Investigations of Diabetic Bone Disease

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LITERATURE, REGISTRY, AND CLINICAL STUDIES

BY
JAKOB STARUP LINDE

DISSERTATION SUBMITTED 2015

AALBORG UNIVERSITY
DENMARK

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CV

Jakob Starup Linde was born in 1986 in Ræhr, Denmark. He studied Medicine at Aarhus University from 2006 and graduated in June 2012 as MD. Shortly after he enrolled as a PhD student at the Faculty of Medicine, Aalborg University September 2012 and was employed at the Department of Endocrinology and Internal Medicine, Aarhus University Hospital. He has authored fifteen scientific publications among these eight are as first or shared first authorships and have obtained an H-index of six. He is sub investigator in the EU project, CARING, during the PhD period and have thus been engaged in research collaboration with institutions in Netherlands, Sweden, Norway, and Finland. He is supervisor for research year student Sidse Westberg Rasmussen, student help Emilie Frey Bendix, and project supervisor for PhD student Stine Aistrup Eriksen. In the PhD period he has undertaken educational activities in the areas of endocrinology, neurology, and microbiology as a case facilitator for primarily 2nd and 5th semester students in Medicine or Medici students, Aalborg University. Furthermore, he is a board member from 2013 and from 2014 also academic secretary in the Jutland medical society: Jydsk Medicinsk Selskab.

ENGLISH SUMMARY

Diabetes mellitus is associated with an increased risk of fracture. The risk of a hip fracture is 7 fold increased in patients with type 1 diabetes and 1.4 fold increased in patients with type 2 diabetes. These differences in fracture rates are unexplained as current fracture predictors underestimate fracture risk in both diabetes types. Thus, further understanding of the underlying causes of diabetic bone disease may lead to better fracture predictors and preventive measures in patients with diabetes.

The aim of the thesis was to investigate the relationship between bone turnover markers and diabetes and to investigate disparities in bone status between patients with type 1 and type 2 diabetes.

Two systematic reviews and a meta-analysis were conducted to assess and pool what is known about bone turnover markers in diabetes. To examine the effects of glucose on bone turnover, a state-of-the-art intervention study was conducted. A clinical cross-sectional study was conducted, where both patients with type 1 and type 2 diabetes are examined by sophisticated bone structural scans and biochemical bone turnover markers. A registry-based study utilizing the Danish National Hospital Discharge registry was performed to investigate associations between comorbidities, pharmaceutical use, and biochemical markers and the risk of fracture in patients with type 2 diabetes.

Patients with type 2 diabetes had lower bone turnover markers compared to patients with type 1 diabetes and bone mineral density and tissue stiffness were increased in patients with type 2 diabetes. The bone turnover markers were inversely associated with blood glucose in patients with diabetes and both an oral glucose tolerance test and an intravenous test decreased bone resorption markers in healthy men. In the registry based study; fracture risk in patients with type 2 diabetes were associated with low LDL-cholesterol levels and in the cross-sectional study; sclerostin levels were associated with fractures in patients with type 1 diabetes.

In patients with diabetes, bone turnover and bone strength are associated with clinically relevant measures such as cholesterols and fluctuate on the basis of blood glucose level alterations. Future investigations should apply the advanced techniques of continuous glucose monitoring to map the circadian rhythm of bone turnover and glucose in patients with diabetes. Furthermore, longitudinal studies are needed to confirm whether sclerostin and LDL-cholesterol are fracture predictors.

DANSK RESUMÉ

Diabetes mellitus er forbundet med en øget risiko for knoglebrud. Hoftebrud forekommer 7 gange så hyppigt hos type 1 diabetespatienter og 1,4 gange så hyppigt hos type 2 diabetespatienter. Den øgede risiko for knoglebrud forklares ikke af nuværende prædiktorer, da de undervurderer risikoen i begge diabetes typer. Yderligere forståelse af baggrunden for diabetiskknoglesygdom kan føre til bedre prædiktorer for knoglebrud og præventiv behandling hos diabetespatienter.

