 2.7), measured by Bluehill 3 software. Mechanical properties derived from the curve were ultimate load, ultimate displacement, stiffness and work to failure as published previously (Turner 2006).
Figure 2.6: Setup of the three-point bending machine

**Figure 2.6A** shows three-point bending instrument connected to a computer with Bluehill 3 software that is responsible for measuring mechanical properties of bone. **Figure 2.6B** shows the position of femur on 10 mm-apart grips and a moving crosshead. **Figure 2.6C** shows control panel of the three-point bending instrument that allows vertical movement of load and captor.
Figure 2.7 Representative of force-displacement curve for three-point bending test

As specimen is subjected to an increasing force, displacement initially increases linearly with the load. However, after a critical value of applied force is reached (yield point), bone undergoes deformation with a relatively small increase in the applied load until it reaches the maximum value. Formation of cracks from innumerable microcracks at midshaft of bone results in lower force required to keep bone elongating further, until it finally breaks. Mechanical properties derived from this graph were extrinsic stiffness represented by the slope of linear region of the curve; total absorbed energy (area under the curve); maximum load (peak of the curve on y-axis), maximum displacement (displacement at maximum force); yield (a transition point, above which force begins to cause permanent damage to bone) and the post-yield energy (area under the curve after yield point).
2.6.4 Nanoindentation

Measurement of mechanical strength of whole bone was performed by three-point bending tests while mechanical properties of bone matrix were investigated by nanoindentation (Mabilleau et al. 2013, Mieczkowska et al. 2013). Blocks of embedded bone were polished with a DiaPro Nap-B diamond particle using Struers Tegramin-30 machine (Struers, Denmark) prior to experimental day and left in saline (NaCl, w/v, 0.9%) for rehydration overnight. The polished block was placed on a stable platform and using microscope, an indentation area 3 mm below the growth plate which is equivalent to cortical bone area was selected. Once the indentation area was chosen, the block was transferred under the indenter. 12 indentations were positioned in cortical bone using NHT-TTX (CSM instrument, Peseux, Switzerland) which was equipped with a Berkowitch pyramidal diamond probe as an indenter. A loading/unloading rate of 40 mN/min was applied to the bone and produced indentations that were 900 nm in depth. In order to prevent creeping of bone material, load was maintained for 15 s at its maximum rate. Depending on properties of bone matrix, maximum force applied to reach the required depth reflected the hardness of the bone. The variables derived from this test were maximum force, indentation modulus, hardness and dissipated energy as described previously (Oliver & Pharr, 1992).

Hardness is calculated from this equation;

\[ H_H \frac{F_{\text{max}}}{A_p} \]

Where
F_{\text{max}} is the maximum force
A_p is the projected contact area
Figure 2.8 The nanoindentation system

**Figure 2.8A** shows the position of PMMA block on a stable platform and under the indenter. **Figure 2.8B** shows the pyramidal indentation as an impact of Berkowitch diamond probe on cortical bone.

Indentation modulus is the initial slope of the unloading section of the curve and obtained from the equation below;

\[
E_{II} = \frac{1 - v_i^2}{E_r - \frac{1 - v_i^2}{E_i}}
\]

Where \( E_{r} = \frac{S \sqrt{\pi}}{2\beta \sqrt[4]{A_p(h_p)}} \) in Pascal

Where

\( E_i \) = Elastic modulus of the indenter (1141 GPa)
\( E_r \) = Reduced modulus of the indentation contact
\( v_i \) = Poisson’s ratio of the indenter (0.07)
\( v_s \) = Poisson’s ratio of the sample
The curve illustrates the different period of time during indentation. During loading, a probe is touching and penetrating bone where there is a combination of elastic and deformation properties. When maximum penetration is achieved (900 nm), there is a 15 s plateau phase maintained to avoid creeping deformation or plasticity. Subsequently at the unloading phase, the probe is retracted and the curve reflects elasticity of the sample. Mechanical parameters obtained from this curve are hardness (a measure of resistance to permanent deformation), indentation modulus (initial slope of unloading curve), maximum force (load that reached maximum penetration) and dissipated energy (area between loading and unloading curves).

2.6.5 Quantitative backscattered electron imaging (qBEI)

Scanning electron microscope scans sample using a focused beam of electrons which interact with atoms in the specimen and produce various signals. These signals carry information on the composition or topography at the surface of the sample and produce an image. Back-scattered electron (BSE) is one of these signals where beams of electrons are reflected from the sample and the signal is closely related to the atomic number (Z) of the specimen. From assessment on quantitative backscattered electron imaging and in combination with ImageJ software (National Institute of Health, Bethesda, MD), bone mineral density distribution (BMDD) can be determined (Mieczkowska et al. 2013, Roschger et al. 1998).
Prior to the experimental day, polymethylmethacrylate blocks were polished with DiaPro Nap-B diamond particles (Struers, Denmark) to get 1-µm finishing and then coated with carbon. The blocks were observed with a scanning electron microscope (EVO LS10, Carl Zeiss Ltd., Nanterre, France) (Figure 2.10A) which was equipped with a five quadrant semi-conductor backscattered electron detector and at least four images were taken from the cortical bone area. The electron microscope was operated at 20 keV with a 250 pA probe current at working distance of 15 mm. Calibration of the backscattered electron signal (grey scale) was achieved using pure carbon (Z = 6, mean grey level = 25), pure aluminium (Z = 13, mean grey level = 225) and pure silicon (Z = 14, mean grey level = 253) as standards (Micro-analysis Consultants Ltd., St. Ives, UK).

The adjustments in brightness and contrast of those reference materials established a correlation of atomic number (Z)(y-axis) and BSE gray level (x-axis). Using hydroxylapatite, $\text{Ca}_{10}$$\text{(PO}_4)_6$$\text{(OH)}_2$ (Z = 14.06) composition, the BSE gray level histogram was converted to weight percentage of calcium. The integrity of the instrument was checked every 15 min by imaging these standards. The region of interest was imaged at 200x magnification (0.5 µm per pixel) and Using ImageJ software, change in grey levels distribution, also known as bone mineral density distribution (BMDD) was determined from 3 parameters; $\text{Ca}_{\text{peak}}$ which reflects the most frequent calcium content of the bone area, $\text{Ca}_{\text{mean}}$ which is the mean calcium content of the bone area obtained from area under the curve and $\text{Ca}_{\text{width}}$ which is the heterogeneity of mineralisation measured at 50% of maximum Ca level.
Figure 2.10 Representative of scanning electron microscope (A), PMMA blocks (B) and scanning image of trabecular bone (C)

Figure 2.10A shows scanning electron microscope system that was used in quantitative backscattered electron imaging technique

Figure 2.10B shows proximal longitudinal section of embedded bone in polymethylmethacrylate (PMMA) blocks

Figure 2.10C shows microarchitectural image of trabecular bone captured by scanning electron microscope
2.7 STATISTICAL ANALYSIS

Results are expressed as mean ± SEM together with number of observations. Values were compared by repeated measures ANOVA or one-way ANOVA with Student-Tukey or New-Keuls post hoc test or two-tailed unpaired t-test using GraphPad Prism 5. A P value of < 0.05 was considered to be statistically significant.
Chapter 3

Effects of GIP peptides on SaOS-2 cells \textit{in vitro} and genetic deficiencies of GIP and/or GLP-1 receptors on bone mass \textit{in vivo}
3.1 SUMMARY

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino acid incretin peptide that regulates blood glucose levels by stimulating insulin secretion following food intake. GIP receptors (GIPRs) are predominantly found in pancreas however recently, the presence of active GIPRs has been revealed on bone cells. The physiological actions of GIP are limited as the molecule is rapidly degraded by dipeptidyl peptidase 4 (DPP-4). In order to circumvent DPP-4 activity, stable GIP peptides have been developed. This study investigated the effects of native GIP, and a long-acting GIP analogue namely [D-Ala²]GIP, on biomarkers of bone formation, alkaline phosphatase (AlkP) activity and cyclic adenosine monophosphate (cAMP) generation in human osteoblastic-like SaOS-2 cells. Preliminary in vivo studies on the effects of GIP and its sister incretin, glucagon-like peptide 1 (GLP-1) on bone were also carried out by assessing bone mineral density (BMD) and bone mineral content (BMC) in mice lacking GIP and/or GLP-1 receptors. SaOS-2 cells were incubated with GIP peptides (10⁻¹² - 10⁻⁶ M) for 8 h and both native GIP and [D-Ala²]GIP significantly stimulated IGF-1 secretion (1.2-1.4-fold, P < 0.01) at all concentrations tested. In harmony, both peptides significantly (1.1-1.3-fold, P < 0.01 to P < 0.001) induced TGF-β release, but only [D-Ala²]GIP was effective at the lowest concentration (10⁻¹² M) tested. Native GIP and [D-Ala²]GIP significantly increased (1.6-2.3-fold, P < 0.05 to P < 0.01) cAMP production, with [D-Ala²]GIP being more potent. AlkP activity in SaOS-2 cells was enhanced after 24 h incubation with [D-Ala²]GIP (1.1-fold, 10⁻¹₀ – 10⁻⁶ M, P < 0.01) and native GIP (1.1-fold, 10⁻⁶ M, P < 0.01) when compared to control cultures. Moreover, following 48 and 72 h incubation, [D-Ala²]GIP was significantly (P < 0.05 to P < 0.001) more potent than native GIP in terms of augmenting AlkP activity at all concentrations examined. Further investigation of the effects of GIP on bone density and content revealed that GIPR KO mice had significantly reduced (P < 0.01) total BMD as well as at femur (P < 0.001) and tibia (P < 0.01) BMD. In parallel, BMC at those regions was also decreased (P < 0.05 to P < 0.001). BMD and BMC in GLP-1R KO mice were not significantly different compared to normal mice. The absence of both incretin receptors markedly decreased (P < 0.05) BMD and BMC at the femur while only BMD was significantly reduced (P < 0.01) at the tibia. The data demonstrates that
GIP has clear anabolic effects on bone cells and this is supported by the appearance of impaired bone quality in GIPR- and double incretin receptor-deficient mice.

3.2 INTRODUCTION

Glucose-dependent insulinoitropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are released in response to food ingestion. The primary effect of these hormones is to enhance the secretion of insulin in a glucose-dependent manner and hence, control postprandial glucose concentrations. Interestingly, previous studies reveal the presence of functional GIP receptors, but not GLP-1 receptors on bone (Bollag et al. 2000, Zhong et al.2007), suggesting a direct action of GIP on skeleton. GIPRs are predominantly found in pancreatic tissue, however the expression of GIP receptors are also detected in other tissues such as bone (Baggio & Drucker 2007).

The first evidence of GIP receptors on bone was reported in normal rat bone as well as in established human osteoblastic-like osteosarcoma cell lines, the SaOS-2 and MG-63 (Bollag et al. 2000). Previous studies have shown that exposure to GIP results in an increase in bone-forming biomarkers, collagen type 1 expression and alkaline phosphatase activity from osteoblast-derived cells (Bollag et al. 2000). Furthermore, a lower percentage of apoptotic bone cells was seen in the presence of GIP in vitro (Tsukiyama et al 2006), indicating the protective effects of GIP on bone cells. Further examination in GIPR KO mice found that deficiencies in GIPR signalling not only reduced bone size and mass, but also detrimentally affected bone microarchitecture and strength (Xie et al. 2005). Conversely, increased bone mass was seen in transgenic mice with overexpression of GIP (Xie et al. 2007). A more recent study demonstrated that the reduction of bone strength in GIPR-deficient mice is associated with alterations of material properties of bone matrix (Mieczkowska et al. 2013).

Bone remodeling is a complex process which involves communication between osteoblasts and osteoclasts, where old bone is removed by osteoclasts and replacement of new bone by osteoblasts (Matsuo & Irie 2008). Although extensive studies have been conducted in determining the effects of GIP on osteoblastic cells, much less information is available on the effects of GIP on bone-resorbing osteoclast
cells. GIP has been shown to inhibit osteoclast activity and differentiation (Zhong et al. 2007) and in agreement with these findings, bone-resorbing biomarkers were reduced in GIP-overexpressing transgenic mice (Xie et al. 2007), indicating the positive action of GIP in reducing bone resorption. Similar to its glucoregulatory action in pancreas, GIP exerts its effects on bone through activation of cAMP- and Ca\textsuperscript{2+}-dependent pathways (Bollag et al. 2000). Collectively, these studies show that GIP has dual actions on bone turnover by enhancing bone formation and reducing bone resorption.

GIP has only short half-life in rodents and humans (Deacon et al. 2000, Kieffer et al. 1995). The intact biologically active peptide is rapidly degraded by dipeptidyl peptidase 4 (DPP-4). To circumvent this, DPP-4-resistant GIP molecules with prolonged bioactivity have been developed (Hinke et al. 2002, Irwin et al. 2005a). [D-Ala\textsuperscript{2}]GIP is recognised as an enzyme-resistant GIP agonist which improves glucose tolerance and insulin release in high-fat fed mice (Hinke et al. 2002, Lamont & Drucker 2008). This study was undertaken to investigate and characterise the potential beneficial effects of stable [D-Ala\textsuperscript{2}]GIP on the release of TGF-\textbeta and IGF-1, cAMP production and alkaline phosphatase activity in human osteoblastic-derived SaOS-2 cells as compared to native GIP(1-42). Effects of genetic deficiencies of GIPRs and GLP-1Rs on metabolic parameters, total and regional bone mineral density (BMD) and bone mineral content (BMC) were also assessed.
3.3 MATERIALS AND METHODS

3.3.1 Peptides

Native GIP(1-42) and [D-Ala²]GIP(1-42) were purchased from GL Biochem Ltd. (Shanghai, China) and the peptides were characterised by MALDI-TOF mass spectrometry as described in Section 2.1.2. Identity of the peptides was confirmed by comparing values for experimental mass obtained from MALDI-TOF and calculated theoretical masses.

3.3.2 Maintenance of SaOS-2 cells

SaOS-2 cells were maintained as described in Section 2.2.1. Culture medium was changed every 3 days and the cells were utilised at 80% confluency. The assays were run in duplicate and repeated 2-3 times according to previous studies (Bollag et al. 2000, Zhong et al. 2003).

3.3.3 Animals and study design

3.3.3.1 GIPR KO mice

In order to characterise the mice, glucose tolerance tests (18 mmol/kg bw glucose alone or in combination with 25 nmol/kg bw GIP) were performed on GIPR KO and respective control mice (from C57BL/6J colony) as explained in Section 2.3.8. Following GTT, mice were anaesthetised with isoflurane and pentobarbitol sodium (10 μl/g b.w) and the whole body was scanned using DEXA imaging.

3.3.3.2 GLP-1R KO mice

Glucose tolerance tests (18 mmol/kg bw glucose alone or in combination with 25 nmol/kg bw of GLP-1) were conducted as described in Section 2.3.8. GLP-1R KO mice and its controls (both from the C57BL/6J background) were then injected with isoflurane and pentobarbitol sodium (10 μl/g b.w) prior to whole body scanning by DEXA imaging.
3.3.3.3 Double Incretin receptor (DIR) KO mice

Mice were characterised from glucose tolerance tests (18 mmol/kg bw glucose alone or in combination with GIP or GLP-1 (each at 25 nmol/kg bw)) as outlined in Section 2.3.8. All mice were administered with isoflurane and pentabarbitol sodium (10 μl/g bw) prior to DEXA scanning.

3.3.4 Measurement of TGF-β

SaOS-2 cells were seeded at a density of 2 x 10^5 cells/well and incubated in media supplemented with 10% FBS until confluent. 24 h prior to addition of GIP peptides, media was changed with fresh 0.1% FBS-containing media. On the day of experimentation, media was removed and 1 ml of GIP peptides (10^{-12} - 10^{-6} M) was added to each well. Plates were then incubated for 8 h. After which, media was collected and TGF-β released in the supernatant measured using a TGF-β Immunoassay kit (Quantikine, R&D Systems). The concentrations of TGF-β were calculated from a standard curve of recombinant human TGF-β ranging from 0 to 2000 pg/ml.

3.3.5 Measurement of IGF-1

SaOS-2 cells were seeded at a density of 2 x10^5 cells/well in 6-well plates and allowed to reach confluency in 10% FBS-containing media. Media was changed with fresh media supplemented with 0.1% FBS 24 h prior to addition of various peptides. Media was removed, followed by an addition of 1 ml of GIP peptides (10^{-12} - 10^{-6} M). Plates were then incubated at 37°C for 8 h. After which, media was collected and IGF-1 released in the supernatant measured using IGF-1 Immunoassay kit (Quantikine, R&D Systems). The concentrations of IGF-1 in the samples was determined from a standard curve of recombinant human IGF-1 in the range of 0 - 60 ng/ml.
3.3.6 Measurement of bone alkaline phosphatase activity

SaOS-2 cells were seeded at a density of $1 \times 10^5$ cells/well in 6-well plates and cultured for 72 h in growth media. 24 h prior to addition of peptides, media was replaced with 2% FBS-containing media. Cells were incubated with varying concentrations of GIP or related analogue ($10^{-6}$ - $10^{-12}$ M) in media supplemented with 2% FBS, 1% penicillin/streptomycin. After the desired incubation period (24, 48 or 72 h), cells were washed 3 times with PBS (Oxoid, England). 250 µl of 0.2% Nonidet-40 (NP-40) (Sigma) was then added into each well and plates were left on an orbital shaker for 10 min. Cells were then scraped using a cell scraper (Costar). The cellular material was homogenized by 3 cycles of freeze (-70°C)-thawing (37°C). The mixture of cells/0.2% NP-40 was collected in 500 µl eppendorf tubes and snap-frozen in liquid nitrogen. The tubes were then placed on a shaker for 16 h at 4°C and for a final disruption and homogenisation of cells, contents of the tubes were sonicated using a Soniprep 150 Plus ultrasonic disintegrator for 15s. The sonicated samples were then centrifugated (15 min, 13000g, 4°C) and supernatant was collected for alkaline phosphatase activity and total protein contents as previously explained (Mahoney et al. 2008).

3.3.6.1 Alkaline phosphatase determination

Samples (50 µl) were added to 96-well plates in duplicate and alkaline phosphatase activity was indirectly determined using 4-methyl umbelliferyl phosphate (Sigma) as the substrate. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 100 µl of 0.6 M Na$_2$CO$_3$ (Sigma-Aldrich). Alkaline phosphatase cleaves the phosphate group of the non-fluorescent 4-methylumbelliferyl phosphate (MUP) and generates highly fluorescent and stable 4-methyl umbelliferone (MU). Fluorescence was measured at excitation wavelength of 360 nm and an emission wavelength of 450 nm (with cut off 435 nm) using a FlexStation 3 (Molecular Devices). Alkaline phosphatase activity was then calculated from a standard curve (0-1000 pmol) of 4-methyl umbelliferone (Aldrich).
3.3.6.2  Total protein determination

Total protein content in samples used to assay alkaline phosphatase activity was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). Briefly, 25 μl of sample and standard were added to a 96-well plate in duplicate and 200 μl of reagent AB (50:1) added. The plates were incubated at 37°C for 30 min and left for 5 min at room temperature. The plates were spectrophotometrically read at 562 nm using a microplate reader (Molecular Devices) and total protein content was calculated according to a bovine serum albumin standard curve (0-1500 μg/ml). Alkaline phosphatase activity was normalised against total protein content per well and was expressed as pmol 4-MU/μg protein.

3.3.7  Measurement of cyclic AMP

SaOS-2 cells were seeded at a density of 5 x 10^4 cells in 96-well plates and cultured in α-MEM 1X media (Invitrogen) supplemented with 10% FBS (Lonza), penicillin and streptomycin for 24h to allow attachment to plate. Prior to experimentation, cells were washed with HBSS (Invitrogen). Cells were then incubated with various concentrations (10^{-12}-10^{-6} M) of GIP and longer-acting GIP, [D-Ala^2]GIP supplemented with 200 μM of 3-isobutyl-1-methylxanthine (IBMX). The plate was left for 40 min at 37°C and after that, cells were washed 3 times with 150 μl of PBS. Cells were then lysed with 150 μl cell lysis buffer (R&D Systems) and a freeze (-20°C)-thaw (37°C) cycle was carried out. The contents were then collected into 500 μl eppendorf tubes and centrifugated at 600 g for 10 min (4°C). Supernatants were collected and cAMP was measured using a cAMP assay kit (R&D Systems). The concentration of cAMP was calculated from a standard curve of cAMP ranging from 0 to 240 pmol/ml.

3.3.8  Measurement of plasma glucose concentration

Plasma glucose was determined using an Analox GM-9 glucose analyser as explained in Section 2.4.1.
3.3.9 Measurement of body composition, bone density and mineral content by DEXA scanning

Unconscious mice were placed on a specimen tray of the DEXA scanner and the whole body was scanned. Bone mineral density (BMD), bone mineral content (BMC), lean mass, fat mass and percentage of total fat were measured as explained in Section 2.5.

3.3.10 Statistical analysis

Data were analysed using repeated measures one-way or two-way ANOVA with Newman-Keuls post hoc tests or two-tailed t-tests using PRISM 5.0. Data are expressed as mean ± S.E.M and a P value < 0.05 was considered statistically significant.