Afhandlingens formål var at undersøge sammenhængen mellem knogleomsætningsmarkører og diabetes samt at undersøge, om knoglestatus er forskellig mellem patienter med type 1 og type 2 diabetes.

For at vurdere hvad der allerede vides om knogleomsætningsmarkører hos diabetespatienter blev der foretaget litteraturgennemgange med kvalitativ analyse samt kvantitativ analyse i form af en meta-analyse. Effekten af glukose på knogleomsætningsmarkører blev undersøgt ved dels at tilsætte glukose til blodprøver samt ved udførelsen af et interventionsstudie. Knogleomsætning og knoglestruktur blev undersøgt i et tværsnitsstudie, hvor både type 1 og type 2 diabetespatienter blev undersøgt med avancerede knoglestrukturelle skanninger og biokemiske knogleomsætningsmarkører. Et registerstudie der anvender det danske Landspatientregister belyste sammenhængen mellem knoglebrud hos type 2 diabetespatienter med andre sygdomme, lægemiddelforbrug og biokemi målt i forbindelse med behandling og kontrol af diabetes.

Ved undersøgelsen havde type 2 diabetespatienter lavere niveauer af knogleomsætningsmarkører sammenlignet med type 1 diabetespatienter, mens både knoglemineraltæthed og knoglevævsstivhed var højere blandt type 2 diabetespatienter. De målte knogleomsætningsmarkører faldt med stigende blodsukkerniveauer i diabetespatienterne. Desuden reduceredes niveauet af en af de målte knoglenedbrydningsmarkører i raske mænd ved både oralt indtag af glukose og infusion af glukose i en blodåre. Registerundersøgelsen viste at knoglebrud i type 2 diabetespatienter var forbundet med lave niveauer af LDL kolesterol, medens der i tværsnitsstudiet var en sammenhæng mellem knogleomsætningsmarkøren sclerostin og knoglebrud hos type 1 diabetespatienter.

Hos diabetespatienter findes der sammenhænge mellem knogleomsætning og knoglestyrke og vigtige behandlingsmæssige markører som kolesteroltal og blodsukkerniveauer. Fremtidige undersøgelser bør anvende kontinuerlig glukosemonitorering til at kortlægge døgnrytmen af knogleomsætning og blodsukkerniveauer hos diabetespatienter. Desuden er longitudinellestudier nødvendige for at bekræfte, om sclerostin og LDL kolesterol er frakturprædiktorer.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

25 OHD: 25 hydroxy vitamin D
1,25 OHD: 1,25 dihydroxy vitamin D
aBMD: areal Bone Mineral Density
AGE: Advanced Glycation End-products
AP: Alkaline phosphatase
ATC: Anatomical Therapeutic Chemical Classification system
BAP: Bone specific alkaline phosphatase
BMD: Bone Mineral Density
BMI: Body Mass Index
BMS: Bone material strength
CICP: Collagen type 1C propeptide
CTX: C-terminal cross-linked telopeptide of type-I collagen
CV: Coefficient of variation
DPP-IV: Dipeptidyl peptidase IV
DPD: Deoxypyridinoline
DXA: Dual energy x ray absorptiometry
eGFR: estimated Glomerular filtration rate
FGF-23: Fibroblast growth factor 23
FRAX: Fracture Risk Assessment Tool
GLP-1: Glucagon-like peptide 1
GLP-1 RA: Glucagon Like Peptide-1 receptor agonists
GLP-2: Glucagon-like peptide 2
HbA1c: Glycated hemoglobin A1c
HR: Hazard Ratio
HDL: High-density lipoprotein
HRpQCT: High Resolution peripheral Quantitative Computed Tomography
ICD: International Classification of Diseases
ITD: Insulin treated diabetes
IGF-1: Insulin-like Growth Factor -1
IIGI: Isoglycemic intravenous glucose infusion
IVGTT: Intravenous glucose tolerance test
LDL: Low-density lipoprotein
LRP-5: Low-density lipoprotein receptor related protein 5
LRP-6: Low-density lipoprotein receptor related protein 6
MRI: Magnetic resonance imaging
NITD: Non-insulin treated diabetes
NOS: Newcastle Ottawa Scale
NPU: Nomenclature for Properties and Units
NTX: N-terminal cross-linked telopeptide of type-I collagen
OC: Osteocalcin
OGTT: Oral glucose tolerance test
OPG: Osteoprotegerin

OR: Odds ratio
QCT: Quantitative computed tomography
P-: Plasma
P1CP: Procollagen type 1 carboxyl terminal propeptide
P1NP: Procollagen type 1 amino terminal propeptide
PTH: Parathyroid hormone
RANKL: Receptor Activator of Nuclear factor Kappa beta Ligand
S-: Serum
T1D: Type 1 diabetes
T2D: Type 2 diabetes
TBS: Trabecular Bone Score
TRAP: Tartrate resistant acid phosphatase
U-: Urine
ucOC: Undercarboxylated osteocalcin
vBMD: volumetric Bone Mineral Density.

1. INTRODUCTION

1.1 DIABETIC BONE DISEASE

Diabetes and osteoporosis are common diseases both worldwide and in Denmark (1, 2). Diabetes is associated with morbidity and mortality, but also an increased risk of fracture. Patients with diabetes are especially at risk of hip fractures, which may cause individual distress but also relates to an increased risk of death. Paradoxically, the normally used methods to predict fracture underestimate the fracture risk in patients with diabetes. Furthermore, the evidence is sparse whether the bone remodeling is altered in patients with diabetes. Thus, the mechanism, prediction, and prevention of diabetic bone disease are unrevealed. This study will review the current knowledge of diabetic bone disease and add to it by investigating bone turnover markers, bone structural scans, and fracture predictors in patients with diabetes.

1.2 OSTEOPOROSIS

Osteoporosis is a highly prevalent disease with an estimated 200 million individuals suffering worldwide from it (3, 4). In Denmark close to 500.000 individuals have osteoporosis. However, only 130.000 are treated or diagnosed with osteoporosis. Thus, missed diagnoses are common (1, 5). The total cost of osteoporotic fractures was 1.5 billion EUR in Denmark in 2011 with hip fracture being the most expensive fracture type (6). Thus, osteoporotic fractures account for a large part of health expenses in Denmark. Until the occurrence of a low energy fracture osteoporosis is asymptomatic, this makes preventive measures difficult. Osteoporosis is usually associated with aging due to an increased bone resorption compared to bone formation, and for women a dramatic loss of bone at the menopause makes them more susceptible to osteoporosis (7). The decrease in bone content is related to an increased bone resorption, which is not balanced by an equal increase in bone formation (7). Diabetes may present a secondary cause of osteoporosis and even weak associations between diabetes and osteoporosis will lead to a significant number of fractures among the 300.00 patients with diabetes in Denmark. Other secondary causes of osteoporosis includes disuse osteoporosis due to immobilization, malabsorption or low intake of vitamin D and calcium, bone toxic substances as smoking and certain pharmaceuticals as glucocorticoids and antiepileptics, and an altered bone turnover as observed in hyperparathyroidism, hyperthyroidism, and pituitary diseases. Inflammatory diseases as rheumatoid arthritis and inflammatory bowel disease are also associated with osteoporosis, which may be due to use of pharmaceuticals (glucocorticoids) and inflammatory cytokines (8).

It is possible to measure the decrease in bone by the bone mineral density (BMD), which predicts fracture risk (9). By screening individuals at risk preventive measures are thus possible. In certain patient subgroups, as patients with diabetes and patients with renal failure, BMD is less useful (10, 11). The age of the general population increases, and elderly subjects are more susceptible to fractures (12) which increase

mortality and immobilization (13). Therefore, it is important to take measures to prevent osteoporosis and fractures.