3.4 RESULTS

3.4.1 Dose-dependent effects of native GIP and [D-Ala²]GIP on TGF-β1 and IGF-1 release from SaOS-2 cells

Native GIP and [D-Ala²]GIP dose-dependently stimulated TGF-β release from SaOS-2 cells (Figure 3.1). As compared to control, [D-Ala²]GIP significantly induced TGF-β release at all concentrations tested (10⁻¹² – 10⁻⁶ M, P < 0.01) while stimulatory effect of native GIP was seen only at higher concentration (10⁻¹⁰ – 10⁻⁶ M, P < 0.01). Similarly, IGF-1 was also shown to increase in the presence of native GIP and [D-Ala²]GIP (Figure 3.2). Both native and stable GIP demonstrated to induce higher IGF-1 release than control incubations at all concentrations employed (10⁻¹² – 10⁻⁶ M, P < 0.01). However, the stimulatory effect on TGF-β and IGF-1 release was not significantly different between native and long-acting GIP.
3.4.2 Dose-dependent effects of native GIP and [D-Ala²]GIP on alkaline phosphatase activity in SaOS-2 cells

Both GIP(1-42) and [D-Ala²]GIP enhanced AlkP activity during a 24 h incubation in SaOS-2 cells (Figure 3.3). Long-acting [D-Ala²]GIP increased alkaline phosphatase activity at concentrations of 10⁻¹⁰ M and above (P < 0.05) while native GIP was only effective at 10⁻⁶ M (P < 0.01) compared to controls. No significant differences were observed between GIP(1-42) and [D-Ala²]GIP in stimulating alkaline phosphatase activity during 24 h incubation time. Stimulation by both GIP peptides continued to elevate the activity of alkaline phosphatase during 48 and 72 h incubations. Compared to respective control, 48 and 72 h exposure of SaOS-2 to [D-Ala²]GIP significantly increased the activity of the bone-related protein at all concentrations tested (Figure 3.4 & 3.5). Native GIP demonstrated increased alkaline phosphatase activity only at 10⁻⁶ M (P < 0.01) and higher concentrations after 48 h and the lowest effective concentration after 72 h incubation was 10⁻¹⁰ M (P < 0.05). There were significant differences seen in bone-specific alkaline phosphatase activity at all concentrations employed between native GIP and [D-Ala²]GIP after 48 h (P < 0.01) and 72 h (P < 0.01) incubation periods (Figure 3.4 & 3.5).

3.4.3 Dose-dependent effects of native GIP and [D-Ala²]GIP on cAMP production in SaOS-2 cells

As shown in Figure 3.6, native GIP and [D-Ala²]GIP significantly stimulated cAMP production in SaOS-2 cells compared to controls. The lowest effective concentration of [D-Ala²]GIP was 10⁻⁸ M (P < 0.05) while for native GIP this was 10⁻⁶ M (P < 0.01). [D-Ala²]GIP significantly enhanced cAMP generation at concentration of 10⁻⁶ M (P < 0.05) when compared to GIP(1-42).

3.4.4 Comparison of body composition in GIPR, GLP-1R, DIR KO and normal mice

Body weight and percentage total fat mass (Figure 3.7 & 3.10) of GIPR- and GLP-1R-deficient mice not significantly different compared to normal mice. Similar to the
single KO mice, the absence of both incretin receptors had no significant effect on body weight and total fat mass in DIR KO mice (Figure 3.13).

### 3.4.5 Comparison of glucose tolerance in GIPR, GLP-1R, DIR KO and normal mice

Intraperitoneal administration of glucose alone modestly reduced the glycaemic excursion and plasma glucose AUC in GIPR KO mice compared to normal controls (Figure 3.8). In the presence of exogenous GIP, normal mice had a significant reduction (37% decrease, \( P < 0.05 \)) in plasma glucose AUC values, whilst it was unchanged in GIPR-deficient mice. As shown in Figure 3.11, deficiency in GLP-1R signalling significantly impaired glucose tolerance as seen by a notable increase (2.6 fold, \( P < 0.001 \)) in plasma glucose AUC values of GLP-1 KO mice compared to wild type mice. Glucose supplemented with GLP-1 significantly reduced (32% decrease, \( P < 0.05 \)) plasma glucose AUC value in normal mice, but not in GLP-1R KO mice. Similarly, DIR KO mice exhibited a significant elevation (1.5 fold, \( P < 0.05 \)) in plasma glucose level when given glucose load compared to sex- and age-matched controls (Figure 3.14). Wild type mice received exogenous GIP or GLP-1 had noticeable reductions (30-57% decrease, \( P < 0.05 \)) in plasma glucose AUC values, while the parameter was unchanged in DIR KO mice.

### 3.4.6 Comparison of bone mineral density and mineral content in GIPR, GLP-1R, DIR KO and normal mice

As shown in Figure 3.9, bone mineral density (BMD) and bone mineral content (BMC) as assessed by DEXA were lowered in GIPR knockout (KO) mice when measured as total as well as at femur, lumbar and tibia areas. Significant reductions were seen in total (BMD: 8% decrease, \( P < 0.01 \); BMC: 13% decrease, \( P < 0.05 \)), femur (BMD: 16% decrease, \( P < 0.001 \); BMC: 15% decrease, \( P < 0.001 \)) and tibia (BMD: 14% decrease, \( P < 0.01 \); BMC: 15 decrease, \( P < 0.001 \)) of GIPR-deficient mice. In contrast, deficiencies in GLP-1Rs did not adversely affect either parameter and although total BMC appeared to be reduced, the reduction did not reach statistical significance (Figure 3.12). Total, femoral, lumbar and tibial BMD (Figure 3.15A) were all decreased in DIR KO mice with significant reductions only found at
femur (8% decrease, P < 0.05) and tibia (27% decrease, P < 0.01). No differences were observed at total, lumbar and tibial BMC but femoral BMC was markedly reduced in DIR KO mice (11% decrease, P < 0.05, Figure 3.15B).

3.5 DISCUSSION

Bone turnover is controlled by numerous hormones and growth factors through both endocrine and paracrine signaling. Following food ingestion, bone resorption biomarkers are rapidly changed (Henriksen et al. 2003), suggesting a role of the gastrointestinal tract (GIT) in bone remodeling. Glucose-dependent insulino tropic polypeptide (GIP) is one of hormones released from the GIT and in addition, GIP receptors are found on bone cells (Bollag et al. 2000, Zhong et al. 2007), suggesting a direct role of GIP on bone. Although GIP receptors are widely distributed, most studies on GIP are focus on its potential to stimulate glucose-mediated pancreatic insulin release. To date, only a few studies have been carried out to establish the effects of GIP on bone and the mechanism of action of GIP on the skeleton remain largely unknown.

The potential of GIP as therapeutic peptide is restricted by the fact that it is a substrate for dipeptidyl peptidase 4 (DPP-4) and once release into circulating blood, DPP-4 cleaves alanine residue at second position of biologically intact GIP, generating the truncated metabolite of GIP, GIP(3-42), with no biological activity (Baggio & Drucker 2007). In order to circumvent DPP-4 action, several N-terminally-protected GIP analogues have been generated (Hinke et al. 2002, Irwin et al. 2005a). From a series of N-terminal modifications of GIP peptide, [D-Ala\(^2\)]GIP has been identified as one of the most promising analogues (Hinke et al. 2002). The potential anabolic effect of native GIP on bone cells has been shown in earlier studies (Bollag et al. 2000, Bollag et al. 2001, Zhong et al. 2007). However, the effects of prolonged activation of the GIPR by stable GIP molecules on the skeleton are less well known.

Receptor binding studies have shown the presence of GIP receptors on osteoblastic-like cell lines, namely SaOS-2 and MG-63 (Bollag et al. 2000) and therefore, the SaOS-2 cell line was chosen in this study. Previously, GIP was reported to stimulate
transforming growth factor-β (TGF-β) release from osteoblastic-derived cells the MG-63 (Zhong et al. 2003). TGF-β is a product of osteoblasts and found abundantly in bone matrix (Robey et al. 1987). TGF-β plays an important role in bone formation by stimulating osteoblastic proliferation and differentiation of MSCs (Filvaroff et al. 1999, Janssens et al. 2005, Lee et al. 2002, Tang et al. 2009). As expected, TGF-β concentrations significantly increased in the presence of native GIP and its stable analogue in SaOS-2 cells. However, the stimulatory effect of GIP was only observed at lower concentrations (10^{-10} M) in our study, probably due to the longer incubation period employed as compared to previous studies. In line with its prolonged half-life, [D-Ala²]GIP significantly stimulated TGF-β release at the lowest concentration tested (10^{-12} M), indicating enhanced bioactivity.

Another growth factor that is found in bone matrix is insulin-like growth factor-1 (IGF-1). The role of IGF-1 in bone is not limited to stimulation of preosteoblastic proliferation and differentiation (Giustina et al. 2008; Xian et al. 2012), but also to enhanced production of bone matrix and bone mineralisation (Zhang et al. 2002), leading to a further increase in osteoblastic function and bone formation. Similar to TGF-β release, 8-hour incubation of SaOS-2 cells with native GIP and [D-Ala²]GIP resulted in significant elevations of IGF-1 levels in comparison to control cultures. However, there were no significant differences seen in stimulatory effects of the two GIP peptides in terms of IGF-1 secretion.

Osteoblastic-related protein, alkaline phosphatase is widely used as a biomarker for bone formation (Eastell & Hannon 2008). In our study, alkaline phosphatase activity appeared to increase following 24 h incubation with GIP peptides. When the incubation period was extended to 48 and 72 h, the dose-dependent stimulatory effect of [D-Ala²]GIP on alkaline phosphatase activity was at least 30% more prominent than native GIP. Interestingly, beneficial effect of [D-Ala²]GIP appeared less prominent at 72, as opposed to 48 h incubations. A similar pattern on alkaline phosphatase activity was seen when GIP was incubated for 6 days with the osteoblastic-like MG-63 cell line (Bollag et al. 2000). One possible explanation for this is cell death which could be due to exhaustion of the alkaline phosphatase signaling pathway. However, a cell viability assay was not conducted to confirm
this. Alkaline phosphatase is an osteoblastic-related protein actively involved in bone mineralisation by reducing extracellular inorganic pyrophosphate, a suppressor of hydroxyapatite crystals (Golub & Boesze-Battaglia 2007, Hessle et al. 2002). The fact that GIP can significantly induce alkaline phosphatase activity, indicates the hormone could regulate bone mineralisation.

The GIPR is a member of the seven-transmembrane domain-spanning receptors, also known as G-protein coupled receptor (GPCR) superfamily and activation of the receptor by its ligand stimulates adenylyl cyclase, which subsequently increases intracellular cAMP levels (Baggio & Drucker 2007). Therefore, it is not surprising to see that in the presence of GIP, cAMP generation was enhanced in osteoblastic-derived cells, as previously reported (Bollag et al. 2000). Consistent with this earlier study, we found dose-dependent increases in cAMP levels in cultured SaOS-2 cells with both GIP peptides at concentration as low as 10^-8 M. Furthermore, the cAMP response to the DPP-4 resistant [D-Ala^2]GIP was noticeably increased (approx. 28% increase) at 10^-6 M compared to native GIP, further confirming increased potency of [D-Ala^2]GIP. Unlike native GIP, modification at the N-terminal of [D-Ala^2]GIP protects this analogue from DPP-4 activity. The increase in [D-Ala^2]GIP stability permits longer GIP receptor activation and as a result, there is a significant rise in cAMP production at higher [D-Ala^2]GIP concentration.

We used mice that were genetically deficient in GIP, GLP-1 or both receptors to assess the effects of incretins on bone in vivo. Assessment of body composition found that body weight and fat content were not affected in mice lacking either GIPRs, GLP-1Rs or both receptors. Intraperitoneal administration of glucose induced modest reduction in the glycaemic excursion in GIPR KO mice compared to normal littermates. In agreement with previous observations, GLP-1R and DIR KO mice exhibited a significant impairment of glucose homeostasis (Preitner et al. 2004, Scrocchi et al. 1996). In the present study, GIPR KO mice had better glucose tolerance than GLP-1R KO mice when compared with their respective controls. The reason could be that there is a compensatory mechanism of insulin secretion in GIPR KO mice, as previously reported (Pamir et al. 2003). In order to confirm the genetic background of the KO mice, glucose supplemented with either GIP and/or GLP-1 was intraperitoneally injected into wild type and KO mice.
Bone mineral density (BMD), measured by DEXA, is frequently used to determine bone health and it represents bone mass of that particular individual (Cummings et al. 2002). In the present study, suppression of GIPRs alone clearly altered BMD and BMC in mice when measured at total bone and at regions of interest. Importantly, similar observations have been seen in age-matched GIPR KO mice from a different genetic background in a previous study (Xie et al. 2005) and this is further strengthened by recent findings that observed reduced mechanical properties of bone matrix in GIPR-deficient mice on the same background as mice in the current study (Mieczkowska et al. 2013). In the study done by Xie et al., serum biomarkers were also assessed and it was found that osteocalcin and alkaline phosphatase levels were significantly reduced in mice lacking GIPR. Osteocalcin is produced by osteoblasts (Lee et al. 2000) and a reduction in osteocalcin levels indicates that osteoblast activity is reduced, which in turn reflects a decrease in rate of bone formation. As discussed earlier, alkaline phosphatase plays an important role in bone mineralisation (Golub & Boesze-Battaglia 2007) and reduction in alkaline phosphatase concentrations shows less mineralisation of bone. Therefore, inactivation of GIPR signaling causes disruption in the molecular processes of bone formation, leading to bone impairments as seen in GIPR KO mice.

On the other hand, deficiency in GLP-1Rs appeared to have no effect on BMD and BMC in our study. In contrast, previous studies reported that bone densitometry, morphology and mechanical properties were modified in mice lacking GLP-1Rs, leading to reduced bone strength (Mabilleau et al. 2013, Yamada et al. 2008). However, bone quality is determined not only by bone mass, but also on the microstructural and material properties of bone matrix (Seeman & Delmas 2006). Thus, investigation of bone morphology and mechanical strength in other studies confirm the positive effects of GLP-1R signaling on bone integrity. According to Yamada and colleagues, modifications in bone quality of GLP-1R-deficient mice were due to enhanced bone resorbing activity as demonstrated in increased number of osteoclasts and elevated markers for bone resorption, deoxypyridinoline (Yamada et al. 2008). Single KO of either GIPR or GLP-1R diminished bone microarchitecture and strength by attenuating bone formation or enhancing bone resorption. Therefore, it would be expected that simultaneous inactivation of both incretin receptors signaling pathways would severely compromise skeletal integrity,
as observed by pronounced reductions of bone mass in DIR KO mice in the present study.

In the present study, we conclude that GIP has beneficial effects on osteoblastic cells. In addition, the presence of functional GIP and GLP-1 receptors is essential to maintain normal bone remodeling as bone impairments were demonstrated in mice lacking incretin receptors. Diabetes is linked to bone loss (Vestergaard 2011) and this leads to subsequent complications such as fracture. GIPRs are expressed on bone, forming a direct interaction of GIP to bone cells and it would be interesting to examine the effects of DPP-4-resistant GIP analogues on bone strength and quality in animal models of diabetes. Although the presence of its receptors on bone cells is uncertain, the sister incretin of GIP, glucagon-like peptide-1 (GLP-1) is also suspected to positively regulate bone turnover. Furthermore, GLP-1R-deficient mice appear to have reductions in bone mass and bone mineral density, increased bone resorption as well as reduced mechanical properties and bone strength (Mabilleau et al. 2013, Yamada et al. 2008). Therefore, it would be interesting to assess the possible impact of clinically established GLP-1 mimetics on bone microstructure and strength in animal model of diabetes.
Figure 3.1: Effect of increasing concentrations of GIP peptides on TGF-β release from SaOS-2 cells

SaOS-2 cells were grown in 0.1%-FBS-containing media and stimulated with indicated concentrations of peptides. Cultures were incubated for an additional 8 h and TGF-β levels were measured using recombinant human TGF-β Immunoassay kit. Values are mean ± SEM for n=4 and experiment was repeated twice. **P < 0.01, ***P < 0.001 compared with control group.
Figure 3.2: Effect of increasing concentrations of GIP peptides on IGF-1 release from SaOS-2 cells

SaOS-2 cells were grown in 0.1%-FBS-containing media and stimulated with indicated concentrations of peptides. Cultures were incubated for an additional and IGF-1 levels were measured using recombinant human IGF-1 Immunoassay kit. Values are mean ± SEM for n=4 and experiment was repeated twice. ***P < 0.001 compared with control group.
SaOS-2 cells were grown in 6-well plates and stimulated with indicated concentrations of peptides. After 24 h, the reaction was stopped and AlkP production was indirectly measured using 4-methyl umbelliferyl phosphate as substrate. The values were normalised against total amount of protein/well using BCA protein kit. Values are mean ± SEM for n=6 and experiment was repeated three times. *P < 0.05, **P < 0.01, *** P < 0.001 vs control.
SaOS-2 cells were grown in 6-well plates and stimulated with indicated concentrations of peptides. After 48 h, the reaction was stopped and AlkP production was indirectly measured using 4-methyl umbelliferyl phosphate as substrate. The values were normalised against total amount of protein/well using BCA protein kit. Values are mean ± SEM for n=6 and experiment was repeated three times. **P < 0.01, ***P < 0.001 vs control; ΨP < 0.05, ΨΨP < 0.01, ΨΨΨP < 0.001 vs native GIP.
Figure 3.5: 72 h stimulation of alkaline phosphatase activity by various concentrations of GIP peptides in SaOS-2 cells

SaOS-2 cells were grown in 6-well plates and stimulated with indicated concentrations of peptides, with replenishment of fresh media every 24 h. After 72 h, the reaction was stopped and AlkP production was indirectly measured using 4-methyl umbelliferyl phosphate as substrate. The values were normalised against total amount of protein/well using BCA protein kit. Values are mean ± SEM for n=6 and experiment was repeated three times. *P < 0.05, **P < 0.01, ***P < 0.001 vs control; ΨP < 0.05, ΨΨP < 0.01 vs native GIP.
Figure 3.6: Effects of different concentrations of GIP peptides on cAMP generation in SaOS-2 cells

SaOS-2 cells were seeded and induced with different concentrations of GIP peptides for 40 min. cAMP release was then measured using a commercially available cAMP assay kit. Values are mean ± SEM for n=4 and experiment was repeated twice. *P < 0.05, **P < 0.01 vs control. ψ P < 0.05 vs GIP.
Figure 3.7: Body weight and percentage of total fat in normal and GIPR KO mice

Body weight (A) and total fat (B) of 27 week-old mice was measured by dual energy X-ray absorption before necropsy. Values are expressed as mean ± S.E.M for 5 mice per group.
Figure 3.8: Comparison of glucose tolerance in normal and GIPR KO mice

A)

Plasma glucose concentrations (A) were measured prior to and after intraperitoneal administration of glucose alone (18 mmol/kg, control) or in combination with GIP (25 nmol/kg body weight) in normal and GIPR KO mice. Plasma glucose AUC values (determined by using mean value at 0 min as a baseline) for 0-60 min post-injection are also shown (B). Injection time is indicated by the arrow (0 min). Values are mean ± SEM for 5 mice. *P < 0.05 compared with normal glucose alone.
Figure 3.9: Comparison of (A) bone mineral density and (B) bone mineral content of total bone area, femur, tibia and lumbar regions in normal and GIPR KO mice.

A)

BMD (g/cm³)

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B)

BMC (g)

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BMD and BMC were measured by dual energy X-ray absorption before necropsy. Values are mean ± SEM for 5 mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared with normal group.
Figure 3.10: Body weight and percentage of total fat in normal and GLP-1R KO mice

Body weight (A) and total fat (B) of 43 week-old mice was measured by dual energy X-ray absorption before necropsy. Values are expressed as mean ± S.E.M for 4 mice per group.
Figure 3.11: Comparison of glucose tolerance in normal and GLP-1R KO mice

A) Plasma glucose concentrations (A) were measured prior to and after intraperitoneal administration of glucose alone (18 mmol/kg, control) or in combination with GLP-1 (25 nmol/kg body weight) in normal and GLP-1-r KO mice. Plasma glucose AUC values (determined by using mean value at 0 min as a baseline) for 0-60 min post-injection are also shown (B). Injection time is indicated by the arrow (0 min). Values are mean ± SEM for 4 mice. *P < 0.05, ***P < 0.001 compared with normal + glucose alone, ΔΔΔP < 0.01 compared with normal + glucose + GLP-1.
Figure 3.12: Comparison of (A) bone mineral density and (B) bone mineral content of total bone area, femur, tibia and lumbar regions in normal and GLP-1R KO mice

A)

BMD (g/cm³)

Control  GLP-1R KO

B)

BMC (g)

Control  GLP-1R KO

BMD and BMC were measured by dual energy X-ray absorption before necropsy. Values are mean ± SEM for 4 mice.
Figure 3.13: Body weight and percentage of total fat in normal and DIR KO mice

A)

Body weight (g)

Normal  |  DIR KO

0  |  30

B)

Percentage of total fat (%)

Control  |  DIR KO

0  |  25

Body weight (A) and total fat (B) 29 week-old mice was measured by dual energy X-ray absorption before necropsy. Values are expressed as mean ± S.E.M for 4-5 mice per group.
Figure 3.14: Comparison of glucose tolerance test in normal and DIR KO mice

Plasma glucose concentrations (A) were measured prior to and after intraperitoneal administration of glucose alone (18 mmol/kg, control) or in combination with GIP or GLP-1 (each at 25 nmol/kg body weight) in normal and DIR KO mice. Plasma glucose AUC values (determined by using mean value at 0 min as a baseline) for 0-60 min post-injection are also shown (B). Injection time is indicated by the arrow (0 min). Values are mean ± SEM for 4-5 mice. *P < 0.05 compared with normal + glucose alone; △P < 0.05, △△P < 0.01 compared with respective normal group.
Figure 3.15: Comparison of (A) bone mineral density and (B) bone mineral content of total bone area, femur, tibia and lumbar regions in normal and DIR KO mice.

BMD and BMC were measured by dual energy X-ray absorption before necropsy. Values are mean ± SEM for 4-5 mice. *P < 0.05, **P < 0.01 compared with normal group.
Chapter 4

Recovery of cortical bone strength at the tissue level by [D-Ala²]GIP and Liraglutide in streptozotocin-induced insulin-deficient diabetic mice
4.1 SUMMARY

Impaired insulin production has adverse effects on bone integrity, consequently there is increased bone fracture risk in diabetic individuals. There is now accumulating evidence suggesting that the gut hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) play a role in the maintenance of bone strength and quality. This study assessed the effects of subchronic administration of the stable incretin receptor mimetics, [D-Ala²]GIP and Liraglutide, on metabolic control and bone structural integrity in streptozotocin (STZ)-induced insulin-deficient diabetic mice. STZ (150 mg/kg bw) was used to induce diabetes (blood glucose level > 12 mmol/l), five days prior to commencement of treatment intervention. Diabetic mice then received once-daily i.p injection of saline (0.9% w/v NaCl), [D-Ala²]GIP or Liraglutide (each at 25 mmol/kg bw) for 21 days. [D-Ala²]GIP and Liraglutide had no effects on body weight, water intake, non-fasting plasma glucose and insulin concentrations. Although no significant differences were seen in plasma glucose concentrations compared to saline STZ controls, glucose tolerance had a tendency to be improved in [D-Ala²]GIP- and Liraglutide-treated mice. This slight improvement was due to a significant augmentation of glucose-stimulated plasma insulin levels in mice that received either [D-Ala²]GIP (2.3-fold, P < 0.05) or Liraglutide (3.3-fold, P < 0.01). On the other hand, treatment with [D-Ala²]GIP and Liraglutide did not affect insulin sensitivity in diabetic mice. In general, streptozotocin-induced diabetic mice displayed severe impairments of cortical bone as seen through reductions of cortical mineral content, cortical thickness and mechanical properties of whole bone and bone matrix, whilst trabecular bone was unaffected. Treatment with [D-Ala²]GIP or Liraglutide did not affect whole body or individual bone masses. Similarly, no changes were seen in mineral content of cortical and trabecular bone in diabetic mice when assessed by qXRI. Parameters for trabecular bone microstructure as determined by microCT were unchanged with [D-Ala²]GIP or Liraglutide therapy. Three-point bending tests revealed no dramatic changes in whole-bone strength between treated and nontreated diabetic mice. However, defects in mechanical tissue properties of diabetic mice as examined by nanoindentation were ameliorated by [D-Ala²]GIP and Liraglutide where there were increases observed in hardness (35-49% increase, P < 0.01),
indentation modulus (31% increase, \( P < 0.001 \)), maximum load (35-43% increase, \( P < 0.001 \)) and dissipated energy (41% increase, \( P < 0.01 \)) compared to diabetic controls. Importantly, mechanical tissue responses were elevated to levels similar to normal mice. Nonetheless, the improvements in intrinsic material properties were independent of bone mineralisation. In conclusion, three-week administration of long-acting incretin hormones improved glucose tolerance and enhanced recovery of cortical bone strength at the tissue-level in STZ-induced insulin-deficient diabetic mice.

4.2 INTRODUCTION

Insulin deficiency has detrimental effects on bone, resulting in higher bone fragility as reported in diabetes (Hofbauer et al. 2007). A role for gastrointestinal tract (GIT) in controlling bone turnover has been suggested as serum markers for bone resorption are reduced following feeding (Henriksen et al. 2003). Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are two gut hormones suspected to be implicated in bone turnover. GIP and GLP-1 are incretin peptides that regulate postprandial glucose homeostasis by enhancing glucose-mediated insulin release from islet beta-cells (Yabe & Seino 2011). However, native GIP and GLP-1 are rapidly degraded by dipeptidyl peptidase-4 (DPP-4), resulting in a short half-life (approx. 3-5 minutes) (Baggio & Drucker 2007). In order to protect them from DPP-4 activity, stable GIP and GLP-1 analogues have been synthesised (Hinke et al. 2002, Irwin et al. 2005a, Knudsen et al. 2000, O’Harte et al. 2002).

The GLP-1-based mimetic drug, Liraglutide is an established antidiabetic drug already available on the market since 2010 (Peterson & Pollom 2010). Liraglutide exhibits 97% sequence homology to human GLP-1 and it differs from native GLP-1 through substitution of Lys\(^{34}\) with Arg and addition of C-16 acyl chain at Lys position 26 (Knudsen et al. 2000). This GLP-1 agonist is proven to be resistant to DPP-4 and not subjected to renal clearance through its ability to bind to albumin. Therefore, it is suitable for once daily administration (Agerso et al. 2002, Knudsen et al. 2000). There is also growing interest in targeting sister incretin of GLP-1, GIP, as potential therapeutic agent for management of T2DM. Several N-terminally protected GIP peptides have been developed and examined for glucose-lowering and
insulin-releasing activities, and [D-Ala²]GIP has been reported to be one of the most promising molecule (Hinke et al. 2002).

Interestingly, GIPRs and GLP-1Rs are widely distributed outside the pancreas, suggesting their extrapancreatic actions (Baggio & Drucker 2007). Functional GIPRs are evidenced on bone cells that are responsible for bone formation and bone resorption, osteoblasts and osteoclasts, respectively (Bollag et al. 2000, Zhong et al. 2007). Deficiencies of GIPR signalling in mice resulted in significant reductions of bone mass, reduced biomarkers for bone formation and increased bone resorption biomarkers (Tsukiyama et al. 2006, Xie et al. 2005). In parallel, decreased bone resorption biomarkers were seen in transgenic mice overexpressing GIP (Xie et al. 2007). Furthermore, GIP inhibited pit formation by mature osteoclasts and as a result, osteoclastic bone resorptive activity was reduced (Zhang et al. 2007). Collectively, the data from Chapter 3 and previous studies suggested the anabolic role of GIP in enhancing bone formation and inhibiting bone resorption. Therefore, GIP may present a therapeutic approach for bone-related diseases (Irwin et al. 2010).

Despite inconsistencies in previous studies on the expression of GLP-1Rs on bone (Bollag et al. 2000, Mabilleau et al. 2013, Nuche-Berenguer et al. 2010a), bone histomorphometrical analyses found increased numbers of osteoclasts and bone resorption activity in GLP-1R-deficient mice (Yamada et al. 2008). Further examination showed that genetically-modified GLP-1R knockout mice exhibited significantly lowered material properties and consequently, reduced bone strength compared to normal mice (Mabilleau et al. 2013). Taken together, the data indicate the importance of GLP-1 and GLP-1R signaling on normal bone function.

In Chapter 3 of this thesis, native GIP and [D-Ala²]GIP have been shown to have anabolic effects on bone in vitro and genetic deficiencies in incretin receptors lead to alterations of BMD and BMC. Based on previous studies, it is suggested that incretin therapies not only improve glucose homeostasis in diabetes but additionally, they could improve diabetic bone abnormalities. Furthermore, it would be interesting to identify the main target of gut hormones on bone and characterise their impact on bone regulation. Therefore, in the present study, we employed [D-Ala²]GIP and Liraglutide to examine the effects of once-daily administration on metabolic
parameters and more importantly on bone microstructure, cortical geometry and bone strength in STZ-induced insulin-deficient diabetic mice.

4.3 MATERIALS AND METHODS

4.3.1 Synthesis of (D-Ala²)GIP and Liraglutide

[D-Ala²]GIP and Liraglutide were purchased from GL Biochem Ltd. (Shanghai, China) at 97.4% and 95.8% purity, respectively. The peptides were characterised by MALDI-TOF mass spectrometry (Table 2.1) as described in Section 2.1.2.

4.3.2 Animals and study design

The chronic effect of the GIPR agonist, [D-Ala²]GIP and the GLP1R mimetic, Liraglutide on metabolic control and bone quality were examined in streptozotocin (STZ)-induced diabetic mice. Young male NIH Swiss mice (n=30, 8 weeks old) initially received 150 mg/kg bw of STZ in citrate buffer to develop diabetes. Normal mice (n=6) were also injected with citrate buffer alone. 6 days post-STZ introduction, the mice were divided into three groups (n=10) and started receiving once daily injection of [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg bw) or saline vehicle (0.9% NaCl) for 21 days, whilst normal mice received saline vehicle (0.9% NaCl) once daily for the same period of time. Body weight, food consumption, fluid intake, non-fasting blood glucose and insulin concentrations were monitored at regular intervals. Intraperitoneal glucose tolerance test (18 mmol/kg bw, 18-h fast) and non-fasting insulin sensitivity test (25 U/kg bw) were performed at the end of the study as outlined in Section 2.3.8 and 2.3.9, respectively. No adverse effects were seen following administration of [D-Ala²]GIP and Liraglutide. All experiments were carried out according to UK Home Office Regulations (UK Animals Scientific Procedures Act 1986).
4.3.3 Measurement of plasma glucose and insulin

Plasma glucose was determined using an Analox GM-9 glucose analyser as explained in Section 2.4.1, while insulin RIA was carried out as described in Section 2.4.2 to determine plasma insulin concentrations.

4.3.4 Measurement of body composition, bone density and mineral content by DEXA scanning

Unconscious mice were placed on a specimen tray of DEXA scanner and the whole body was scanned. Bone mineral density (BMD), bone mineral content (BMC), lean mass, fat mass and percentage of total fat of ROIs were determined as explained in Section 2.5.

4.3.5 Assessment of bone quality and strength

Bone microarchitectural and mechanical strength tests were carried out on excised femurs or tibias from all groups of mice at the end of the study.

4.3.5.1 Quantitative X-ray Imaging (qXRI)

Each femur was scanned and imaged together with two standards, 1.5 mm-thick steel and polyester which represent grey level 255 and 0, respectively. Bone mineral content was determined as detailed in Section 2.6.1.

4.3.5.2 X-ray microcomputed tomography (µCT)

Tibias were positioned and scanned as described in Section 2.6.2 and parameters that were obtained from the assessment were trabecular bone mass, trabecular bone thickness, numbers of trabecular bone, trabecular separation, cortical bone diameter, bone marrow diameter, cortical thickness and cross-sectional moment of inertia.
4.3.5.3 Three-point bending test

Femurs were individually kept in test tubes containing saline (NaCl, 0.9%) for rehydration and left overnight at 4°C. The test was carried out as described in Section 2.6.3 and four mechanical properties were evaluated; ultimate load, ultimate displacement, stiffness and total absorbed energy (work-to-failure).

4.3.5.4 Nanoindentation

Prior to experimentation, polymethylmethacrylate blocks were polished with diamond particles to obtain a smooth surface and the blocks were then left in saline (NaCl, 0.9%) at 4°C for 15 h. Nanoindentation was performed as outlined in Section 2.6.4 and maximum force, hardness, indentation modulus and dissipated energy were computerized from force-penetration depth curve.

4.3.5.5 Quantitative backscattered electron imaging (qBEI)

The polymethylmethacrylate blocks were polished with diamond particles, followed by carbon-coating process. Subsequently, the blocks were observed under scanning electron microscope as detailed in Section 2.6.5.

4.3.6 Statistical analysis

Data were analysed using repeated measures one-way or two-way ANOVA with Tukey post hoc tests or two-tailed t-tests using PRISM 5.0. Data are expressed as mean ± S.E.M and a P value < 0.05 was considered statistically significant.
4.4 RESULTS

4.4.1 Effects of once-daily administration of [D-Ala²]GIP or Liraglutide on metabolic parameters in streptozotocin-induced diabetic mice.

As shown in Figure 4.1, body weight of diabetic mice significantly decreased (30% decrease, P < 0.001) from day 4 post-STZ injection compared to normal mice. Neither [D-Ala²]GIP nor Liraglutide administration altered body weight of STZ-induced diabetic mice (Figure 4.1). As compared to lean mice, diabetic mice demonstrated increased cumulative food intake which appeared significant (P < 0.05) on day 18 while water intake continued to increase (P < 0.05) from day 4 onwards. No differences were observed in food intake (Figure 4.2) and water intake (Figure 4.3) in saline-, Liraglutide- and [D-Ala²]GIP-treated diabetic mice. STZ treatment significantly increased plasma glucose levels (5-fold, P < 0.001, Figure 4.4A) and the increase was accompanied by a significant reduction in insulin concentrations (4-fold, P < 0.001, Figure 4.4B) compared to lean littermate mice. Treatment with [D-Ala²]GIP or Liraglutide for 21 days did not improve non-fasting plasma glucose concentrations and insulin levels compared to untreated STZ mice (Figure 4.4).

4.4.2 Effects of once-daily administration of [D-Ala²]GIP or Liraglutide on glucose tolerance and glucose-induced plasma insulin release in streptozotocin-induced diabetic mice.

Daily administration of [D-Ala²]GIP or Liraglutide induced no significant effects on glycaemic response at every time point tested following glucose loading (Figure 4.5A). Although overall improvements in glucose tolerance were observed, as seen by the reduction of plasma glucose AUC (0-60 min) in diabetic mice treated with [D-Ala²]GIP or Liraglutide, the differences did not reach statistical significance. On the other hand, plasma insulin concentration in response to glucose loading significantly increased 15 minutes post-injection in Liraglutide- treated mice compared to saline control group (48% increase, P < 0.05, Figure 4.6A). No differences were seen in plasma insulin levels at any time point in mice treated with [D-Ala²]GIP compared to the control group (Figure 4.6A). Overall plasma insulin response as measured by
integrated AUC (0-60 min) were augmented in mice treated with [D-Ala₂]GIP (57% increase, P < 0.05) and Liraglutide (70% increase, P < 0.01) compared with saline controls (Figure 4.6B).

4.4.3 Effects of once-daily administration of [D-Ala²]GIP or Liraglutide on insulin sensitivity in streptozotocin-induced diabetic mice.

Treatment with [D-Ala²]GIP or Liraglutide had no significant effect on blood glucose concentrations following exogenous administration of insulin (Figure 4.7). The overall effect, shown from blood glucose AUC (0-60min) values, was enhanced in mice receiving [D-Ala²]GIP or Liraglutide. However, the beneficial effect was not significant compared to saline-treated diabetic controls (Figure 4.7B).

4.4.4 Effects of [D-Ala²]GIP and Liraglutide on total body fat, bone mineral density (BMD) and bone mineral content (BMC) in streptozotocin-induced diabetic mice.

Once daily administration of [D-Ala²]GIP or Liraglutide for 21 days had no significant effect on percentage of total body fat compared to streptozotocin-induced diabetic mice (Figure 4.8). All three STZ groups exhibited decreased total body fat mass (55 - 60% decrease, P < 0.001) compared to lean mice. Total BMD and BMC were not significant in all four groups, whilst BMD and BMC at specific bone regions of interest appeared to be slightly decreased in diabetic mice compared to normal littermates, but the differences did not reach significance (Figure 4.10-4.12). Total as well as BMD and BMC at femur, tibia and lumbar regions in mice injected with [D-Ala²]GIP or Liraglutide showed no differences compared with saline-treated diabetic and normal mice (Figure 4.9-4.12).

4.4.5 Effects of [D-Ala²]GIP and Liraglutide on femoral mineral content in streptozotocin-induced diabetic mice.

Femoral mineral content, represented by mean grey level, was determined by quantitative x-ray imaging at cortical (Figure 4.13) and trabecular (Figure 4.14) bone. Grey level at cortical bone in diabetic mice was significantly decreased (9%
decrease, P < 0.01, Figure 4.13B) compared to normal littermates. However, there were no significant differences seen in GLmean values between [D-Ala²]GIP-, Liraglutide- and saline-treated STZ mice. As seen in Figure 4.14A, trabecular bone in STZ-induced diabetic mice was slightly modified. However, bone mineral content was not significantly different in diabetic and normal mice (Figure 4.14B). Additionally, no apparent changes were observed with [D-Ala²]GIP or Liraglutide treatment (Figure 4.14).

4.4.6 Effects of [D-Ala²]GIP and Liraglutide on trabecular bone morphology and cortical bone geometry in streptozotocin-induced diabetic mice.

Trabecular bone morphology and cortical bone geometry were investigated using high resolution microCT. Trabecular bone volume, thickness, numbers and separation were not affected by STZ-induced diabetes as there were no significant differences between STZ-treated and normal mice (Figure 4.15). [D-Ala²]GIP and Liraglutide therapy did not change these parameters. On the other hand, cortical bone appeared to be altered in diabetic mice. Diabetic control mice had increased diameter of bone marrow (18% increase, P < 0.05, Figure 4.16B) accompanied by reduction in cortical thickness (17% decrease, P < 0.001, Figure 4.16C) as compared to normal mice. Cortical bone diameter was unchanged, whilst CSMI values were lowered, but not significant in STZ-mice. Once daily administration of [D-Ala²]GIP or Liraglutide over 21 days had no effect on cortical diameter, thickness or CSMI, whilst bone marrow diameter in Liraglutide-treated diabetic mice was slightly reduced compared to diabetic mice that received saline vehicle (Figure 4.16B).

4.4.7 Effects of [D-Ala²]GIP and Liraglutide on whole-bone mechanical properties in streptozotocin-induced diabetic mice.

As assessed by three-point bending test, diabetic mice demonstrated reduced ultimate force, ultimate displacement, stiffness and work-to-failure but only the differences in ultimate force (20% decrease, P < 0.01, Figure 4.17A) and stiffness (22% decrease, P < 0.01, Figure 4.17C) were significant compared to lean littermates. None of the parameters investigated were markedly different in [D-Ala²]GIP-, Liraglutide- and saline-treated diabetic groups (Figure 4.17).
4.4.8 Effects of [D-Ala²]GIP and Liraglutide on nanomechanical properties of cortical bone matrix in streptozotocin-induced diabetic mice.

As compared to normal mice, a significant reduction was seen in hardness (30% decrease, $P < 0.01$), indentation modulus (18% decrease, $P < 0.001$), maximum load (24% decrease, $P < 0.001$) and dissipated energy (20% decrease, $P < 0.05$) of cortical bone in STZ-induced diabetic controls. Treatment with Liraglutide or [D-Ala²]GIP increased hardness (35-49% increase, $P < 0.01$, Figure 4.18A), indentation modulus (31% increase, $P < 0.001$, Figure 4.18B), maximum load (35-43% increase, $P < 0.001$, Figure 4.18C) and dissipated energy (39% increase, $P < 0.01$, Figure 4.18D) compared to control diabetic mice. Furthermore, elevations in all four parameters in treated diabetic mice reached levels similar to lean littermates (Figure 4.18).

4.4.9 Effects of [D-Ala²]GIP and Liraglutide on bone mineral density distribution in streptozotocin-induced diabetic mice.

All three variables of bone mineral density distributions; $Ca_{\text{peak}}$, $Ca_{\text{mean}}$ and $Ca_{\text{width}}$ at cortical parts of the tibia in diabetic mice were similar to lean controls. Treatment with [D-Ala²]GIP or Liraglutide had no significant effects on BMDD (Figure 4.19).
4.5 DISCUSSION

Bone loss in insulin-deficient diabetic patients can lead to severe complications such as bone fracture and mortality (Gulcelik et al. 2011, Zhokouskaya et al. 2014). Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are well-known incretin hormones that are involved in the regulation of glucose homeostasis postprandially (Yabe & Seino 2011). The primary action of these peptides is on pancreas to glucose-dependently stimulate islet beta-cell insulin secretion which leads to reduction of blood glucose concentrations after food ingestion (Baggio & Drucker 2007). The widespread expression of incretin receptors in brain, adipose tissue and bone (Bollag et al. 2000, Goke et al. 1995, Usdin et al. 1993, Yip et al. 1998, Zhong et al. 2007) suggests multiple actions of incretins at extrapancreatic sites. However, biological actions of incretins are limited as the gut hormones are rapidly inactivated by DPP-4 enzyme (Baggio & Drucker 2007). In order to protect against N-terminal cleavage of incretins, structurally modified incretin agonists have been developed (Hinke et al. 2002, Irwin et al. 2005a, Knudsen et al. 2000, O’Harte et al. 2002).

In Chapter 3, native GIP and its stable analogue [D-Ala²]GIP were shown to have positive effects on stimulating bone-formation biomarkers. Additionally, initial assessment of bone quality found that animal model of incretin KO mice had altered bone mass. These findings supported previous studies that showed deficiencies in GIPR function resulted in reduced bone microarchitecture and strength (Mieczkowska et al. 2013, Xie et al. 2005). On the other hand, less studies on GLP-1 action on bone have been published. To date, GLP-1 is believed to positively affect bone as GLP-1R-deficient mice were observed to have higher bone-resorbing activity and impaired bone quality (Mabellieau et al. 2013, Nuche-Berenguer et al. 2009, Yamada et al. 2008). In view of these observations, the present study examined the effects of once-daily administration of long-acting [D-Ala²]GIP and Liraglutide peptides on metabolic parameters, bone micromorphology and bone strength and quality in streptozotocin-induced insulin-deficient diabetic mice.