1.21 BONE STRUCTURE, CELLS, AND TURNOVER

Bone consists of mineralized matrix (hydroxyapatite), non-mineralized matrix (collagen), and cells (osteoclasts, osteoblasts, and osteocytes). The hydroxyapatite crystals provide mechanical resistance and additional strength to the collagen structures, whereas the network of collagen, mainly type I collagen, contributes with stability and elasticity (14). Bone turnover consists of a combination of bone resorption and bone formation as presented in Figure 1 (15-18).

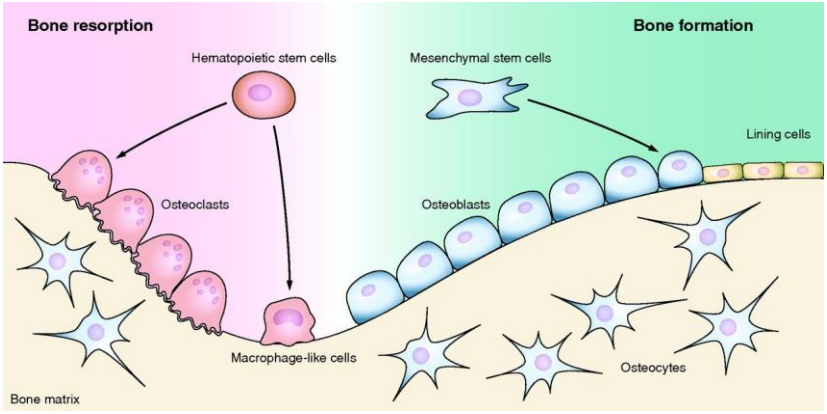

Figure 1. *Bone remodeling (From Imai et al. permission is not required)* (18) .

Osteoclasts are cells which are able to resorp bone and osteoblasts are cells capable of forming new bone whereas the function of osteocytes may be regulatory. Osteoclasts are multinucleated giant cells differentiated from monocyte/macrophage precursor cells and resorb bone under tight regulation (19). Osteoclasts resorb bone by adhering to the underlying bone and creating a sealing zone where acidic proteases are secreted, which degrade bone (19, 20). The degradation products; collagen, calcium, and phosphate are processed within the osteoclast and released to the circulation (19). Osteoblasts derive from mesenchymal stem cells and are bone forming cells and regulate osteoclast differentiation (21). Osteoblasts regulate osteoclasts by secretion of Receptor Activator of Nuclear factor Kappa beta Ligand (RANKL) and osteoprotegerin (OPG). During bone formation osteoblasts produce collagen type I, other non-collagenous proteins, and insulin-like growth factor-1 (IGF-1) that recruit additional osteoblasts during formation. Following bone resorption, osteoblasts migrate to the resorption area and create non-mineralized matrix and regulate the following mineralization (21). Osteocytes are thought to be encased osteoblasts in the mineralized bone which becomes less active as they stop synthesizing matrix. They may function as sensors of mechanical loading and may both regulate bone formation and bone resorption (22) by secretion of sclerostin,

OPG, and RANKL and furthermore regulate the phosphate balance by secreting fibroblast growth factor-23 (FGF-23) (23). Thus, osteocytes, osteoblasts, and osteoclasts communicate through several pathways as presented in Figure 2. These pathways are further described below.

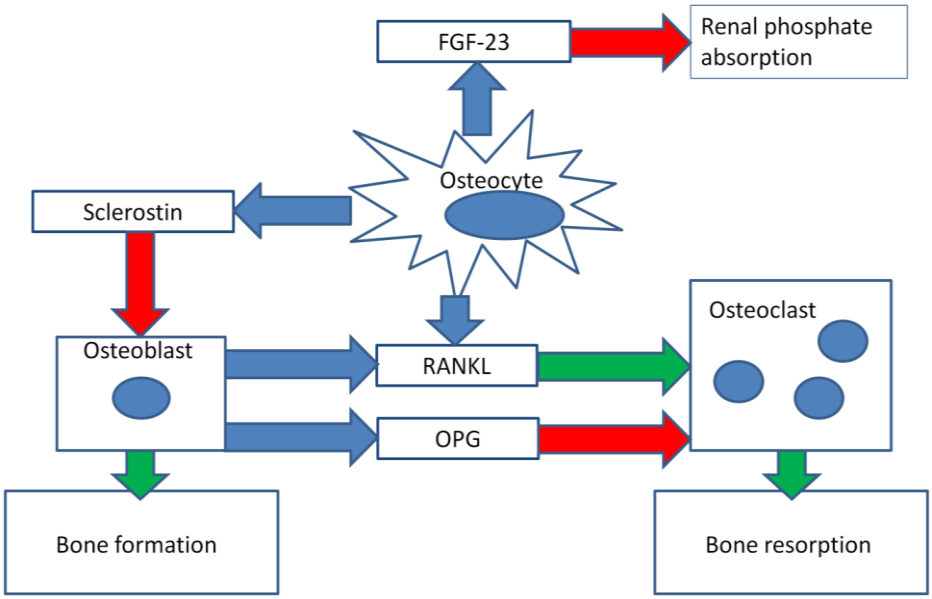

Figure 2. *Overview of the interactions between osteocytes, osteoblasts, and osteoclasts. Red arrows are inhibitory actions, green arrows stimulatory actions, and blue arrows secreted products.*

1.22 REGULATORS OF BONE METABOLISM

1.221 PTH AND VITAMIND D

Parathyroid hormone (PTH) and vitamin D are important hormones of bone turnover. PTH is produced in the parathyroid glands as a response to low blood calcium levels (24). PTH releases calcium to the bloodstream by different mechanisms; 1) increasing bone resorption in an osteoclast mediated mechanism 2) stimulating the tubular reabsorption of calcium 3) decreasing the reabsorption of phosphate in the kidneys, and activating vitamin D from the 25 hydroxy form to 1,25 dihydroxy D vitamin (1,25 OHD) (24). Vitamin D is produced in the skin by exposure to sunlight, but is also a part of the dietary intake through meats, fish, milk, and eggs (24). 1,25 OHD increases absorption of calcium from the intestine and reabsorption in the kidneys, stimulates differentiation of osteoclasts, and controls mineralization in the bone (24).

1.222 RANKL/OPG SYSTEM

RANKL is mainly produced by the osteoblasts and binds to the RANK on osteoclastic precursor cells, which promotes differentiation, activation, and survival of osteoclasts (25). Both PTH and 1,25 vitamin D increases the production of RANKL. OPG is a decoy receptor to RANKL and inhibits the activation of RANK (25, 26). The RANK/RANKL/OPG system is illustrated in Figure 3. A RANKL antibody is used as pharmacological treatment of osteoporosis and decreases the risk of fractures in osteoporotic women (27). Through the interaction with primarily osteoclasts the RANKL/OPG system has important effects on bone resorption and is a valuable target in osteoporosis treatment. Whether the RANKL/OPG system is affected by diabetes is unknown, but it may contribute to the mechanisms of bone deterioration. RANKL and OPG have been investigated in patients with diabetes compared to controls. Serum (s-) RANKL levels are not different when comparing patients with type 1 diabetes (T1D) and patients with type 2 diabetes (T2D) with controls (28-30), whereas s-OPG is increased in patients with T1D and T2D compared to controls (26, 28-32). However, one study reports decreased s-OPG in young patients with T1D compared to controls (33). My study will address the RANK system and bone in diabetes.