Streptozotocin (STZ) is a chemical that is toxic to pancreatic beta cells (Lenzen 2008) and is extensively used to induce hyperglycaemia and hypoinsulinemia in
mouse (Coe et al. 2013) and rat (Horcajada-Molteni et al. 2001) models. In line with observations from other studies, STZ introduction leads to hyperglycaemia and hypoinsulinemia 2 days post STZ delivery, and glucose and insulin levels remained extremely high and low, respectively, compared to lean mice. Body weight was also reduced in STZ-treated rodents (Graham et al. 2011, Lu et al. 1998, Motyl & McCabe 2009) and in agreement with previous reports, the reduction in body weight resulted from a decrease in fat mass (Coe et al. 2013). Loss of body weight was however independent of water and food intake as both parameters continued to elevate until the end of experimental period and this is consistent with diabetic hyperphagia and polydipsia.

Once daily intraperitoneal administration of [D-Ala²]GIP or Liraglutide for 21 days had no effect on body weight or feeding behavior in diabetic mice. This is not surprising as a high single dose of STZ used in our protocol causes rapid beta-cell destruction which is unlikely to be corrected by the relatively short period of treatment employed. Additionally, there were no changes in non-fasted plasma glucose and insulin levels in STZ-induced diabetic mice, as opposed to other studies which reported improved glycaemic excursion with Liraglutide and [D-Ala²]GIP therapy (Porter et al. 2010, Porter et al. 2011). This could simply be due to frequency of drug administered and different level of severity of diabetic model employed. As such, other studies used twice-daily injection in high-fat dietary induced obesity and insulin resistance mice, whilst our study opted for once-daily treatment in more severe model of STZ-induced diabetes.

Glucose tolerance had tendency to be improved as seen by reductions of plasma glucose concentrations and significantly augmented glucose-induced plasma insulin levels after 21 days administration of Liraglutide or [D-Ala²]GIP in diabetic mice. However, due to the extremely high plasma glucose concentrations (approx. 25 mmol/l) in 18-h fasted STZ-induced diabetic mice, significant glucose-stimulated plasma insulin release was insufficient to fully alleviate hyperglycaemia. Non-fasted plasma insulin concentrations were seemingly low throughout the experimental period (Figure 4.4). However, higher levels of plasma insulin were observed when an exogenous glucose load was intraperitoneally administered (Figure 4.6) at the end
of the study, suggesting glucose-dependent insulin-stimulating effect of these analogues. Indeed, Liraglutide has previously been shown to improve beta cell mass and function (Tamura et al. 2015). Although not as effective as GLP-1 agonist, [D-Ala²]GIP has a tendency to also increase beta cell mass (Maida et al. 2009). Unlike human beta-cells, murine pancreatic beta cells also have the potential to regenerate following STZ administration (Bonner-Weir et al. 1981, Wang et al. 1994) which could partially explain the increase in insulin production observed in STZ-induced diabetic mice when compared to db/db mice.

As reported previously, rodent models of STZ-induced diabetes display bone loss (Botolin & McCabe 2007, Coe et al. 2013, Horcajada-Molteni et al. 2001, Nyman et al. 2011). In our study, initial assessment of bone was performed using DEXA and similar to previous observations, bone loss was seen in diabetic mice. Although the differences in bone mineral density and content did not reach significance, BMD and BMC were clearly reduced at femoral, tibial and lumbar regions of mice with pharmacologically induced diabetes in comparison to normal controls. In line with what we have seen in DEXA scanning, GLmean which represents mineral content was also reduced at cortical, but not trabecular bone of diabetic mice. Insulin has anabolic effects on bone (Cornish et al. 1996, Thomas et al. 1998) and it is most likely that hypoinsulinemia in STZ-induced diabetic mice leads to bone defects. In addition, hyperglycaemia is associated with formation of advanced glycation end (AGE) products (Saito & Marumo 2010) and AGEs are reported to inhibit the function of bone-forming osteoblast cells and induce bone resorption activities by osteoclasts, resulting in excessive bone loss (Alikhani et al. 2007, McCarthy et al. 1997, Miyata et al. 1997, Yamagishi 2011).

In contrary to available literature (Nyman et al. 2011), only cortical bone was severely affected in streptozotocin-induced diabetic mice. An explanation for this could be the different duration of diabetes in each study. Bones were excised and preserved after 4 weeks of hyperglycaemia in the current study, whilst the minimum duration of diabetic bones examined in aforementioned study was 10 weeks. In the present study, cortical bone thickness was significantly reduced and this reduction was accompanied by a significant increase in bone marrow diameter with an unchanged outer bone diameter. However, Nyman and colleagues did not assess
bone marrow diameter in their study. Yet, others propose that diabetic bone loss is due to an increase in bone marrow adiposity which was supported by elevations in biomarkers of adipocytes in mouse models of STZ-induced diabetes (Botolin et al. 2005). More importantly, adipocyte volume in bone marrow was also reported to increase in osteoporotic human bone in iliac crest bone biopsies (Justesen et al. 2001), indicating the similarity in rodent and human bone loss. Therefore, the changes in cortical geometry in this study were probably due to less bone formation, resulting from enhanced differentiation of progenitors of mesenchymal stem cells to bone marrow adipogenesis. In addition to changes in cortical thickness, CSMI, which is a biomechanical indicator of the ability of bone to resist bending, was also reduced in diabetic mice (Nyman et al. 2011). Similarly, a modest reduction in resistance of cortical bone to bending was seen in our work study. Chronic administration of \( [D{-}Ala^{2}]GIP \) did not improve bone microstructure and morphology in STZ-induced diabetic mice. However, the diameter of bone marrow was slightly improved with Liraglutide therapy.

Bone integrity is characterised by both bone quantity and strength. While quantity of bone is dependent on skeletal mass, bone strength is defined by a combination of all factors that contribute to resistance in skeletal fracture, such as microstructure, geometry as well as quality of minerals and collagens (Licata 2009). Assessment of bone microstructure with microCT revealed defective cortical bone, with unaffected trabecular bone in diabetic mice. Apart from improvement in marrow diameter by Liraglutide, neither \( [D{-}Ala^{2}]GIP \) nor Liraglutide therapy had favourable effects on the other variables measured for microstructure and morphology. Therefore, further examination by three-point bending and nanoindentation were done to assess the biomechanical properties of cortical bone in diabetic mice. At the whole-bone level, STZ-induced diabetic mice had alterations in mechanical properties and this was shown by the reductions of ultimate force and stiffness. In parallel, size-independent material properties of bone matrix were also reduced in diabetic mice as compared to normal littermates. The abnormalities in bone strength are not surprising as hyperglycaemia provides a suitable condition for irreversible non-enzymatic modifications of bone collagen, which in turn alter bone matrix structural and mechanical properties and can lead to high bone fragility (Saito & Marumo 2010).
Treatment with [D-Ala$^2$]GIP or Liraglutide for 21 days did not induce noticeable changes of size-dependent mechanical properties measured of cortical long bone in diabetic mice. In contrast, material properties at the tissue-level were restored to lean control levels by [D-Ala$^2$]GIP and Liraglutide. Our findings are consistent with a previous study that observed increased bone material properties with another stable GIP analogue, N-AcGIP, in rats and that the primary target of GIP is at cortical bone (Mabilleau et al. 2014). Insulin deficiency contributes to bone loss and we hypothesised that augmented insulin concentrations by GIP and GLP-1 may have beneficial effects on bone. However, daily circulating insulin levels were very low and not improved by the treatments in this experiment. Therefore, we can say that the beneficial effects of [D-Ala$^2$]GIP or Liraglutide on bone are independent of the insulin axis. These improvements were also not dependent on calcium deposition, as bone mineral density distribution was similar in treated and nontreated diabetic mice.

One could argue that duration of the study should be extended to observe more significant effects on bone microarchitecture. The opportunity to study STZ diabetic mice depends on the extent of beta cell destruction, which indicates severity of diabetes and its complications. In the present study, single injection of high-dose STZ was used to initiate immediate insulin-producing beta cell destruction and to demonstrate clear effects of insulin-deficient diabetes to bone. Due to the rapid onset and severity of beta cell loss in this experimental model, it was not possible to keep STZ diabetic mice for longer period without implementing insulin therapy, which itself may affect bone. Additionally, potential effects of STZ on incretin function need to be considered. GLP-1 and GIP primarily enhances insulin release from beta cells and the destruction of islet beta cells by STZ indirectly reduces the capacity of incretins in stimulating insulin production. Apart from that, no evidence for a direct toxicity of STZ on incretins has been reported. In the present study, treatment started on day 6 after STZ introduction and due to a short biological half-life of STZ (5-15 min) (Eleazu et al. 2013), this glucoseamine derivative may have been fully metabolised when incretin-based analogues were administered.

As mentioned earlier, there are multiple factors that influence bone strength such as bone mass, microstructure, morphology, mineralisation and organic phase. As the first four factors were not improved in our study, changes in organic status which
was not assessed in this study could be the reason of recovery of material properties of bone matrix. Indeed, a previous study found that increased tissue-level mechanical properties of bone were accompanied by increased collagen maturity by N-AcGIP (Mabilleau et al. 2014). However, the exact mechanism by which GIP regulates bone mineral and organic materials is still unknown. On the other hand, activation of GLP-1R was reported to modulate bone through a calcitonin-pathway in rodent (Yamada et al. 2008) and interestingly, calcitonin has been previously shown to inhibit the expression and activity of proteins responsible for extracellular matrix degradation (Sondergaard et al. 2006). Therefore, it is possible that improvements in bone matrix hardness and stiffness seen with Liraglutide therapy in our study could be due to calcitonin.

In this study, we reveal that STZ-diabetic mice exhibited impaired cortical geometry as well as reduced bone mechanical properties, ultimately resulting in low bone strength. It was also demonstrated that both stable incretin peptides had no effect on cortical mass and micromorphology in diabetic mice. However, a recovery process in bone strength was seen through improvement of intrinsic tissue material properties in STZ-induced diabetic mice treated with [D-Ala²]GIP and Liraglutide. With regards to GIP and GLP-1 analogues employed, both [D-Ala²]GIP and Liraglutide had equal potential in optimising bone quality at the tissue level. Additionally, it would be interesting to investigate the osteoprotective effects of prolonged and simultaneous activation of GIP and GLP-1 receptors in genetically-induced animal models of diabetes.
Figure 4.1: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on body weight in STZ-induced diabetic mice

Body weight was measured at 2-3 day intervals, starting 6 days prior to STZ delivery (150 mg/kg b.w, indicated by the arrow) until 26 days post STZ-injection. Mice were treated once daily with saline vehicle (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w) for 21 days (horizontal black bar). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice. ***P < 0.001 compared with saline normal group.
Figure 4.2: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on cumulative food intake in STZ-induced diabetic mice

Food intake was measured at 2-3 day intervals, starting 6 days prior to STZ delivery (150 mg/kg b.w, indicated by the arrow) and until 26 days post-STZ injection. Mice received daily treatment with saline vehicle (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w) for 21 days (horizontal black bar). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice. *P < 0.05, **P < 0.01 compared with saline normal group.
Figure 4.3: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on fluid intake in STZ-induced mice

Water intake was measured at 2-3 day intervals, starting 6 days prior to STZ delivery (150 mg/kg b.w, indicated by the arrow) until 26 days post-STZ injection. Mice were injected once daily with saline vehicle (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w) for 21 days (horizontal black bar). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice. *P < 0.05, ***P < 0.001 compared with saline normal group.
Figure 4.4: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on non-fasting plasma glucose (A) and insulin (B) in STZ-induced diabetic mice.

Mice were injected once daily with saline vehicle (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w) for 21 days (horizontal black bar). Lean littermates were used for comparative purposes. Blood was collected 3 days prior to STZ delivery (indicated by arrow) and every 5 days thereafter. Values are expressed as mean ± S.E.M for 6-8 mice. ***P < 0.001 compared with saline normal group.
Figure 4.5: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on glucose tolerance in STZ-induced diabetic mice

A)  

Tests were conducted following 21 days once daily treatment with saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). A) Glucose alone (18 mmol/kg b.w) was administered at time 0 (indicated by the arrow) in 18 h fasted mice and plasma glucose concentrations prior to and 15, 30 and 60 min after glucose administration were recorded. Plasma glucose AUC values (determined by using mean value at 0 min as a baseline) are also shown (B). Values are mean ± S.E.M for 6-8 mice per group.
Figure 4.6: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on glucose-induced insulin release in STZ-induced diabetic mice

Tests were conducted following 21 days once daily treatment with saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). A) Glucose alone (18 mmol/kg b.w) was administered at time 0 (indicated by the arrow) in 18 h fasted mice and plasma insulin concentrations prior to, and 15, 30 and 60 min after glucose administration were recorded. Plasma insulin AUC values (determined by using mean value at 0 min as a baseline) are also shown (B). Values are mean ± S.E.M for 6-8 mice per group. *P < 0.05, **P < 0.01 compared with saline STZ-induced diabetic group.
Figure 4.7: Effects of once daily administration of \([D-{\text{Ala}}^2]\)GIP or Liraglutide on insulin sensitivity in STZ-induced diabetic mice

A)

![Graph showing plasma glucose levels over time for different treatments.]

B)

![Graph showing plasma glucose AUC values for different treatments.]

Tests were performed after once daily treatment with saline (0.9%, w/v, NaCl), \([D-{\text{Ala}}^2]\)GIP or Liraglutide (each at 25 nmol/kg b.w) for 21 days. A) Insulin (25 U/kg b.w) was administered at time 0 (indicated by the arrow) in non-fasted mice and plasma glucose concentrations prior to, and 15, 30 and 60 min after glucose administration were recorded. B) Plasma glucose AUC values (determined by using mean value at 0 min as a baseline) for 0-60 min post-injection. Values represent the means ± S.E.M for 6-8 mice per group.
Figure 4.8: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on total body fat as measured by DEXA scanning in STZ-induced diabetic mice

Body fat was measured by dual energy X-ray absorption after once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w) for 21 days. Lean littermates were used for comparative purposes. Values are expressed as mean ± S.E.M for 6-8 mice per group. ***P < 0.001 compared to saline normal group.
Figure 4.9: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on A) total bone mineral density (BMD) and B) total bone mineral content (BMC) in STZ-induced diabetic mice.

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w.). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice.
Figure 4.10: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on lumbar A) bone mineral density (BMD) and B) bone mineral content (BMC) in STZ-induced diabetic mice

A)

Parameters were assessed by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice.
Figure 4.11: Effects of once daily administration of \([D-Ala^2]GIP\) or Liraglutide on femoral A) bone mineral density (BMD) and B) bone mineral content (BMC) in STZ-induced diabetic mice.

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9\%, w/v, NaCl), \([D-Ala^2]GIP\) or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice.
Figure 4.12: Effects of once daily administration of [D-Ala\textsuperscript{2}]GIP or Liraglutide on tibial A) bone mineral density (BMD) and B) bone mineral content (BMC) in STZ-induced diabetic mice.

A)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tibial BMD (g/cm\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal + saline</td>
<td>0.06</td>
</tr>
<tr>
<td>STZ-induced + saline</td>
<td>0.06</td>
</tr>
<tr>
<td>STZ-induced + (D-Ala\textsuperscript{2})GIP</td>
<td>0.06</td>
</tr>
<tr>
<td>STZ-induced + Liraglutide</td>
<td>0.06</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tibial BMC (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal + saline</td>
<td>0.02</td>
</tr>
<tr>
<td>STZ-induced + saline</td>
<td>0.02</td>
</tr>
<tr>
<td>STZ-induced + (D-Ala\textsuperscript{2})GIP</td>
<td>0.02</td>
</tr>
<tr>
<td>STZ-induced + Liraglutide</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9\%, w/v, NaCl), [D-Ala\textsuperscript{2}]GIP or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice.
Figure 4.13: Effects of once daily administration of \([D-Ala^2]GIP\) or Liraglutide on femoral cortical bone mineral content in STZ-induced diabetic mice
Cortical bone mineral content was measured by quantitative x-ray microradiograph following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. A) Digital x-ray images of femoral cortical bone were measured 3-4 mm below growth plate. B) Mean grey level. Values are mean ± S.E.M for 6-8 mice. **P < 0.01 compared to saline normal group.
Figure 4.14: Effects of once daily administration of [D-Ala$^2$]GIP or Liraglutide on femoral cancellous bone mineral content in STZ-induced diabetic mice

A)
Cancellous bone mineral content was measured by quantitative x-ray microradiograph following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. A) Digital x-ray images of femoral cancellous bone which was measured 2 mm below growth plate. B) Mean grey level. Values are mean ± S.E.M for 6-8 mice.
Figure 4.15: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on trabecular bone mass and microarchitecture in STZ-induced diabetic mice

A)

B)

**Figure Legend:**
- **A)**
  - Normal + saline
  - STZ-induced + saline
  - STZ-induced + (D-Ala²)GIP
  - STZ-induced + Liraglutide
  - BV/TV (%)

- **B)**
  - Normal + saline
  - STZ-induced + saline
  - STZ-induced + (D-Ala²)GIP
  - STZ-induced + Liraglutide
  - Tb.Th (µm)
Parameters were obtained from microtomography scan of tibia following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice.
Figure 4.16: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on cortical bone geometry in STZ-induced diabetic mice

A)  

- Normal + saline
- STZ-induced + saline
- STZ-induced + (D-Ala²)GIP
- STZ-induced + Liraglutide

B)  

- Normal + saline
- STZ-induced + saline
- STZ-induced + (D-Ala²)GIP
- STZ-induced + Liraglutide

* *
Images of cortical bone were obtained from a microtomography scan of tibia following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. Cortical bone was located 3 mm below growth plate and diameter of bone (A), diameter of bone marrow (B), cortical thickness (C) and cross-sectional moment of inertia (D) were measured using ImageJ software and calculated as described in Section 2.7.3. Values are mean ± S.E.M for 6-8 mice. *P < 0.05, ***P < 0.001 compared to normal saline group.
Figure 4.17: Effects of once daily administration of [D-Ala\textsuperscript{2}]GIP or Liraglutide on whole-bone mechanical properties of femoral cortical bone in STZ-induced diabetic mice

A)

- Normal + saline
- STZ-induced + saline
- STZ-induced + (D-Ala\textsuperscript{2})GIP
- STZ-induced + Liraglutide

B)

- Normal + saline
- STZ-induced + saline
- STZ-induced + (D-Ala\textsuperscript{2})GIP
- STZ-induced + Liraglutide
Parameters were obtained from 3-point bending test following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice. *P < 0.05, **P < 0.01 compared with saline normal group.
Figure 4.18: Effects of once daily administration of [D-Ala²]GIP or Liraglutide on intrinsic mechanical tissue response of cortical bone in STZ-induced diabetic mice

A)

- Normal + saline
- STZ-induced + saline
- STZ-induced + (D-Ala²)GIP
- STZ-induced + Liraglutide

B)

- Normal + saline
- STZ-induced + saline
- STZ-induced + (D-Ala²)GIP
- STZ-induced + Liraglutide
Parameters were measured by nanoindentation following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared with STZ-induced diabetic control group.
Figure 4.19: Effects of once daily administration of [D-Ala²]GIP or liraglutide on cortical bone mineral density distribution (BMDD) in STZ-induced diabetic mice

A)

- Normal + saline
- STZ-induced + saline
- STZ-induced + (D-Ala²)GIP
- STZ-induced + Liraglutide

B)

- Normal + saline
- STZ-induced + saline
- STZ-induced + (D-Ala²)GIP
- STZ-induced + Liraglutide
Bone mineralisation at cortical bone was measured by quantitative backscattered electron imaging following 21 days once daily administration of saline (0.9%, w/v, NaCl), [D-Ala²]GIP or Liraglutide (each at 25 nmol/kg b.w). Lean littermates were used for comparative purposes. Values are mean ± S.E.M for 6-8 mice.
Chapter 5

Simultaneous activation of GIP, GLP-1 and glucagon receptors by [D-Ala$^2$]GIP-oxyntomodulin positively affects cortical and trabecular bone properties and improves bone mechanical strength in genetically-induced C57 BL/KsJ diabetic ($db/db$) mice
5.1 SUMMARY

Bone abnormalities have appeared as new complications of diabetes and although bone mineral density is unaffected or modestly increased in type 2 diabetes mellitus, patients are still prone to bone fracture. Gut hormones, glucose-dependent insulinitropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are primarily known to control glucose homeostasis but recently, the incretins are also suspected to participate in bone regulation. Stimulation of GIP and GLP-1 receptors ameliorates defective cortical bone strength by improving tissue material properties in animal models of STZ-induced insulin-deficient diabetes. However, the effects of co-activation of both receptors together with glucagon receptors on bone quality are unknown. This study assessed the effects of daily treatment of triple GIP, GLP-1 and glucagon acting agent, [D-Ala²]GIP-Oxymodulin ([D-Ala²]GIP-Oxm) on metabolic control, bone microarchitecture and strength in genetically-induced diabetic (db/db) mice. C57 BL/KsJ diabetic (db/db) mice received once-daily injection of saline (0.9% w/v NaCl) or [D-Ala²]GIP-Oxm (50 mmol/kg bw) for 21 days. [D-Ala²]GIP-Oxm had no effect on body weight, water intake and accumulative food intake. Non-fasting plasma glucose was unchanged despite augmented insulin concentrations (3.5-fold, P < 0.01). No differences were seen in glucose tolerance and insulin sensitivity between treated and untreated diabetic groups. As compared to lean mice, db/db mice exhibited significant increases in total fat mass, reduced bone mineral density (BMD) and bone mineral content (BMC) and severe impairments in mechanical strength and microarchitecture at both cortical and trabecular bone. Administration of [D-Ala²]GIP-Oxm did not affect total, lumbar, femoral and tibial BMD and BMC in db/db mice. In addition, no modifications were observed in GLmean and absorbing material density between groups. As assessed by microCT, trabecular thickness (8% increase, P < 0.005) and cortical bone diameter (4% increase, P < 0.05) were significantly increased in [D-Ala²]GIP-Oxm mice. Bone mechanical assessments demonstrated that mice that were treated with [D-Ala²]GIP-Oxm had augmented maximum displacement (65% increase, P < 0.05) and work-to-fracture (47% increase, P < 0.01), whilst bone matrix hardness (31% increase, P < 0.05) and indentation modulus (9% increase, P < 0.05) were also improved. The changes were independent of bone mineral density distribution. In conclusion, simultaneous activation of GIP, GLP-1 and glucagon receptors by [D-
Ala^3\text{GIP-Oxm} analogue not only improved the material properties of bone matrix, but also the microstructure of cortical and trabecular bone, which in turn contributes to the overall improvement in mechanical response at the whole-bone level in leptin receptor-deficient diabetic (db/db) mice.