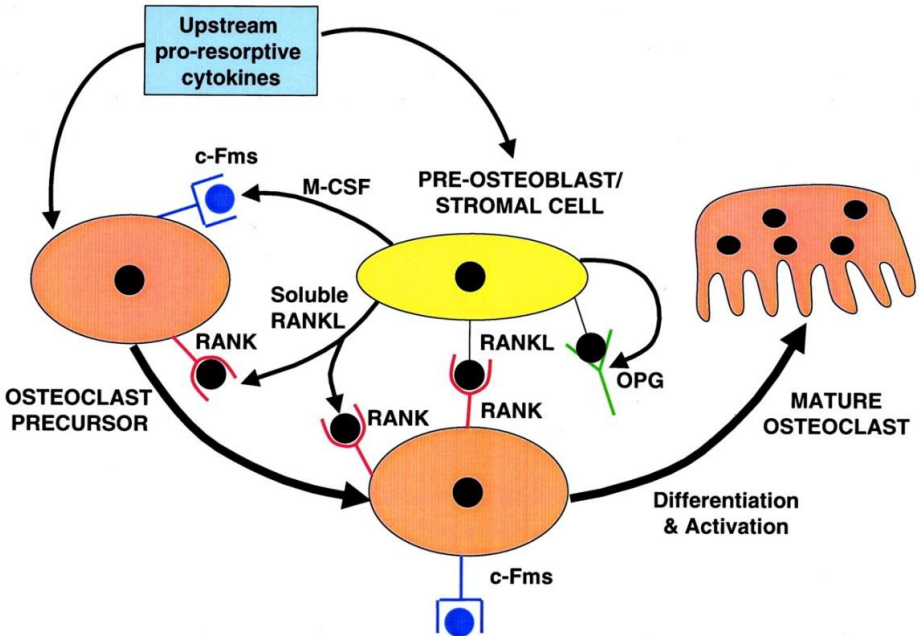

Figure 3. *RANK/RANKL/OPG pathway (From Khosla with permission)* (25).

1.223 WNT PATHWAY

The Wnts are proteins that bind to the low-density lipoprotein receptor related proteins 5 and 6 (LRP-5 and LRP-6). Activation of the receptors prevents

degradation of β-catenin, which alter gene regulation and stimulates osteoblast differentiation, thus the Wnt and LRP-5/LRP-6 signaling is a determinant of bone mass (34, 35). Sclerostin is an important antagonist of Wnts by binding to and inactivating signaling from LRP-5 and LRP-6 (35). Sclerostin antibodies are tested for pharmaceutical use with positive results (36).Wnt may affect glucose metabolism and regulate glucagon-like peptide-1 (GLP-1) secretion. LRP-6 mutations cause hyperlipedemia and diabetes and LRP-5 is essential for glucose induced insulin secretion and normal cholesterol in mice (35). The Wnt pathway may contribute to both bone health and glycemic regulation in patients with diabetes; however few human studies have been conducted. One study reports increased s-sclerostin levels in patients with T2D compared to patients with T1D (37), however age differences are large between the groups. S-sclerostin levels are not different when comparing controls and patients with T1D (37, 38), whereas it is increased in patients with T2D (37, 39). My study will touch on the role of sclerostin, low density lipoprotein (LDL)-cholesterol, and bone status and fracture risk in diabetes.

1.224 FGF-23

FGF-23 is a phosphatonin mainly secreted from the osteocytes, and the highest expression of FGF-23 is in bone. It reduces phosphate levels by limiting reabsorption in the renal tubules and inhibiting the renal production of 1,25 OHD vitamin. Furthermore, it may regulate PTH. In patients with chronic kidney disease FGF-23 levels are elevated, possibly, to preserve normal phosphate levels (40, 41), whereas levels may be normal in patients with diabetes (42). Furthermore, FGF-23 is an inhibitor of bone mineralization the mechanism, however, is not elucidated and the effects may be indirect. FGF-23 may be regulated by other systems, as 1,25 OHD vitamin induces expression of FGF-23 in osteocytes (40, 41). A single study evaluated s-FGF-23 in patients with T2D and controls and found no difference, however characteristics of the included participants were not reported (42). My study will evaluate FGF-23 levels in diabetes and their relationship with bone status.