### 5.2 INTRODUCTION

Obesity has an impact on the quality of the skeleton and despite inconsistencies in reporting the effects of body mass on bone metabolism, bone impairments have been revealed in established rodent models of obesity and insulin resistance (Ealey et al. 2006, Ionova-Martin et al. 2010, Patsch et al. 2011, Williams et al. 2011). Several bone-related hormones are altered in obesity including leptin, the important regulator of body weight (Friedmann & Halaas 1998, Thomas et al. 1999). As such, leptin receptor-deficient (db/db) mice represent a genetic rodent model of spontaneous obesity as well as hyperglycaemia and hyperinsulinemia (Flatt et al. 1983). The leptin receptor is encoded by db gene and a recessive mutation in the gene has been shown to affect the intracellular domain of the single membrane-spanning receptor (Chen et al. 1996). Although leptin appears to bind to its receptor in db/db mice (Tartaglia et al. 1995), defective intracellular signal transduction attenuates leptin function, leading to a severe obesity and diabetes phenotype (Coleman 1978).

In response to nutrient intake, glucose-dependent insulino tropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are released by the gastrointestinal tract. The physiological target of these gut hormones is on pancreatic β cells stimulating insulin secretion and control blood glucose concentrations (Baggio & Drucker 2007) and as such, incretins provide therapeutic potential for the treatment of type 2 diabetes (Drucker 2003). Recently, emerging evidence on the importance of GIP and GLP-1 signaling in normal bone regulation was revealed in genetically-modified knockout and transgenic mice (Mabilleau et al. 2013, Tsukiyama et al. 2006, Xie et al. 2005, Xie et al. 2007, Yamada et al. 2008, Chapter 3). Taken together, these data suggest beneficial effects of GIPR and GLP-1R signaling on bone. Nonetheless, endogenous incretins are a substrate of dipeptidyl peptidase-4 (DPP-4) and the gut hormones are rapidly cleaved by this serine protease enzyme, rendering the inactive truncated form
of these peptides. In order to circumvent DPP-4 activity, several stable long-acting incretin analogues were developed and examined for its glucose-lowering and insulin-releasing properties.

Oxyntomodulin (Oxm) is a 37-amino acid gut molecule, derived from proglucagon family peptide and co-secreted with GLP-1 upon food intake. Oxm exerts its effects by binding to and co-activating both glucagon and GLP-1 receptors (Baggio et al. 2004) to control insulin secretion (Schjoldager et al. 1988). Oxm is also involved in the regulation of energy expenditure and food intake (Cohen et al. 2003). In addition, several N-terminally modified stable GIP peptides have been examined for antidiabetic activity and [D-Ala²]GIP is one of the most promising molecules (Hinke et al. 2002). Based on these observations, [D-Ala²]GIP-Oxyntomodulin, is a recently characterised triple-acting peptide that interacts with GIP, GLP-1 and glucagon receptors has been generated by our research group and has proven to have antidiabetic potential (Bhat et al. 2013). Taking into consideration the capacity of this peptide to activate numerous peptide receptor pathways, [D-Ala²]GIP-Oxyntomodulin is viewed as potential therapeutic agent in management of bone disease in diabetes.

In Chapter 4, the link between diabetes and impairment in skeletal integrity was established in a chemically-induced mouse model. In the present study, we will assess the effects of prolonged and simultaneous activation of GIP, GLP-1 and glucagon receptors on metabolic parameters, bone morphology and strength in mice with genetically-induced obesity and diabetes, namely C57 BL/KsJ diabetic (db/db) mice.
5.3 MATERIALS AND METHODS

5.3.1 Synthesis of [D-Ala$^2$]GIP-Oxyntomodulin

The triple-acting analogue, [D-Ala$^2$]GIP-Oxyntomodulin was purchased from GL Biochem Ltd. (Shanghai, China) at 95% purity. The peptide was characterised by MALDI-TOF mass spectrometry as explained in Section 2.1.2.

5.3.2 Animals and study design

C57 BL/KsJ diabetes (db/db) mice (n=10, 11 weeks old) were divided into two groups that received [D-Ala$^2$]GIP-Oxyntomodulin (50 nmol/kg bw) or saline vehicle (0.9%, w/v, NaCl) for 21 days. Lean mice (n=6) from the same genetic background were also treated with saline vehicle (0.9%, w/v, NaCl) for the same period of time and used for comparative purposes in bone quality assessment. Body weight, food consumption, fluid intake, non-fasting blood glucose and insulin concentrations were monitored regularly during the 21-day experimental period. Glucose tolerance (18 mmol/kg bw, intraperitoneally, 18-h fast) and non-fasting insulin sensitivity tests (50 U/kg bw, intraperitoneally) were carried out at the end of the study as described in Section 2.3.8 and 2.3.9, respectively. All experiments were conducted according to UK Home Office Regulations (UK Animals Scientific Procedures Act 1986).

5.3.3 Measurement of plasma glucose and insulin

Plasma glucose was measured using an Analox GM-9 glucose analyser as described in Section 2.4.1 and plasma insulin concentrations were determined by RIA experiment as outlined in Section 2.4.2.

5.3.4 Measurement of body composition, bone density and mineral content by DEXA scanning

Unconscious mice were placed on a specimen tray of the DEXA scanner and whole body scanning was performed. Bone mineral density (BMD), bone mineral content (BMC), total scanned area, lean mass, fat mass and percentage of total fat of ROIs
were measured as described in Section 2.5. Age-matched lean controls (n=6) with the same genetic background were included for comparative purposes.

5.3.5 Assessment of bone quality and strength

Bone microstructure and strength were assessed on excised femurs and tibias from all groups of mice at the end of the study.

5.3.5.1 Quantitative x-ray Imaging (qXRI)

The diameter of femurs was measured and recorded, followed by scanning processes. The femurs were imaged together with two standards, 1.5 mm-thick steel and polyester which represent grey level 255 and 0, respectively. Bone mineral content was determined as detailed in Section 2.6.1.

5.3.5.2 X-ray microcomputed tomography (µCT)

Tibias were positioned and tomographed as described in Section 2.6.2 and parameters that were obtained from the projections were trabecular bone volume, trabecular bone thickness, numbers of trabecular bone and trabecular separation. Images of cortical bone 3-4 mm below the growth plate were obtained and using ImageJ software, cortical bone diameter and marrow were measured, followed by determination of cortical thickness and cross-sectional moment of inertia as explained in Section 2.6.2.

5.3.5.3 Three-point bending test

Bones were individually kept in test tubes containing saline (NaCl, 0.9%) to rehydrate and left overnight at 4°C. The test was carried out as described in Section 2.6.3 and four mechanical properties were computerised and evaluated; ultimate load, ultimate displacement, stiffness and total absorbed energy (work-to-failure).
5.3.5.4 Nanoindentation

Prior to experimentation, polymethylmethacrylate blocks were polished with a diamond particle to remove the carbon layer previously coated for qBEI and the blocks were left in saline (NaCl, 0.9%) at 4°C for 15 h. Nanoindentation was performed as outlined in Section 2.6.4 and maximum force, hardness, indentation modulus and dissipated energy were measured.

5.3.5.5 Quantitative backscattered electron imaging (qBEI)

The polymethylmethacrylate blocks were polished with diamond particles to give 1-μm finishing, followed by carbon-coating process. Subsequently, the blocks were observed under scanning electron microscope as detailed in Section 2.6.5.

5.3.6 Statistical analysis

Data were analysed using repeated measures one-way or two-way ANOVA with Tukey’s post hoc tests or two-tailed t-tests using PRISM 5.0. Data are expressed as mean ± S.E.M and a P value < 0.05 was considered statistically significant.
5.4 RESULTS

5.4.1 Effects of once-daily administration of [D-Ala²]GIP-Oxm on metabolic parameters in db/db mice.

Treatment with [D-Ala²]GIP-Oxm for 21 days had no significant effect on body weight (Figure 5.1), food intake (Figure 5.2) and water intake (Figure 5.3) in db/db mice. Non-fasting plasma glucose concentrations in [D-Ala²]GIP-Oxm-treated diabetic mice were reduced from day 10 onwards, however the values did not reach statistical significant (Figure 5.4A). On the other hand, plasma insulin levels were significantly increased on day 20 (3.5-fold, P < 0.01) in mice treated with [D-Ala²]GIP-Oxm compared to db/db saline controls (Figure 5.4B).

5.4.2 Effects of once-daily administration of [D-Ala²]GIP-Oxm on glucose tolerance, glucose-induced plasma insulin release and insulin sensitivity in db/db diabetic mice.

Once daily administration of [D-Ala²]GIP-Oxm had no significant effect on glycaemic response at every time point tested following glucose loading (Figure 5.5A). From plasma glucose AUC (0-60 min) values, overall plasma glucose concentrations in db/db mice treated with [D-Ala²]GIP-Oxm were lowered compared to db/db controls, but the differences were not significant (Figure 5.5B). Glucose-stimulated plasma insulin concentrations were slightly higher at every time point recorded (Figure 5.6A) in [D-Ala2]GIP-Oxm-treated mice when compared to controls and as a result, there was an increase in overall plasma insulin levels (Figure 5.6B). Nonetheless, this increase did not reach significance. As seen in Figure 5.7, daily treatment of [D-Ala²]GIP-Oxm had no significant effect on plasma glucose concentrations following exogenous administration of insulin.

5.4.3 Effects of [D-Ala²]GIP-Oxm on percentage body fat, bone mineral density and bone mineral content in db/db mice.

Diabetic (db/db) mice had significantly higher percentage of body fat compared to lean littermates (70% increase, P < 0.001, Figure 5.8). Treatment with [D-Ala²]GIP-
Oxm had no significant effect on total fat mass in db/db mice. There were no differences in BMD and BMC at regions of interest between [D-Ala²]GIP-Oxm and saline-treated diabetic mice (Figure 5.9-5.12). However, diabetic groups of mice exhibited a significant decrease in overall BMD and BMC (BMD: 24% decrease, P < 0.001, Figure 5.9A; BMC: 50 - 59% decrease, P < 0.001, Figure 5.9B) as well as at lumbar (BMD: 33 - 38% decrease, P < 0.001, Figure 5.10A; BMC: 53 - 63% decrease, P < 0.001, Figure 5.10B), femur (BMD: 42% decrease, P < 0.001, Figure 5.11A; BMC: 72% decrease, P < 0.001, Figure 5.11B) and tibia (BMD: 21 - 26% decrease, P < 0.001, Figure 5.12A; BMC: 45% decrease, P < 0.001, Figure 5.12B) compared to normal mice.

5.4.4 Effects of [D-Ala²]GIP-Oxm on cortical and trabecular bone mineral content in db/db mice.

Assessment on bone mineral content was further investigated at both cortical and trabecular femur using quantitative x-ray microradiographs. As can be seen in Figure 5.13, db/db mice had reduced GLmean (15% decrease, P < 0.001, Figure 5.13B) and absorbing material density (11% decrease, P < 0.001, Figure 5.13C) as compared to lean mice. Similarly, GLmean at trabecular bone was also reduced (19% decrease, P < 0.001, Figure 5.14B) which was accompanied by a decrease in absorbing material density (13% decrease, P < 0.001, Figure 5.14C). Treatment with [D-Ala²]GIP-Oxm induced no significant differences in cortical GLmean and absorbing material density of the treated and untreated db/db mice (Figure 5.13). The same parameters were also unchanged at trabecular bone (Figure 5.14).

5.4.5 Effects of [D-Ala²]GIP-Oxm on trabecular bone microarchitecture and cortical bone geometry in db/db mice.

Trabecular bone microarchitecture as well as geometry of cortical bone was determined by high resolution microCT. Bone volume was significantly reduced (70% decrease, P < 0.001, Figure 5.15A) in diabetic db/db mice compared to lean controls. This was accompanied by a reduction in trabecular thickness (15% decrease, P < 0.001). Additionally, there was a significant decrease in trabecular numbers (65% decrease, P < 0.001, Figure 5.15C) and in agreement, there was an
increase in trabecular separation (51% increase, P < 0.001, Figure 5.15D) in diabetic groups compared to normal mice. Treatment with [D-Ala²]GIP-Oxm for 21 days had no effect on trabecular bone volume, trabecular numbers and trabecular separation. Although other parameters were unchanged, trabecular thickness was significantly increased (7% increase, P < 0.05, Figure 5.15B) in mice treated with [D-Ala²]GIP-Oxm compared to the diabetic control group.

As seen in Figure 5.16, there were significant reduction in cortical bone diameter (21% decrease, P < 0.001, Figure 5.16A), bone marrow diameter (21% decrease, P < 0.001, Figure 5.16B), cortical thickness (20% decrease, P < 0.01, Figure 5.16C) and CSMI (61% decrease, P < 0.01, Figure 5.16D) at cortical bone of db/db mice compared to normal controls. [D-Ala²]GIP-Oxm improved cortical bone diameter (4% increase, P < 0.05, Figure 5.16A) in db/db mice while other parameters were unaffected.

5.4.6 Effects of [D-Ala²]GIP-Oxm on whole bone strength in db/db mice.

Extrinsic mechanical properties of cortical bone were assessed by three-point bending. As shown in Figure 5.17, diabetic mice had significantly reduced maximum force (30% decrease, P < 0.01), maximum displacement (72% decrease, P < 0.01), stiffness (47% decrease, P < 0.001) and work-to-failure (71% decrease, P < 0.001) compared to lean controls. Administration of [D-Ala²]GIP-Oxm for 21 days in db/db mice markedly improved ultimate displacement (65% increase, P < 0.05, Figure 5.17B) as compared to the saline-treated db/db group. The increase in maximum displacement was accompanied by an augmentation in work-to-failure (47% increase, P < 0.01, Figure 5.17D). However, no differences were observed in ultimate force and stiffness between db/db mice groups (Figure 5.17A & C).

5.4.7 Effects of [D-Ala²]GIP-Oxm on cortical bone nanomechanical properties in db/db mice.

As can be seen in Figure 5.18, mechanical properties evaluated at the tissue-level were reduced in leptin receptor-deficient db/db mice compared to lean controls. Although significant reductions were observed in hardness (14% decrease, P < 0.01),
indentation modulus (10% decrease, P < 0.05) and dissipated energy (39% decrease, P < 0.01), maximum force was unchanged (Figure 5.18C). Daily treatment of db/db mice with [D-Ala²]GIP-Oxm significantly increased bone hardness (31% increase, P < 0.05, Figure 5.18A) and indentation modulus (9% increase, P < 0.05, Figure 5.18B). In addition, there was slight improvement in dissipated energy (Figure 5.18D), but it was not significant compared to saline-treated diabetic group. [D-Ala²]GIP-Oxm had no effect on maximum force in db/db mice (Figure 5.18C).

5.4.8 Effects of [D-Ala²]GIP-Oxm on cortical bone mineral density distribution in db/db mice.

There were no differences seen in Ca_{peak} and Ca_{mean} of diabetic and normal mice. However, diabetic db/db mice displayed significant increase in Ca_{width} (17% increase, P < 0.05, Figure 5.19C) compared to lean controls. Treatment of [D-Ala²]GIP-Oxm over 21 day period in db/db mice did not affect these three parameters of mineral density distribution.
5.5 DISCUSSION

In this study, an established genetically-induced mouse model of obesity and diabetes, the C57 BL/KsJ diabetic (db/db) mouse was employed to examine not only the glucose-lowering and insulinotropic properties of [D-Ala²]GIP-Oxm, but also other aspects that related to bone microstructure and strength. In agreement with earlier observations, db/db mice displayed progressive body weight gain, hyperglycaemia and hyperinsulinemia (Coleman 1978, Flatt et al. 1983). The increase in body weight was associated with a significant increase in fat mass. In contrast to a recent study in high-fat fed mice, [D-Ala²]GIP-Oxm administration for 21 days had no effect on body weight in db/db mice (Bhat et al. 2013). On the other hand, non-fasting plasma insulin concentrations were noticeably increased on day 20 in treated db/db mice.

When comparison is made between non-fasting plasma insulin levels in the present study and STZ study (Chapter 4), insulin concentrations were surprisingly lowered in db/db mice to similar levels to STZ mice. An explanation for this could be due to insulin-producing beta cell degeneration as the db/db mice age. It is also well-established that following STZ administration, pancreatic beta cells can undergo regeneration from replication of surviving intra-islet beta cells (Bonner-Weir et al. 1981, Wang et al. 1994) and as a result, increased insulin production in STZ mice. [D-Ala²]GIP-Oxm therapy also failed to improve glycemic response to glucose load in db/db mice, unlike previous study in high-fat mice (Bhat et al. 2013). This difference is possibly due to the severity of the mouse model used in our study as even after food was withdrawn for 18 h, plasma glucose levels in db/db mice were still very high at around 22.5 mmol/L, whereas 18-h fasted high-fat fed mice in Bhat’s study (2013) had much lower plasma glucose concentrations of approximately 10.0 mmol/L. In order to standardize the data for metabolic parameters of db/db mice, lean group was not included. The lean controls were only used for comparative purposes in bone assessment.

Insulin is known to induce bone formation (Cornish et al. 1996) and in Chapter 4, we clearly revealed that hypoinsulinemia in STZ-diabetic mice contributes to bone loss. Therefore, the leptin receptor-deficient model which exhibited hyperinsulinemia or
insulin resistance was used in this study. To date, only Bhat and colleagues have examined glucose-lowering and insulin-releasing activity of the recently-developed peptide, [D-Ala²]GIP-Oxm (Bhat et al. 2013) with no reports on the impact of the peptide on skeletal microstructure and strength. In the present study, we found that total, femur, tibia and lumbar bone mineral density and bone mineral content were significantly decreased in db/db mice and this supported earlier observations that also found reduced BMD and BMC in mice lacking leptin receptors (Ealey et al. 2006). In agreement with DEXA scanning, GLmean and absorbing material density, which represents bone mineral content, were also reduced at both femoral cortical and trabecular bone.

One of the possible contributing factors to the reductions of bone mass in db/db mice is loss in leptin signaling. Osteoblasts and adipocytes are derived from the same progenitors, marrow stromal cells (MSCs), and interestingly, leptin receptors were proven to be expressed on human MSCs (Thomas et al. 1999). In addition, the presence of leptin dose-dependently enhanced osteoblastic protein expression and reduced biomarkers for mature adipocytes in cultures of MSCs, emphasising the importance of leptin in controlling differentiation pathways of MSC precursors either into osteoblasts or adipocytes (Thomas et al. 1999). As a result of defective leptin signal transduction, differentiation of precursors is shifted to adipogenesis, hence grossly increased body weight and bone loss, leading to bone impairments. Furthermore, leptin has previously been reported to inhibit the production of bone-resorbing osteoclast cells (Holloway et al. 2002). Thus, deficiency in leptin signaling may enhance generation of osteoclasts and increase osteoclastic bone resorptive activity, resulting in further bone loss. Once daily administration of [D-Ala²]GIP-Oxm did not have a remarkable effect on BMD, BMC, GLmean or absorbing material density of db/db mouse bone when compared to saline-treated db/db controls. This is not surprising as examinations of bone by DEXA and qXRI were conducted at the macrostructure level of the skeleton and with the short period of treatment, any improvements in bone health may not be seen by these methods.

Further assessments performed on the microstructure and morphology of specific areas of bone by microCT revealed that db/db mice had reduced trabecular bone volume, which contributed to a decrease in trabecular thickness. In line with
previous literature, bone mass reduction was also accompanied by less numbers of trabecular and higher trabecular separation (Williams et al. 2011). In addition, midshaft cortical diameter, bone marrow, cortical thickness and CSMI in db/db mice were all markedly reduced compared to lean littermates, suggesting that cortical bone was also severely compromised. Microstructural examination demonstrated that not only cortical bone diameter in db/db mice was increased with 21-day therapy with [D-Ala²]GIP-Oxm, but the regimen also significantly elevated trabecular thickness. Taken together, these improvements contribute to increased bone mass.

In Chapter 4, cortical bone area, bone mechanical and intrinsic material properties were severely affected in STZ-diabetic mice. Importantly, material properties were restored in diabetic mice that received [D-Ala²]GIP or Liraglutide once daily. Similar to STZ-induced diabetic models, db/db mice displayed impaired cortical bone properties that lead to decreased bone strength. The effects of leptin receptor deficiency were further evaluated on whole-bone strength by three-point bending tests and femurs of db/db mice required less breaking force than lean control mice, indicating lower bone strength. Nanoindentation, which is independent of bone geometry and porosity (Rho et al 1997), was carried out to further strengthen the findings in three-point bending. Consistent with previous observations, hardness, indentation modulus and dissipated energy were reduced in db/db mice, suggesting that mechanical properties of bone matrix were adversely affected (Williams et al. 2011). In order to determine if these modifications resulted from changes in calcium distribution, qBEI was performed, but no significant differences in Ca_{peak} and Ca_{mean} was observed in all three groups. Although db/db mice exhibited a significant increase in cortical Ca_{width} in comparison to lean mice, which represents heterogeneity of calcium deposition, no alteration of quantity or frequency of calcium mineral means overall mineralisation was unchanged.