1.3 DIABETES MELLITUS

Diabetes mellitus is a highly prevalent disease not only in Denmark with 320.000 registered patients with diabetes (43), but also worldwide with an estimate of 387 million suffering from the condition in 2014 (2). This number is increasing towards 592 million individuals suffering from diabetes in 2035 (2). This is of great concern as diabetes mellitus is related to increased morbidity and mortality (44). As with osteoporosis, diabetes is a costly disease. In 2012 the US spent 245 billion dollars on direct medical costs and reduced productivity (45). In 2006 Denmark spent 4.2 billion EUR in direct and indirect costs (46). Diabetes mellitus is in general divided into two types: T1D and T2D (47). Patients with T1D are defined by a complete deficiency of insulin caused by pancreatic β-cell destruction and in many cases it may be serological confirmed by the presence of autoantibodies against the pancreatic islets (47). The more prevalent T2D is defined by insulin resistance and

an inadequate compensatory insulin secretion resulting in a functional insulin deficiency and hyperglycemia (47). A common phenotypic trait of patients with T2D is obesity, whereas patients with patients with T1D usually are normal weight; however not all patients with T2D exhibit this trait. The obesity itself causes insulin resistance (47). Due to a remaining production of insulin in patients with T2D they may go undiagnosed for several years in which the hyperglycemic conditions may affect various tissues (47). Thus, at the time of diagnosis of patients with T2D long term complications may be observed (47). Although the diabetes types differ in characteristics they are diagnosed by the same criteria. The diagnosis of diabetes is based on the measurement of increased glycated hemoglobin A1c (HbA1c) levels, increased fasting plasma (p-) glucose levels or increased two hour p-glucose value after an oral glucose tolerance test (OGTT) (47).

1.31 DIABETES COMPLICATIONS

Diabetes is characterized by hyperglycemia, due to insulin resistance and/or reduced beta cell function, which causes long term damage to specific organs (47). Long term complications to diabetes include microvascular disease; nephropathy, neuropathy, and retinopathy, and macrovascular disease; stroke, acute myocardial infarction, and peripheral vascular complications (47). Another recently added complication to diabetes is fracture (11, 48). Diabetes complications are related to advanced glycation end-products (AGE). AGE are compounds formed by non-enzymatic reactions between sugars and amine residues on lipids, protein, and nucleic acids. In patients with diabetes AGE are formed as a result of hyperglycemia, oxidative stress, and lipids and accumulate within various organs including kidney, retina, and atherosclerotic plaques (49). These AGEs may also accumulate in bone and contribute to the diabetic bone disease.

1.4 DIABETES AND FRACTURE

Recently, research has intensified on the relationship between diabetes and fracture. An association is found between an increased risk of fracture and diabetes mellitus (11, 48). In a meta-analysis *Vestergaard* reports a relative risk of hip fracture risk of 6.94 for patients with T1D and of 1.38 for patients with T2D (11). Further, this study reveals a discrepancy between BMD levels and fracture risk. BMD is increased in patients with T2D and slightly decreased in patients with T1D, but not to the extent that explains the observed fracture risk (11). When patients with T2D are stratified by gender the increased fracture risk is still apparent in both genders (48). Later studies confirm these findings in patients with T2D (50, 51) and a recent study revealed a lifelong increased fracture incidence in patients with T1D (52). The Fracture Risk Assessment Tool (FRAX) that uses common risk factors including BMD provides 10 year fracture risk estimates (53). When applying FRAX to diabetes it underestimates both hip fracture risk and major osteoporotic fracture risk in patients with T2D (54) (Figure 4), thus common used fracture predictors seem of poor use in diabetes.