Most importantly, femurs excised from db/db mice that received [D-Ala²]GIP-Oxm appeared to have higher cortical resistance to fracture, supported by significant increments in maximum displacement at maximum force, and amount of work required to break the bone as compared to saline-treated db/db controls. The increase in whole-bone strength was associated not only by elevations in hardness and stiffness at cortical tissue level, but also at organ level where there was
enhancement of bone mass from increases in cortical and trabecular bone diameter and thickness, respectively. Improvement in material properties of bone matrix is significant as intrinsic mechanical response was identical to the response in lean mice. Similar to what we have seen with [D-Ala$^2$]GIP and Liraglutide therapy, the anabolic effects of [D-Ala$^2$]GIP-Oxm on bone are independent of mineralisation distribution with respect to calcium content, as no differences were seen in bone mineral density distribution. Activation of GIPR or GLP-1R has beneficial effects on bone as discussed in Chapter 3 and 4 and therefore, it is not surprising to see that co-activation of both incretin receptors had pronounced beneficial effects on restoration of bone quality in db/db mice. On the other hand, the effect of activation of glucagon receptors on bone is relatively unknown. Additionally, histomorphometrical and gene expression studies are needed to confirm the participation of osteoblasts and/or osteoclasts, and to establish the possible signaling pathways of this long-acting Oxm analogue on bone.

In conclusion, this study reveals that both cortical and trabecular bone of db/db mice are detrimentally altered. Moreover, the DPP-4 resistant [D-Ala$^2$]GIP-Oxm analogue elicits significant beneficial effects on both types of bone by increasing trabecular thickness, cortical diameter and bone strength at the tissue and organ level. The positive effects of triple-activation of GIPR, GLP-1R and glucagon receptors by [D-Ala$^2$]GIP-Oxm on bone quality in db/db mice provides additional evidence to include incretins in therapeutic strategies for bone-related diseases. Additionally, it would be interesting to study and characterise the potential actions of established incretin-based therapies on bone quality in other diabetic mouse models such as the high-fat dietary induced obesity and insulin resistance.
Figure 5.1 : Effects of once daily administration of [D-Ala²]GIP-Oxm on body weight in db/db mice

Body weight was measured every 2 days, starting 4 days prior to treatment. Mice were treated once daily with saline vehicle (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) for 21 days (horizontal black bar). Values are mean ± S.E.M for 5-6 mice.
Figure 5.2: Effects of once daily administration of [D-Ala²]GIP-Oxm on cumulative food intake in db/db mice

Food intake was measured every 2-3 days, starting 4 days prior to treatment. Mice received daily treatment with saline vehicle (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) for 21 days. Values are mean ± S.E.M for 5-6 mice.
Figure 5.3 : Effects of once daily administration of [D-Ala²]GIP-Oxm on fluid intake in db/db mice

Water intake was measured 4 days prior to treatment. Mice were injected once daily with saline vehicle (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) for 21 days (horizontal black bar). Values are mean ± S.E.M for 5-6 mice.
Figure 5.4: Effects of once daily administration of [D-Ala\(^2\)]GIP-Oxm on non-fasting blood glucose and plasma insulin concentrations in db/db mice

A)

![Graph A]

B)

![Graph B]

Mice were injected once daily with saline vehicle (0.9%, w/v, NaCl) or (D-Ala\(^2\))GIP-Oxm (50 nmol/kg b.w) for 21 days (horizontal black bar). Blood was collected at 5-day intervals. Values are expressed as mean ± S.E.M for 5-6 mice. **P < 0.01 compared with db/db control group.
Figure 5.5: Effects of once daily administration of [D-Ala²]GIP-Oxm on glucose tolerance in db/db mice

A)

Tests were conducted following 21 days once daily treatment with saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Glucose alone (18 mmol/kg b.w) was intraperitoneally administered at time 0 (indicated by the arrow) in 18 h fasted mice and concentrations of plasma glucose recorded at 15, 30 and 60 min post-injection. Plasma glucose AUC values (determined by using mean value at 0 min as a baseline) are also shown (B). Values are mean ± S.E.M for 5-6 mice per group.
Figure 5.6: Effects of once daily administration of [D-Ala²]GIP-Oxm on glucose-induced insulin release in db/db mice

Tests were conducted following 21 days once daily treatment with saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Glucose alone (18 mmol/kg b.w) was intraperitoneally administered at time 0 (indicated by the arrow) in 18 h fasted mice and concentrations of plasma insulin recorded at 15, 30 and 60 min post-injection. Plasma insulin AUC values (determined by using mean value at 0 min as a baseline) are also shown (B). Values are mean ± S.E.M for 5-6 mice per group.
Figure 5.7: Effects of once daily administration of [D-Ala$^2$]GIP-Oxm on insulin sensitivity in db/db mice

A) 

Tests were performed after once daily treatment with saline (0.9%, w/v, NaCl) or (D-Ala$^2$)GIP-Oxm (50 nmol/kg b.w) for 21 days in db/db mice. A) Insulin (50 U/kg b.w) was administered at time 0 (indicated by the arrow) in non-fasted mice. B) Blood glucose AUC values (determined by using mean value at 0 min as a baseline) for 0-60 min post-injection. Values represent the means ± S.E.M for 5-6 mice per group.
Figure 5.8: Effects of once daily administration of [D-Ala²]GIP-Oxm on total body fat in db/db mice

Body fat was measured by dual energy X-ray absorption after once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) for 21 days in db/db mice. Lean mice were used for comparative purposes. Values are expressed as mean ± S.E.M for 5-6 mice per group. ***P < 0.001 compared with lean control group.
Figure 5.9: Effects of once daily administration of [D-Ala²]GIP-Oxm on A) total bone mineral density (BMD) and B) total bone mineral content (BMC) in db/db mice.

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Values are mean ± S.E.M for 5-6 mice. **P < 0.001 compared to lean control group.
Figure 5.10: Effects of once daily administration of [D-Ala5]GIP-Oxm on lumbar A) bone mineral density (BMD) and B) bone mineral content (BMC) in db/db mice

Parameters were assessed by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala5)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Values are mean ± S.E.M for 5-6 mice per group. ***P < 0.001 compared with lean control group.
Figure 5.11: Effects of once daily administration of [D-Ala²]GIP-Oxm on femoral A) bone mineral density (BMD) and B) bone mineral content (BMC) in db/db mice

A)

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Values are mean ± S.E.M for 5-6 mice. ***P < 0.001 compared with lean control group.
Figure 5.12: Effects of once daily administration of [D-Ala²]GIP-Oxm on tibial A) bone mineral density (BMD) and B) bone mineral content (BMC) in db/db mice

Parameters were measured by dual energy X-ray absorption following 21 days of once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Values are mean ± S.E.M for 5-6 mice. ***P < 0.001 compared with lean control group.
Figure 5.13: Effects of once daily administration of [D-Ala²]GIP-Oxm on femoral cortical bone mineral content in db/db mice

A)

B)

Lean control
db/db control
(D-Ala²)GIP-Oxm

GL mean

***

164
Cortical bone mineral content was measured by quantitative x-ray microradiograph following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. A) Digital x-ray images of femoral cortical bone with 16- color look-up table. B) Mean grey level. C) Cortical absorbing mineral density. Values are mean ± S.E.M for 5-6 mice. ***P < 0.001 compared with lean control.
Figure 5.14: Effects of once daily administration of [D-Ala\textsuperscript{2}]GIP-Oxm on femoral cancellous bone mineral content in db/db mice

A)

B)

Lean control
db/db control
(D-Ala\textsuperscript{2})GIP-Oxm

GL mean

150
100
50
0

***

***

***
Cancellous bone mineral content was measured by quantitative x-ray microradiograph following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. A) Digital x-ray images of femoral cancellous bone with 16-color look-up table B) Mean grey level. C) Trabecular absorbing mineral density. Values are mean ± S.E.M for 5-6 mice. ***P < 0.001 compared with lean control.
Figure 5.15: Effects of once daily administration of [D-Ala²]GIP-Oxm on trabecular bone mass and microarchitecture in db/db mice

A)

B)
Parameters were obtained from microtomography scan following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala³)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Values are mean ± S.E.M for 5-6 mice. **P < 0.01, ***P < 0.001 compared with lean control group, △P < 0.05 compared with db/db control group.
Figure 5.16: Effects of once daily administration of [D-Ala²]GIP-Oxm on cortical bone geometry in db/db mice

A)

B)

Lean control
db/db control
(D-Ala²)GIP-Oxm
Bones were scanned using high resolution microtomography following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Parameters were measured 3 mm below growth plate at cortical bone, A) Diameter of bone, B) diameter of bone marrow, C) cortical thickness and D) cross-sectional moment of inertia. Values are mean ± S.E.M for 5-6 mice. ***P < 0.001 compared with lean control. ∆P < 0.05 compared to db/db saline control.
Figure 5.17: Effects of once daily administration of [D-Ala²]GIP-Oxm on mechanical properties of femoral cortical bone in db/db mice

A)

B)
Parameters were obtained from three-point bending test following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Values are mean ± S.E.M for 5-6 mice. **P < 0.01, ***P < 0.001 compared with lean control group, ΔP < 0.05, ΔΔP < 0.01 compared with db/db control group.
Figure 5.18: Effects of once daily administration of [D-Ala²]GIP-Oxm on intrinsic tissue properties of cortical bone in db/db mice

A)

- Lean control
- db/db control
- [D-Ala²]GIP-Oxm

B)

- Lean control
- db/db control
- [D-Ala²]GIP-Oxm

**Figure shows bar graphs comparing hardness and indentation modulus of different groups.**
Parameters were measured by nanoindentation following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala$^2$)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Values are mean ± S.E.M for 5-6 mice. *P < 0.05, **P < 0.01 compared with lean control. $^\Delta$P < 0.05 compared with db/db control.
Figure 5.19: Effects of once daily administration of [D-Ala\(^2\)]GIP-Oxm on cortical bone mineral density distribution (BMDD) in db/db mice

A) 

B)
Bone mineralisation parameters were measured by quantitative backscattered electron imaging following 21 days once daily administration of saline (0.9%, w/v, NaCl) or (D-Ala²)GIP-Oxm (50 nmol/kg b.w) in db/db mice. Lean mice were used for comparative purposes. Values are mean ± S.E.M for 5-6 mice. *P < 0.05, **P < 0.01 compared with lean control.
Chapter 6

Effects of the GLP-1R agonist Exendin-4 and the DPP-4 inhibitor Sitagliptin on bone strength and quality in high-fat fed mice
Several currently available treatments for type 2 diabetes mellitus (T2DM) have been reported to negatively affect bone. The incretin-based therapies including, GLP-1 mimetics and DPPIV inhibitors, are new classes of drugs introduced for the treatment of T2DM. Although these agents are largely known to improve glycaemic control, their long-term effects on bone quality have not yet been fully established. This study examined the effects of daily treatment with the well-established GLP-1 mimetic, Exendin-4 (25 mmol/kg bw, i.p) and DPP-IV inhibitor, Sitagliptin (50 mmol/kg bw, orally) on metabolic control, bone mineral density, microarchitecture and strength in high-fat fed mice. Exendin-4 and Sitagliptin had no effect on body weight of high-fat fed mice. Reductions of non-fasting glucose (Exendin-4: 58% decrease, P < 0.01; Sitagliptin: 41% decrease, P < 0.001) were accompanied by significant increases of non-fasting insulin (Exendin-4: 83% increase, P < 0.01; Sitagliptin: 55% increase, P < 0.01) in treated compared to nontreated mice. Glucose tolerance (22-32%, decrease, P < 0.05) and insulin sensitivity (50-57% increase, P < 0.001) were improved with Exendin-4 and Sitagliptin treatment. As compared to lean mice, diabetic high-fat mice exhibited significant increases in total fat mass (27% increase, P < 0.01). However, no significant differences in fat mass of Exendin-4 or Sitagliptin treated mice when compared to high-fat controls. Administration of Exendin-4 or Sitagliptin did not affect total, lumbar, femoral and tibial BMD and BMC in high-fat fed mice. In parallel, bone mineral content, as determined by GLmean and absorbing material density at both cortical and trabecular bones, did not change between Exendin-4- or Sitagliptin-treated and their saline-treated diabetic controls. Administration of Exendin-4 marginally increased trabecular bone volume which contributed to the increase in trabecular numbers and reduction in trabecular separation. These changes however, did not reach statistical significance when compared to saline controls. Similarly, Sitagliptin treatment did not improve parameters measured at trabecular bone in high-fat fed mice. From assessment by microCT, high-fat diet altered cortical bone geometry resulting in marked increases in cortical bone diameter (10% increase, P < 0.05), bone marrow (18-25% increase, P < 0.001) and CSMI (49% increase, P < 0.05). These parameters were not improved by Exendin-4 or Sitagliptin therapy. In three-point bending tests, augmented resistance to fracture was observed (32% increase, P < 0.01) in diabetic
mice treated with Exendin-4 but not Sitagliptin, while ultimate force, ultimate displacement and stiffness remained unchanged in both treated groups. From this study, it is concluded that Exendin-4 improved whole-bone mechanical strength, whilst Sitagliptin has neutral effects on bone in high-fat fed mice.

6.2 INTRODUCTION

Bone remodeling is a continuous process throughout life and is coordinated by bone-forming osteoblasts and bone-resorbing osteoclasts (Matsuo & Irie 2008). In this dynamic organ, maintenance of normal osteoblast-osteoclast communication is vital as imbalance in bone formation and resorption will reduce bone quality, leading to increased risk of fracture (Seeman 2002). Diabetes is closely associated with bone impairments and type 2 diabetes mellitus patients are highly susceptible to bone fragility fractures, despite having normal or elevated bone mineral density (Vestergaard, 2007). Emerging evidence on further bone loss with existing antidiabetic therapies such as thiazolidinediones (TZDs) (Schwartz et al. 2006) has lead to major concern on the safety of currently available diabetes treatment on bone. As newly-introduced class of antidiabetic drugs, the impact of incretin-based therapies on bone strength and quality remain largely unknown.

Glucagon-like peptide-1 (GLP-1) is released in response to nutrient ingestion and stimulates glucose-dependent insulin secretion (Baggio & Drucker 2007). GLP-1 also plays a role in delaying gastric emptying, inhibiting glucagon release and promoting satiety (Drucker & Nauck 2006). Furthermore, administration of GLP-1 stimulates pancreatic beta-cells proliferation and reduces islet apoptosis (Li et al. 2003, Xu et al. 1999). Therefore, the collective actions of GLP-1 make this molecule a promising therapeutic approach for type 2 diabetes mellitus (T2DM). As such, GLP-1-based therapies have now been developed for T2DM and the first approved drug in this class was Exendin-4 (Exenatide) (Lovshin & Drucker 2009). Exendin-4 shares 53% sequence homology to mammalian GLP-1, and substitution of alanine with glycine at position 2 of the peptide protects the molecule from rapid degradation by the ubiquitous serine protease, dipeptidyl peptidase-4 (DPP-4).
DPP-4 inhibitor-based drugs were later developed to directly inhibit the actions of DPP-4 to degrade incretin hormones (Barnett 2006). As a result, this prolongs the glucoregulatory and insulinotropic actions of both incretin peptides, namely GLP-1 and its sister incretin glucose-dependent insulinotropic polypeptide (GIP). Treatment with DPP-4 inhibitors enhances beta-cells regeneration and survival in STZ-induced diabetes model of mice (Pospisilik et al. 2003) and a more recent study found preservation of islet beta-cell mass and increase in pancreatic insulin content in isolated islets from high-fat fed mice treated with Sitagliptin (Mu et al. 2006). Sitagliptin (Januvia), was released onto the market in 2006 as orally administered drug for once-daily administration (Lovshin & Drucker 2009). Sitagliptin was proven to improve glycaemic control either in monotherapy or in combination with metformin or thiazolidinedione treatment (Aschner et al. 2006, Charbonnel et al. 2006, Rosenstock et al. 2006). However, the effect of drugs such as Sitagliptin on bone turnover, strength and quality in diabetes remain largely unknown.

In the present study we assessed the capacity of Exendin-4 and Sitagliptin to improve aspects of metabolic control, bone micromorphology and whole-bone mechanical strength in animal dietary-induced high-fat fed mice that present with overt obesity and insulin resistance.
6.3 MATERIALS AND METHODS

6.3.1 Animals and study design

Male NIH Swiss mice (n=16, 8 weeks old, Harlan Ltd., Blackthorn, UK) were individually housed in an air-conditioned room at 22 ± 2 with 12 h light : 12 h dark cycle, had free access to drinking water and high fat diet (45% fat, 20% protein, 35% carbohydrate; Special Diet Service, Essex, U.K.) for 16 weeks prior to the start of the studies. The mice were divided into two groups that received twice daily saline vehicle (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg bw) intraperitoneally for 52 days. For Sitagliptin studies, mice were maintained on high-fat diet for 20 weeks prior to start of the long-term studies. The mice were divided into two groups that received once daily saline vehicle (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg bw) orally for 21 days. Lean mice also injected with saline vehicle and used for comparative purposes for both studies. Body weight, non-fasting blood glucose and insulin concentrations were observed at regular intervals. Glucose tolerance test (18 mmol/kg bw, i.p, 18-h fast) and non-fasting insulin sensitivity test (25 U/kg bw, i.p) were performed at the end of the study as explained in Section 2.3.8 and 2.3.9, respectively. All experiments were performed according to UK Home Office Regulations (UK Animals Scientific Procured Act 1986).

6.3.2 Measurement of body composition, bone density and mineral content by DEXA scanning

Unconscious mice were placed on a specimen tray and the whole body was imaged by DEXA scanner. Bone mineral density (BMD), bone mineral content (BMC), lean mass, fat mass and percentage of total fat of ROIs were measured as explained in Section 2.5. Age-matched lean controls (n=6-8) with the same genetic background were included for comparative purposes.
6.3.3 Assessment of bone mineral content by quantitative x-ray Imaging (qXRI)

The diameter of femurs was measured and recorded, followed by the scanning processes. The femurs were imaged together with two standards, 1.5 mm-thick steel and polyester which represent grey 255 and 0, respectively. Bone mineral content was determined as detailed in Section 2.6.1.

6.3.4 Assessment of trabecular bone morphology and cortical geometry by X-ray microcomputed tomography (µCT)

Tibias were tomographed as described in Section 2.6.2 and parameters that were obtained from the projections include bone mass, trabecular bone thickness, numbers of trabecular bone and trabecular separation. Cortical bone was located from a series of cross-sectional 3D images and ImageJ was used to determined cortical bone diameter and bone marrow diameter. Subsequently, cortical thickness and cross-sectional moment of inertia were calculated based on equations given in Section 2.6.2.

6.3.5 Evaluation of extrinsic mechanical strength of bones

Femurs were individually kept in test tubes containing saline (NaCl, 0.9%) and left overnight at 4°C. Three-point bending tests was carried out as described in Section 2.6.3, and four mechanical properties were evaluated; ultimate load, ultimate displacement, stiffness and total absorbed energy (work-to-failure).

6.3.6 Statistical analysis

Data were analysed using repeated measures one-way or two-way ANOVA with Tukey’s post hoc tests or two-tailed t-tests using PRISM 5.0. Data are expressed as mean ± S.E.M and a P value < 0.05 was considered statistically significant.
6.4 RESULTS

6.4.1 Effects of twice-daily administration of Exendin-4 on metabolic parameters, glucose tolerance and insulin sensitivity in high-fat fed Swiss TO mice.

As shown in Table 6.1, twice-daily treatment of Exendin-4 for 52 days had no effect on body weight of high-fat fed mice. However, reduced non-fasting plasma glucose concentration (58% decrease, P < 0.01) and increased non-fasting insulin level (83% increase, P < 0.01) were observed in Exendin-4-treated high-fat mice compared to saline diabetic controls. In addition, significant improvement in glucose tolerance test (22%, P < 0.05) and insulin sensitivity (57%, P < 0.05) was also observed.

6.4.2 Effects of once-daily administration of Sitagliptin on metabolic parameters, glucose tolerance and insulin sensitivity in high-fat fed Swiss TO mice.

As seen in Table 6.2, once-daily treatment with Sitagliptin for 21 days had no effect on body weight of high-fat fed mice. Non-fasting plasma glucose concentrations were significantly reduced (41% decrease, P < 0.001) and this was accompanied by noticeable increases in non-fasting insulin levels (55% increase, P < 0.01) compared to diabetic controls. Improvement in glucose tolerance (32%, P < 0.001) and insulin sensitivity (50%, P < 0.001) was also observed.

6.4.3 Effects of Exendin-4 and Sitagliptin on total body fat mass, bone mineral density and bone mineral content in high-fat fed NIH Swiss mice.

High-fat diet significantly increased total body fat (27% increase, P < 0.01) of mice compared to lean littermate controls (Figure 6.1). Exendin-4 treatment for 52 days had no significant effect on percentage total body fat mass in high-fat diabetic mice. Bone densitometry which measured by bone mineral density (BMD) and bone mineral content (BMC) revealed that total BMD (Figure 6.2A) and BMD at lumbar (Figure 6.3A), femur (Figure 6.4A) and tibia (Figure 6.5A) were similar between high-fat diet and normal chow mice. Despite unchanged whole body BMC, tibial
BMC was markedly increased (14% increase, P < 0.01, Figure 6.5B) by high-fat feeding. Treatment with Exendin-4 had no effect on BMD and BMC of high-fat fed mice when compared with high-fat controls (Figure 6.2-6.5).

Similar to high-fat mice in the Exendin-4 study, there was an increase (24% increase, P < 0.05, Figure 6.11) in percentage total body fat in mice consuming high-fat diet compared to mice feeding on normal chow in the Sitagliptin study. A modest reduction was seen in body fat mass of high-fat mice administered with Sitagliptin (Figure 6.11) but it was not significant when compared to saline high-fat controls. As compared to normal littermates, total BMD was unchanged in high-fat mice (Figure 6.12A). However, high-fat diabetic mice had increased total BMC (12% increase, P < 0.05, Figure 6.12B). BMD at the lumbar region of high-fat diabetic mice was marginally higher than controls, however, the difference did not reach statistical significance (Figure 6.13A). On the other hand, lumbar BMC of high-fat mice was significantly increased (22% increase, P < 0.05, Figure 6.13B) when compared to lean controls. Femoral and tibial BMD and BMC were similar in high-fat and normal mice (Figure 6.14 & 6.15). Sitagliptin had no significant effect on BMD and BMC at whole body, femur, tibia and lumbar regions in high-fat mice (Figure 6.12-6.15).

### 6.4.4 Effects of Exendin-4 and Sitagliptin on cortical and trabecular bone mineral content in high-fat fed NIH Swiss mice.

As assessed by quantitative x-ray microradiographs, bone mineral content was not affected by high fat diet. GLmean and absorbing mineral density values at both cortical and trabecular were similar in lean and high-fat mice. In addition, treatment with either Exendin-4 (Figure 6.6 & 6.7) or Sitagliptin (Figure 6.16 & 6.17) did not show any significant differences when compared with high-fat controls.

### 6.4.5 Effects of Exendin-4 and Sitagliptin on trabecular bone microstructural morphology and cortical bone geometry in high-fat fed NIH Swiss mice.

Microarchitecture of tibia was assessed by microCT. For the Exendin-4 study, high-fat mice exhibited significantly lowered bone volume (44% decrease, P < 0.01, Figure 6.8A), reduced trabecular numbers (45% decrease, P < 0.05, Figure 6.8C) and
increased trabecular separation (36% increase, P < 0.01, Figure 6.8D) compared with normal mice, while trabecular thickness (Figure 6.8B) was unchanged. Twice-daily administration of Exendin-4 slightly increased bone mass and trabecular numbers, while trabecular separation was reduced compared with high-fat fed control group (Figure 6.8). The differences however did not reach statistical significance (Figure 6.8). At cortical bone, as shown in Figure 6.9, high-fat fed mice exhibited increased bone diameter (10% increase, P < 0.05, Figure 6.9A), bone marrow diameter (25% increase, P < 0.001, Figure 6.9B) and CSMI (49% increase, P < 0.05, Figure 6.9D) compared to lean mice, whilst cortical thickness (Figure 6.9C) was unchanged. Exendin-4 treatment had no effect on cortical bone geometry of high-fat mice (Figure 6.9A-D).

In the Sitagliptin study, high-fat mice had a significant reduction in trabecular bone volume (28% decrease, P < 0.01, Figure 6.18A) and trabecular numbers (30% decrease, P < 0.01, Figure 6.18A), whilst trabecular thickness (Figure 6.18B) and separation (Figure 6.18D) were not significantly different than lean mice. Treatment with Sitagliptin did not change any of these parameters. As compared to lean controls, the high-fat mice displayed significantly increased cortical bone diameter (13% increase, P < 0.001, Figure 6.19A), bone marrow diameter (22% increase, P < 0.01, Figure 6.19B) and CSMI (52% increase, P < 0.001, Figure 6.19D), whilst cortical thickness was not significantly different (Figure 6.19C). Cortical thickness and CSMI were slightly lowered (Figure 6.19C & 6.19D) in Sitagliptin-treated high-fat mice, but the differences did not reach significance when compared to high-fat controls.

6.4.6 Effects of Exendin-4 and Sitagliptin on mechanical properties of cortical bone in high-fat fed NIH Swiss mice.

Mechanical properties of cortical bone were investigated using three-point bending tests. As seen in Figure 6.10, maintenance on high-fat diet did not affect ultimate force, ultimate displacement and stiffness in all three groups. However, work-to-fracture was significantly reduced (34% decrease, P < 0.01) as compared to lean controls. Exendin-4 therapy increased (32% increase, P < 0.01) the work required to break the bone compared to diabetic controls (Figure 6.10D). Most importantly, the
improvement reached a similar work-to-failure level as lean mice. Similar to the Exendin-4 experiment, work-to-fracture in high fat controls from the Sitagliptin study was reduced although the reduction was not significantly different compared to lean mice (Figure 6.20D). Sitagliptin did not influence mechanical properties of bone tissue as no differences were observed during three-point bending tests (Figure 6.20).

6.5 DISCUSSION

Glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors are new classes of drugs introduced for treatment of type 2 diabetes mellitus (Lovshin & Drucker 2009). The target of incretin-based therapies is to sustain improvements in glycaemic control by prolonging or upregulating native incretin action (Drucker et al. 2010). Diabetic individuals are more susceptible to fractures as a result of decreased bone quality (Vestergaard 2011). Furthermore, short-term use of several existing antidiabetic drugs such as the thiazolidinediones (TZDs), are reported to cause bone mass deterioration in rodents (Rzonca et al. 2004, Soroceanu et al. 2004) and humans (Schwartz et al. 2006, Yaturu et al. 2007). However, little is known on the possible effects of clinically approved incretin-based treatments on bone strength and quality. Therefore, the present study assessed the impact of chronic administration of Exendin-4 and Sitagliptin on bone microstructure and mechanical strength in high-fat fed mice with dietary-induced obesity and insulin resistance.

In agreement with previous observations, high-fat fed rodents exhibit significant body weight gain, developed mild hyperglycaemia and hyperinsulinemia with diminished glucose tolerance and insulin response to glucose loading (Porter et al. 2010). Increase in body weight of high-fat mice is associated with a higher percentage of body fat mass as determined by DEXA. Chronic administration of Exendin-4 or Sitagliptin had no beneficial effect on body weight. However, the regimens significantly reduced non-fasting plasma glucose levels and augmented plasma insulin concentrations, as observed in previous studies (Gault et al. 2010, Gault et al. 2015). As compared to Sitagliptin study, non-fasting plasma glucose concentrations of HF control at the end of Exendin-4 study were doubled. Due to protocol of the separate studies, HF control group in Exendin-4 study had longer
access (36 weeks) to high-fat diet compared to HF controls in Sitagliptin study (24 weeks). In general, excessive consumption of high-fat diet contributes to higher accumulation of saturated fats and increased release of inflammatory cytokines and other factors that can disrupt normal metabolic functions. As a result of extended intake of high-fat food, the HF mice in Exendin-4 study had deteriorated metabolic abnormalities and worsening of insulin resistance. Following administration of exogenous glucose or insulin, glucose tolerance and insulin sensitivity were also improved by Exendin-4 and Sitagliptin treatment, in agreement with others (Lamont & Drucker 2008).

Diabetes-related bone loss compromises skeletal integrity and consistent with earlier observations, high-fat feeding also adversely affects bone microstructure and strength in mice (Cao et al. 2009, Ionova-Martin et al. 2010). Bone densitometry by DEXA showed that despite unchanged BMD, BMC in high-fat fed mice was actually increased compared to normal mice. Although we did not measure the length of long-bone, previous studies report that consumption of high-fat diet contributes to larger bone size, hence the increase in BMC (Ionova-Martin et al. 2010). However, estimation of bone mineral content and mineral density at trabecular and cortical bone of mice in the current study by qXRI revealed no significant differences. More importantly, neither Exendin-4 nor Sitagliptin therapy improved these parameters of bone mass.

Bone microstructural assessment demonstrated that high-fat dietary-induced diabetic mice exhibited lower trabecular bone mass, which resulted from a reduction of trabecular bone volume and trabecular numbers, when compared with normal mice. Importantly, the impairments in trabecular bone have also been previously observed by Cao and colleagues and in their study, the reduction in structural parameters at trabecular bone was accompanied by increased RANKL to OPG ratio which was assessed in cultured osteoblasts derived from high-fat fed mice (Cao et al. 2009). RANKL plays major role in osteoclastogenesis by activating c-FOS and TRAF6 molecular pathways (Lamothe et al. 2007, Takayanagi et al. 2002) and the function of RANKL is tightly regulated by a soluble decoy receptor secreted by osteoblasts, OPG (Kostenuik 2005). Increase in the RANKL to OPG ratio would enhance osteoclast activity and overall bone. The findings are further strengthened by
elevation of the serum biomarker for bone resorption, tartrate-resistant acid phosphatase (TRAP) in high-fat mice (Cao et al. 2009).

Chronic administration of Exendin-4 or Sitagliptin failed to restore trabecular bone mass in high-fat diabetic mice. This is surprising as Exendin-4 has previously been shown to have significant beneficial effects on trabecular bone of streptozotocin-induced and insulin-resistant diabetic mice (Nuche-Berenguer et al. 2010b). However, despite no significant differences in these trabecular bone parameters, cancellous bone of high-fat diabetic mice was improved by Exendin-4 therapy, as there were modest augmentations of bone volume and trabecular numbers as well as reduction in trabecular separation. Indeed, it is likely that if a longer period of Exendin-4 treatment was employed, normal trabecular bone mass may have been restored. The neutral effect of Sitagliptin on trabecular bone of high-fat fed male mice is unsurprising as similar observations have been reported previously (Kyle et al. 2011).

As bone quality is determined by multiple factors, mechanical bone strength was also assessed. Mechanical testing is normally conducted at the bone midshaft of long bone which is mostly composed of cortical bone and this method directly evaluates the quality of compact bone. Previously, Cao and colleagues found that 14-week access to high-fat diet alters trabecular bone only, with no prominent changes at cortical bone (Cao et al. 2009). Interestingly, we found that cortical bone of high-fat diet mice was also detrimentally modified. Our findings support previous observations that reported a larger cortical femoral in high-fat fed mice (Ionova-Martin et al. 2010) as also seen by the increase of outer cortical bone diameter in our study. The significant elevation of cortical bone diameter was accompanied by increased bone marrow diameter with unchanged cortical thickness when examined by microCT. In a previous study, histological examination demonstrated the presence of an abundance of adipocytes in the bone marrow area of high-fat mice, and it is possible that expansion of marrow cavity seen in our study is related to an increased number of adipocytes (Halade et al. 2010).

However, there were small inconsistencies in the effects of high-fat diet on mechanical properties of cortical bone in the two separate studies within this
Chapter. As such, in the Exendin-4 study, untreated high-fat diabetic had general reduction in mechanical bone strength which associated with significantly reduced work to fracture as compared to normal mice. Although the same parameter was also slightly reduced in saline-treated diabetic mice with the Sitagliptin study, the reduction did not reach statistical significance. This is probably due to different durations the mice were maintained on the high-fat diet as the Exendin-4 study was longer, allowing further damage to cortical bone by high-fat feeding. As assessed by microCT, cortical bone increased in size, but despite having larger bone, size-dependent bone strength as assessed by three-point bending test was diminished in diabetic mice, indicating decreased bone quality.

Cortical bone from Exendin-4 treated high-fat mice was more resistant to fracture as compared to diabetic control mice and most importantly, the energy required to break femurs was similar to lean controls. In a study carried out by Nuche-Berenguer et al., they found that Exendin-4 treatment increased OPG to RANKL expression and that it also normalised the ratio of LRP5 to DKK1 (Nuche-Berenguer et al. 2010b). Elevation of OPG is associated with reduced osteoclastogenesis as it binds to key mediator for osteoclast differentiation, RANKL and thus, attenuating RANKL bioactivity (Khosla 2001). On the other hand, LRP5 is an activator of canonicical Wnt signaling and its function is blocked by DKK1 (Johnson et al. 2004). Therefore, normalisation of LRP5 to DKK1 in diabetic mice could correct this Wnt signaling pathway together with downstream genes involved in osteoblastic bone formation. This could be the mechanism to explain the beneficial effect of Exendin-4 observed in the current study. Unlike Exendin-4, Sitagliptin treatment had no beneficial effect on whole-bone mechanical strength in high-fat dietary mice. This likely reflects the low circulating levels of incretin achieved with DPP-4 inhibitor, as opposed to exogenous injection of relatively large doses of stable incretin mimetics.

In Chapter 4 and 5 of this thesis, pharmacologically- and genetically-induced diabetic mice exhibited bone impairments and once daily administration of stable GIPRs or/and GLP-1Rs agonists were shown to improve bone quality in those models of diabetes. The data in this Chapter show that induction of diabetes by prolonged feeding of high-fat diet also perturbs the microstructure of trabecular and cortical bone, leading to low bone strength. Despite lack of effects on trabecular
bone, there was cortical bone restoration with Exendin-4, but not Sitagliptin, treatment. However, further investigation is required to establish the mechanism of action and elucidate the anabolic effects of Exendin-4 on bone strength.
Table 6.1 Effects of twice-daily administration of Exendin-4 on metabolic parameters in high-fat fed mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HF control</th>
<th>Exendin-4</th>
<th>Lean control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>58.2 ± 2.53</td>
<td>54.1 ± 1.33</td>
<td>42.7 ± 2.08***</td>
</tr>
<tr>
<td>Non-fasting glucose (mM)</td>
<td>22.5 ± 2.67</td>
<td>9.3 ± 2.16**</td>
<td>4.9 ± 0.19***</td>
</tr>
<tr>
<td>Non-fasting insulin (ng/ml)</td>
<td>2.5 ± 0.37</td>
<td>4.6 ± 0.51**</td>
<td>1.8 ± 0.34</td>
</tr>
<tr>
<td>Glucose Tolerance (AUC_{0-60min})</td>
<td>1042.8 ± 101.8</td>
<td>809.62 ± 24.95*</td>
<td>718.55 ± 72.88**</td>
</tr>
<tr>
<td>Glucose Tolerance (AUC_{0-60min})</td>
<td>1166 ± 127.0</td>
<td>1828 ± 155.4*</td>
<td>2192 ± 129.9***</td>
</tr>
</tbody>
</table>

Mice were maintained on high-fat diet for 16 weeks and parameters were recorded after twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w) for 52 days. AUC, area under curve; AAC, area above curve. Data represent means ± S.E.M for 8 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 compared to saline high-fat group.
Figure 6.1: Effects of twice-daily administration of Exendin-4 on total body fat in high-fat fed mice

Body fat was measured by dual energy X-ray absorption after twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w) for 52 days. Values are expressed as mean ± S.E.M for 8 mice per group. **P < 0.01 compared to lean control group.
Figure 6.2: Effects of twice-daily administration of Exendin-4 on A) total bone mineral density (BMD) and B) total bone mineral content (BMC) in high-fat fed mice.

Parameters were measured by dual energy X-ray absorption following 52 days twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w). Values are mean ± S.E.M for 8 mice.
Figure 6.3: Effects of twice-daily administration of Exendin-4 on lumbar A) bone mineral density (BMD) and B) bone mineral content (BMC) in high-fat fed mice.

A)

Parameters were measured by dual energy X-ray absorption following 52 days twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w). Values are mean ± S.E.M for 8 mice.
Figure 6.4: Effects of twice-daily administration of Exendin-4 on femoral A) bone mineral density (BMD) and B) bone mineral content (BMC) in high-fat fed mice.

A) Parameters were measured by dual energy X-ray absorption following 52 days twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w). Values are mean ± S.E.M for 8 mice.
Figure 6.5: Effects of twice-daily administration of Exendin-4 on tibial A) bone mineral density (BMD) and B) bone mineral content (BMC) in high-fat fed mice

Parameters were measured by dual energy X-ray absorption after twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w) for 52 days. Values are expressed as mean ± S.E.M for 8 mice per group. **p < 0.01 compared to lean control group.
Figure 6.6: Effects of twice-daily administration of Exendin-4 on femoral cortical bone mineral content in high-fat fed mice

A)
Cortical bone mineral content was measured by quantitative x-ray microradiograph following 52 days twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w) A) Digital x-ray images of femoral cortical bone with 16-color lookup table B) Mean grey level C) Absorbing material density. Values are mean ± S.E.M for 8 mice.
Figure 6.7: Effects of twice-daily administration of Exendin-4 on femoral cancellous bone mineral content in high-fat fed mice

A)
Cancellous bone mineral content was measured by quantitative x-ray microradiograph following 52 days twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w). A) Digital x-ray images of femoral cancellous bone with 16-color lookup table. B) Mean grey level. C) Absorbing material density. Values are mean ± S.E.M for 8 mice.
Figure 6.8: Effects of twice-daily administration of Exendin-4 on trabecular bone mass and microarchitecture in high-fat fed mice

A)

B)
Parameters were obtained from a microtomography scan following 52 days twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w). Values are mean ± S.E.M for 8 mice. *P < 0.05, **P < 0.01, compared with lean control.
Figure 6.9: Effects of twice-daily administration of Exendin-4 on cortical bone geometry in high-fat fed mice

A)

B)

Lean control | HF control | HF Exendin-4

B.Dm (µm)

Ma.Dm (µm)
Bones were scanned using a high resolution microtomography scanner following 52 days twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w). Parameters were measured 3 mm below growth plate at cortical bone, A) Diameter of bone, B) diameter of bone marrow, C) cortical thickness and D) cross-sectional moment of inertia. Values are mean ± S.E.M for 8 mice. *P < 0.05, ***P < 0.001 compared with lean control.
Figure 6.10: Effects of twice-daily administration of Exendin-4 on mechanical properties of femoral cortical bone in high-fat fed mice

A)

B)
Parameters were obtained from three-point bending test following 50 days twice daily administration of saline (0.9%, w/v, NaCl) or Exendin-4 (25 nmol/kg b.w). Values are mean ± S.E.M for 8 mice. **P < 0.01 compared with high-fat control group.
Table 6.2 Effects of once-daily administration of Sitagliptin on metabolic parameters in high-fat fed mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HF control</th>
<th>Sitagliptin</th>
<th>Lean control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>55.8 ± 1.26</td>
<td>53.6 ± 0.94</td>
<td>45 ± 2.45***</td>
</tr>
<tr>
<td>Non-fasting glucose (mM)</td>
<td>11.4 ± 1.25</td>
<td>6.7 ± 0.41***</td>
<td>6.7 ± 0.59***</td>
</tr>
<tr>
<td>Non-fasting insulin (ng/ml)</td>
<td>2.0 ± 0.43</td>
<td>3.1 ± 0.39**</td>
<td>1.7 ± 0.57</td>
</tr>
<tr>
<td>Glucose Tolerance (AUC_{0-60min}) (mmol/l.min)</td>
<td>614.3 ± 40.1</td>
<td>418.2 ± 74.4***</td>
<td>417.3 ± 31.9***</td>
</tr>
<tr>
<td>Insulin Sensitivity (AAC_{0-60min}) (% change plasma glucose)</td>
<td>1054.4 ± 180.7</td>
<td>1576.9 ± 187.5***</td>
<td>2239.7 ± 173.5***</td>
</tr>
</tbody>
</table>

Mice were maintained on high-fat diet for 20 weeks and parameters were recorded after once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w) for 21 days. AUC, area under curve; AAC, area above curve. Data are presented as means ± S.E.M for 6 mice per group. **P < 0.01, ***P < 0.001 compared to saline high-fat group.
Figure 6.11: Effects of once daily administration of Sitagliptin on total body fat as measured by DEXA scanning in high-fat fed mice

Body fat was measured by dual energy X-ray absorption after once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w) for 21 days. Values are expressed as mean ± S.E.M for 6 mice per group. *P < 0.05, **P < 0.01 compared to lean control group.
Figure 6.12: Effects of once daily administration of Sitagliptin on A) total bone mineral density (BMD) and B) total bone mineral content (BMC) in high-fat fed mice

A)

![Bar chart showing total BMD (g/cm²)]

B)

![Bar chart showing total BMC (g)]

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). Values are mean ± S.E.M for 6 mice. *P < 0.05, **P < 0.01 compared to lean control group.
Figure 6.13: Effects of once daily administration of Sitagliptin on lumbar A) bone mineral density (BMD) and B) bone mineral content (BMC) in high-fat fed mice

A)

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). Values are mean ± S.E.M for 6 mice. *P < 0.05 compared to lean control group.
Figure 6.14: Effects of once daily administration of Sitagliptin on femoral A) bone mineral density (BMD) and B) bone mineral content (BMC) in high-fat fed mice.

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). Values are mean ± S.E.M for 6 mice.
Figure 6.15 : Effects of once daily administration of Sitagliptin on tibial A) bone mineral density (BMD) and B) bone mineral content (BMC) in high-fat fed mice

A)

Parameters were measured by dual energy X-ray absorption following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). Values are mean ± S.E.M for 6 mice.
Figure 6.16: Effects of once daily administration of Sitagliptin on femoral cortical bone mineral content in high-fat fed mice

A)
Cortical bone mineral content was measured by quantitative x-ray microradiograph following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). A) Digital x-ray images of femoral cortical bone with 16-color lookup table B) Mean grey level C) Absorbing material density. Values are mean ± S.E.M for 6 mice.
Figure 6.17: Effects of once daily administration of Sitagliptin on femoral cancellous bone mineral content in high-fat fed mice

A)
Cancellous bone mineral content was measured by quantitative x-ray microradiograph following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). A) Digital x-ray images of femoral cortical bone with 16-color lookup table. B) Mean grey level C) Absorbing material density. Values are mean ± S.E.M for 6 mice.
Figure 6.18: Effects of once daily administration of Sitagliptin on trabecular bone mass and microarchitecture in high-fat fed mice

A) 

![Graph A](image)

B) 

![Graph B](image)
Parameters were obtained from microtomography scan following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). Values are mean ± S.E.M for 6 mice. *P < 0.05, **P < 0.01, compared to lean control group.
Figure 6.19: Effects of once daily administration of Sitagliptin on cortical bone geometry in high-fat fed mice

A)

B.Dm (µm)

Lean control
HF control
HF Sitagliptin

B)

Ma.Dm (µm)

Lean control
HF control
HF Sitagliptin
Parameters were obtained from microtomography scan following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). Values are mean ± S.E.M for 6 mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared to lean control group.
Figure 6.20: Effects of once daily administration of Sitagliptin on mechanical properties of femoral cortical bone in high-fat fed mice

A)

B)
Parameters were obtained from 3-point bending test following 21 days once daily administration of saline (0.9%, w/v, NaCl) or Sitagliptin (50 nmol/kg b.w). Values are mean ± S.E.M for 6 mice.
Chapter 7

General discussion
7.1 Introduction

Diabetes mellitus is now considered as one of the world’s major health problems as rapidly increasing numbers are reported and the burden that this metabolic disorder imposes on society is becoming more severe (International Diabetes Federation 2015). Diabetes is characterised by high blood glucose levels, resulting from defective insulin production and/or utilisation. While T1DM is associated with inability of pancreatic beta cells to produce insulin, T2DM is largely due abnormalities in the secretion and action of insulin (Zimmet et al. 2001). Diabetes has detrimental effects on many other organs, including bone (Bouillon 1991). T1DM patients consistently have low BMD and in contrast, BMD of T2DM patients is either modestly elevated or unchanged (Vestergaard 2007). Nevertheless, both types of diabetes have been linked to increased risk of fragility fractures (Rakel et al. 2008, Vestergaard 2007). With the growing prevalence of diabetes, many diabetic patients will develop bone metabolic abnormalities, leading to increased bone fracture risk (Janghorbani et al. 2007).

Bone impairments have been reported in rodents and humans with diabetes (Vestergaard 2011). Recently, there has been growing interest in understanding diabetes-related metabolic disturbances in bone and discovering new approaches to deal with diabetic bone loss (Moseley 2012). The identification of several risk factors that are associated with bone health in diabetes has been discussed in Sections 1.2.1. Further, it is clear that common molecular signaling contributing to the pathogenesis of bone disorders can be exploited in the development of potential therapeutics. The beneficial actions of GIP and GLP-1 on bone suggest that incretin hormone mimetics can be useful in managing bone abnormalities in diabetes (Ceccarelli et al. 2013, Irwin et al. 2010).

In this thesis, we investigated the potential beneficial effects of incretin-based therapies on bone abnormalities in diabetes. In vitro studies with GIP and its stable analogue on osteoblastic bone cells were conducted in Chapter 3. Additionally, preliminary studies on the effects of GIP and GLP-1 genetic deficiency on bone mass were also carried out in incretin receptor knock-out mice. In subsequent Chapters, the potential effects of different DPP-4 resistant incretin analogues, and a
DPP-4 inhibitor on bone quality, including bone mass, microstructure, geometry and strength was examined in three different types of diabetic mouse models, namely chemically-induced, genetically-induced and environmentally-induced diabetes.

### 7.2 In vitro studies with GIP

GIP is one of the well-characterised incretin hormones that promotes insulin-releasing activity from pancreatic beta-cells in response to food ingestion. In addition to this, functional GIPRs are evidenced on normal human bone, osteoblast-derived cell lines and osteoclasts, indicating direct bone actions of GIP (Bollag et al. 2000, Zhong et al. 2007). Further studies have shown that GIP increased bone formation and reduced bone resorption, suggesting an anabolic effect of GIP on bone (Bollag et al. 2000, Ding et al. 2008, Tsukiyama et al. 2006, Zhong et al. 2003, Zhong et al. 2007). In Chapter 3, the potential effects of native GIP and the stable [D-Ala²]GIP analogue in stimulating bone formation in SaOS-2 cells was assessed. Both peptides significantly increased bone-related growth factors, TGF-β and IGF-1 and the bone formation biomarker, alkaline phosphatase activity. In line with previous studies, cAMP production was also enhanced with native GIP and stable [D-Ala²]GIP, indicating activation of cAMP signaling pathways with GIPR ligands. More importantly, [D-Ala²]GIP was observed to be more potent than the native peptide. Preliminary studies on the effects of incretins on bone mass were also conducted in mice lacking GIP and/or GLP-1 receptors. It was found that bone mass was significantly reduced in GIPR and DIR KO mice, whilst BMD and BMC in GLP-1R KO mice were comparable with lean littermates. In vivo bone assessment in KO mice studies further confirmed the beneficial actions of GIP on bone (Mieczkowska et al. 2013, Xie et al. 2005).

For future studies, other osteoblastic-like cell lines could be employed for in vitro studies with GIP peptides, namely MG-63 (human osteoblastic-like cell line) (Pautke et al. 2004) and MC3T3-E1 (mouse osteogenic cell line) (Sudo et al. 1983). Moreover, MG-63 has already been proven to express functional GIP receptors (Bollag et al. 2000). During bone formation, osteoblasts make unmineralised osteoid, which mainly consists of collagen, which is then followed by bone mineralization.
One of the determinants for bone strength is the degree of bone mineralisation and with the aforementioned cell lines, assessment on the effects of these peptides on mineral deposition could be performed as explained previously (Fratzl-Zelman et al. 1998, Gregory et al. 2004). There are inconsistencies in reports on the presence of receptors of the other well-known incretin, GLP-1 on osteoblasts (Bollag et al. 2000, Mabilleau et al. 2013, Nuche-Berenguer et al. 2010a). However, these contradictory observations are not surprising as they used different osteoblastic cell lines in their studies. Therefore, future work could include receptor binding studies to determine and confirm GLP-1R expression on bone cells, followed by assessment on the potential impact of GLP-1 and its stable mimetics in vitro.

Bone remodeling is a continuous process coordinated by osteoblasts and osteoclasts (Matsuo & Irie 2008). Maintenance of normal molecular signaling in this complex process is vital as imbalance in bone formation and bone resorption may cause bone loss or brittleness, leading to lower bone strength (Seeman 2002). Our studies, together with others have shown that GIP has positive effects on bone-forming osteoblasts (Bollag et al. 2000, Ding et al. 2008, Tsukiyama et al. 2006, Zhong et al. 2003). However, studies on the effects of GIP on osteoclasts in vitro are lacking as only one previous study by Zhong and colleagues report the expression of GIPR on osteoclasts and the direct inhibition of bone resorptive activity by GIP (Zhong et al. 2007). In agreement, another group revealed in a histomorphometrical study that bone formation biomarkers were reduced, coupled with a significant increase in the number of mature osteoclasts in GIPR KO mice, thereby confirming beneficial dual-action of GIP signaling on bone (Tsukiyama et al. 2006). Further examination to establish the potential effects of activation of GIPR and GLP-1R signaling pathways in bone could include determination of osteoclastic proliferation, differentiation, cell death and changes in bone resorption biomarkers in osteoclastic cell cultures.

7.3 Activation of GIPR or GLP-1R on bone quality in pharmacologically induced insulin-deficient diabetic mice

Deficiency in insulin has adverse effects on bone growth (Thrailkill et al. 2005b). In animal studies, a chemical substance that is toxic to islets beta cells such as streptozotocin (STZ), has been readily used to induce insulin-deficient diabetes
models (Coe et al. 2013, Motyl & McCabe 2009). Incretin hormones, with primary physiological action to stimulate insulin secretion from pancreatic beta-cells, have been shown to have beneficial effects on bone (Mabilleau et al. 2013, Xie et al. 2005, Xie et al. 2007, Yamada et al. 2008). The native forms of these peptides have a short half-life as they are rapidly degraded by DPP-4 (Baggio & Drucker 2007). In order to overcome DPP-4 activity, structurally-modified GIPR or GLP-1R agonist such as [D-Ala²]GIP or Liraglutide have been developed (Hinke et al. 2002, Knudsen et al. 2000).

In Chapter 4 of this thesis, normal mice were injected with a single high dose (150 mg/kg bw) of STZ to induce diabetes. Blood glucose concentrations were measured after 2 days to confirm the development of diabetes. Five days post-STZ introduction, diabetic mice received once-daily treatment with DPP-4 resistant [D-Ala²]GIP or Liraglutide for 21 days. The effects of this regimen on metabolic parameters and bone quality were assessed and evaluated. Once-daily administration of [D-Ala²]GIP or Liraglutide had no significant effects on body weight, water intake, non-fasting plasma glucose and insulin concentrations in diabetic mice. Glucose tolerance had tendency to be improved, whilst no augmentation in insulin sensitivity was seen. No prominent changes observed in bone mass, microarchitecture and whole-bone strength with [D-Ala²]GIP or Liraglutide therapy were observed. However, mechanical properties of bone matrix were remarkably improved with both treatments, indicating recovery of bone quality at the tissue level. It is worth highlighting that the target in GIPR or GLP-1R signaling may be on material properties in bone matrix, and that the positive effects of both long-acting analogues are independent of the insulin axis. Overall, this Chapter reveals the potential effects of the GIPR and GLP-1R agonists, [D-Ala²]GIP and Liraglutide, in attenuating insulin-deficient bone impairments in mice.

Additional studies are required to further elucidate the impact of stable GIPR and GLP-1R agonists on bone. These include assessment of bone matrix with other techniques such as Fourier transform infrared imaging (FTIR), which allows investigation of modifications of collagen and minerals status (Paschalis 2012). Furthermore, bone histomorphometry can be performed to examine molecular activities in the bone (e.g osteoblasts and osteoclasts numbers, bone mineralisation)
In parallel, changes in the levels of bone formation and bone resorption serum biomarkers (osteocalcin, PINP, RANKL/OPG and C-terminal telopeptide) as well as protein expression in bone remodeling-associated signaling pathways (Wnt, RUNX2, MMP, amylin and calcitonin) could be assessed. Additionally, a similar long-term study could be conducted with additional groups of diabetic mice treated with the first line drug for osteoporosis and diabetes, a bisphosphonate and metformin, respectively, so that comparison in the efficacy of incretins can be made.

7.4 Simultaneous activation of GIPR and GLP-1R by [D-Ala²]GIP-Oxm on bone quality in leptin receptor-deficient C57 BL/KsJ (db/db) mice

Obesity has a detrimental impact on skeleton quality and impaired bone has been revealed in established rodent models of obesity and insulin resistance (Ealey et al. 2006, Ionova-Martin et al. 2010, Patsch et al. 2011, Williams et al. 2011). Single activation of GIPR or GLP-1R has been shown to improve material properties of bone matrix in STZ-induced insulin-deficient diabetic mice in Chapter 4. However, the effects of co-activation of these receptors on bone quality are uncertain. Chapter 5 of this thesis investigated the effects of simultaneous activation of GIP and GLP-1 receptors by [D-Ala²]GIP-Oxm on metabolic parameters, bone micromorphology and strength in genetically-induced diabetic (db/db) mice. However, it also needs to be borne in mind that [D-Ala²]GIP-Oxm will also activate glucagon receptors. Administration of [D-Ala²]GIP-Oxm for 21 days had no effect on body weight, water intake and accumulative food intake. Non-fasting plasma glucose levels remained high despite a significant increase in insulin concentration on day 20. Additionally, no significant improvements were observed in glucose tolerance and insulin sensitivity between treated and nontreated diabetic groups.

With regards to bone, [D-Ala²]GIP-Oxm therapy had no effects on bone mass as examined radiographically by DEXA and qXRI. However, further studies on bone microarchitecture found that trabecular thickness and cortical bone diameter were significantly elevated in diabetic mice treated with the stable triple-acting peptide. Assessment of biomechanical strength of whole-bone revealed increases in work required to resist fracture and displacement at maximum load applied. This was in
parallel with augmented tissue-level hardness and indentation modulus. From micromorphology and biomechanical evaluation, it was shown that [D-Ala\(^2\)]GIP-Oxm exerts its effect on both trabecular and cortical bone and that simultaneous activation of both incretin receptors improved not only the material properties of bone matrix, but also bone strength at the organ level. Nonetheless, these improvements were not associated with changes in mineral density distribution as no prominent changes were seen in the frequency, quantity or heterogeneity of calcium concentrations in teated and nontreated \(db/db\) mice. To summarise Chapter 5, the triple-acting agonist [D-Ala\(^2\)]GIP-Oxm has beneficial actions on trabecular bone microarchitecture and cortical bone strength at the organ- and tissue-level.

Further studies are needed to investigate possible molecular processes that are activated or inactivated by the binding of [D-Ala\(^2\)]GIP-Oxm to incretin and glucagon receptors. This could be achieved through use of specific GIP, GLP-1 or glucagon antagonists, which have been characterised (Gault et al. 2003b, Peterson & Sullivan 2001). Furthermore, additional FTIR studies together with gene and protein expression analysis, as suggested in Section 7.3, would be of interest.

7.5 Effects of current incretin-based therapies for human T2DM on bone quality in high-fat fed mice

Accumulating evidence has revealed the adverse effects of several existing antidiabetic therapies for T2DM, such as rosiglitazone and pioglitazone on the quality of the skeleton (Rzonca et al. 2004, Schwartz et al. 2006, Soroceanu et al. 2004, Yaturu et al. 2007). As a new class of antidiabetic drugs, the glucose-lowering and insulin-stimulating properties of incretin-based therapies are well established (Ahren 2007, Lovshin & Drucker 2009, Lund et al. 2011). However, less well known are the potential effects of these drugs on bone integrity. Chapter 6 employed well-characterised high-fat diet-induced mouse model of obesity and diabetes to investigate the effects of the GLP-1 mimetic, Exendin-4 and DPP-4 inhibitor, Sitagliptin on bone mass, microarchitecture, geometry and strength. Chronic treatment with Exendin-4 (52 days) or Sitaglipitin (21 days) had no effect on body weight and fat mass of high-fat fed mice. However, glycaemic control was improved as seen by the reduction of non-fasting glucose level. This was accompanied by a
significant increase in non-fasting insulin in Exendin-4- or Sitagliptin-treated compared to nontreated mice. Additionally, glucose tolerance and insulin sensitivity were improved with Exendin-4 and Sitagliptin therapy.

In agreement with previous studies, mice fed on high-fat had impaired bone microarchitecture and strength (Cao et al. 2009, Ionova-Martin et al. 2010). Administration of Exendin-4 or Sitagliptin did not affect bone mass. On the other hand, a modest increase in trabecular bone volume, which was contributed by an increase in trabecular numbers and reduction in trabecular separation was observed with Exendin-4, whilst Sitagliptin treatment did not improve any of parameters measured at trabecular bone in high-fat fed mice. Examination of cortical bone geometry revealed no beneficial effect of Exendin-4 or Sitagliptin therapy. In three-point bending tests, remarkable increased in resistance to bone failure was observed in diabetic mice treated with Exendin-4, but not Sitagliptin, whilst ultimate force, ultimate displacement and stiffness remained unchanged in all treatment groups. From these two separate studies, it is concluded that Exendin-4 improves mechanical bone strength and exerts its main effect at cortical bone, whilst Sitagliptin had neutral effects on bone.

Smaller-scale tissue-level assessment such as nanoindentation could be performed to confirm the findings of the three-point bending test. Similar to previous chapters, FTIR and gene and protein expression studies would also add value to the present studies.

7.6 Strengths and weaknesses

Three different types of diabetic mouse model were employed in this study; STZ-induced insulin-deficient, genetically-induced leptin receptor-deficient (db/db) and finally, the high-fat diet-induced diabetic model. The most significant point to note is that no single animal model can accurately mimic diabetes in man, so it is therefore necessary to study various aetiologies. STZ-induced and HFD-induced diabetic mice came from Swiss TO background, whilst db/db mice were derived from C57 BL/KsJ colony. In comparison to bone density of STZ-induced diabetic mice (Figure 4.9), db/db mice had a significant drop in bone density with respect to its normal
littermates (Figure 5.9). Bone develops in a relatively short period of time in mouse and at the end of first postnatal month, skeletons are almost completely formed (Zoetis et al. 2003). Due to its genetic background, bone formation in \(db/db\) mice has been disrupted since gestation and furthermore, deficiency in postnatal bone osteogenesis as a loss of leptin signaling has been shown in a previous \(db/db\) mice study (Roszer et al. 2014). In contrast, Swiss TO mice were allowed to grow under normal condition and induction of insulin deficiency in STZ-mice was performed after bone modelling was completed. Therefore, bone loss in \(db/db\) was more severe than Swiss TO STZ-diabetic mice.

As discussed in Chapter 5, deficiency in leptin signaling negatively affects skeletal integrity (William et al. 2011). Leptin directly enhances osteoblast and chondrocyte proliferation and differentiation \textit{in vitro} and \textit{in vivo} (Cornish et al. 2002, Thomas et al. 1999) while inhibiting formation of multinucleated mature osteoclast (Cornish et al. 2002, Holloway et al. 2002). Leptin exerts its positive effects on bone through the specific leptin receptor, which has been evidenced on both osteoblasts and chondrocytes (Cornish et al. 2002, Steppan et al. 2000). \textit{In vivo}, peripheral administration of leptin increases bone mass and reduces bone fragility (Cornish et al. 2002, Steppan et al. 2000) in agreement with observations in leptin deficient \(db/db\) mice in this thesis.

In contrast, Karsenty groups reported that intracerebroventricular infusion of leptin in mutant and wild-type mice inhibits osteoblastic bone formation (Ducy et al. 2000) and induces bone resorption (Elefteriou et al. 2005), suggesting a central inhibitory role of leptin on the skeleton. These contradictory findings may be a result of different types of mice used in the studies or route of administration. Of note, leptin is a systemic hormone produced by adipocytes and is abundantly found in human bone marrow environment (Laharrague et al. 1998), proposing that it has important effects on bone. Furthermore, leptin is positively correlated with fat mass, which in turn, has been reported to protect individuals from skeletal fracture (Holecki & Wiecek 2010, Pasco et al. 2001). Regardless of the nature of leptin in bone, those aforementioned experiments establish that leptin can affect bone quality either through the hypothalamus or directly.
In the present study, incretin-based analogues were administered by the intraperitoneal route. Unlike intravenous route which bypasses the need for molecule absorption, intraperitoneal injection of drug into peritoneal cavity is primarily absorbed into portal vein system (Turner et al. 2011). The compounds eventually pass through the liver and undergo hepatic metabolism like oral delivery, before reaching other organs. In terms of incretin effect, intraperitoneal route of administration does not involve gastrointestinal tract and therefore, this method is more similar to intravenous delivery as the incretin effect is avoided. This means that our results are not influenced by possible activation of the incretin axis in these mice.

There is a possibility of cross-reactivity between GIP and GLP-1 agonists, although it is unlikely to occur (Brubaker & Drucker 2002, Thorens 1992). This theory could be tested through use of specific GIP and GLP-1 receptor antagonists (Gault et al. 2003b), or that the analogues could be studied in GIP and GLP-1 receptor transfected cells (Gremlich et al. 1995, Thorens 1993) or receptor KO animals (Preitner et al. 2004, Scrocchi et al. 1996). These mechanistic type studies fall somewhat outside the scope of the current thesis but nevertheless could be very interesting.

7.7 Summary of future studies

This thesis presented data from in vitro studies with GIP and its stable analogue in osteoblastic-derived cells, as well as total and regional bone masses from single or double incretin receptor knock-out mice. In addition, bone quality after chronic treatment with incretin mimetics or DPP-4 inhibitor in three established mouse models of different aetiologies of diabetes was presented. While the data strongly suggests a beneficial effect of incretins on bone, further assessment is required to provide more evidence for the development of incretin-based therapies in the management of bone abnormalities in diabetes. Below is a summary of potential future work:

1) Investigate the effects of GIPR and GLP-1R agonists in different osteoblastic-like cells such as MG-63 (human) and MC3T3-E1 (mouse) cell lines.
2) Assess effects of stable incretins on generation and differentiation of bone-resorbing osteoclastic cells.

3) Establish the mechanisms of action of long-acting GIP and GLP-1 analogues on bone.

4) Conduct long-term studies with DPP-4 resistant GIP and GLP-1 peptides and compare against first line osteoporotic and antidiabetic drugs such as bisphosphonates and biguanides on bone quality in different models of diabetes.

5) Conduct additional ex vivo bone assessments such as Fourier Transform Infrared (FTIR) spectroscopy and histology that have not been covered in this thesis.

6) Evaluate frequency of bone abnormalities in patients treated with GLP-1 mimetic or DPP-4 inhibitor versus other therapies.

7) Assess direct effects of incretin receptor activation on bone characteristics in patients with diabetes.

In conclusion, this thesis highlights incretin-based mimetics as new approach for the management of bone impairments in diabetes. Additionally, this thesis will hopefully provide useful knowledge that can be utilised in future investigations on diabetic bone loss, and in the development of potential therapeutics that will be beneficial for bone-related diseases in general. Ultimately this thesis will act as a forerunner towards clinical trials to reveal the utility of incretin-based drugs for people with diabetes and related bone disorders.
Chapter 8

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