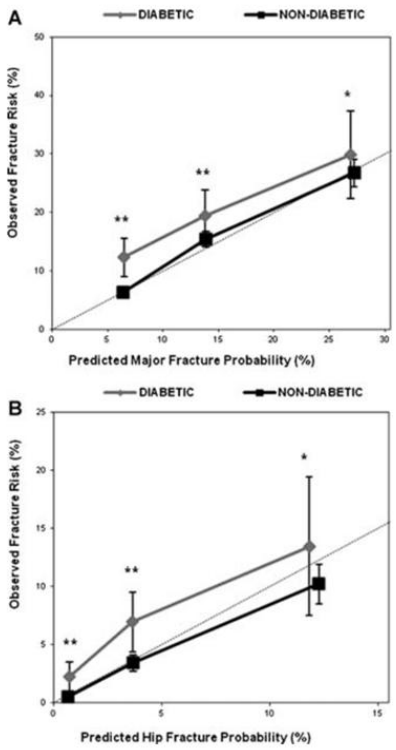

Figure 4. *FRAX prediction of fracture in patients with type 2 diabetes and actual fracture rates. The dotted line indicates the line of identity (perfect concordance). *p < 0.05, **p < 0.001. (from Giangregorio with permission)* (54).

When neither BMD nor other risk factors for osteoporosis are useful predictors, then bone turnover markers, innovative bone scanning techniques, and other variables as patient characteristics, pharmaceutical treatment, and glycemic status may add to the prediction of fractures. Furthermore, it is unknown whether the same factors predict fractures in patients with T1D and T2D.

Using the FRAX model; hip fractures in patients with diabetes compared to non-diabetes subjects are especially increased in those younger than 60 years with a hazard ratio (HR) of 4.67 (55). Although the HR decreased with increasing age the risk remained significantly elevated in diabetes patients (55). Another study using the FRAX model find that BMD and other risk factors predict fractures in old patients with T2D (mean age 73), but suggests using another threshold for BMD in patients with T2D, as the fracture risk is increased at a given BMD or FRAX score compared to non-diabetes individuals (56). However, the greatest fracture risk increase is observed in patients with T2D younger than 60 years old and another BMD threshold may not predict fractures in this group. Other yet unknown predictors of fracture may be of use in patients with diabetes.

A recent study using the Scottish national registry, however, questions previous findings as they find a three times elevated fracture risk in patients with T1D and only a borderline significant increased risk in women with T2D but not in men with T2D (57). Even so, patients with diabetes seem more prone to a fracture, and especially a hip fracture, which is related to increased mortality, morbidity (58), and socioeconomic costs (59). There is a need for a reliable predictor of fracture in patients with diabetes to avoid both suffering for the individual and economical costs for the society.
The increased fracture risk in patients with diabetes may be explained both by deficits in bone biochemical competence which is based on the bone structure; bone matrix, bone mineral, and the collagen composition and an increased rate of falls. Furthermore, falls may be increased due to complications (neuropathy, retinopathy), hypoglycemic events, and pharmaceutical treatment such as anti-hypertensive drugs (Figure 5).

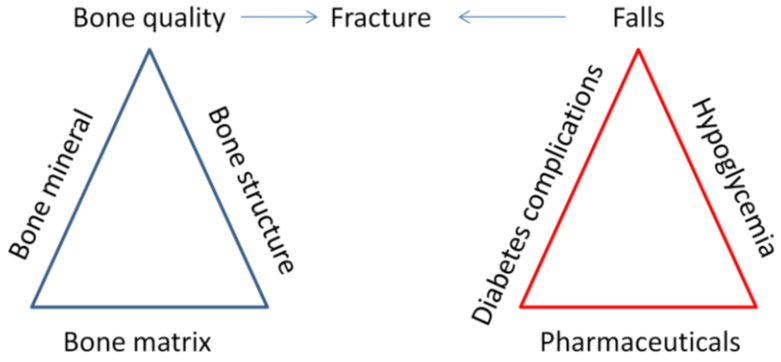

Figure 5. *Factors that relate to falls and bone quality and thereby fractures in patients with diabetes*

1.41 DIABETES, FALLS, AND FRACTURES

An increased risk of fracture may be multi-factorial and caused by both a decrease of bone quality and an increase in risk of falls (60, 61). In diabetes, retinopathy and neuropathy may increase the risk of falls due to poor eyesight and decreased peripheral sensation. A Danish registry based study finds that patients with diabetes with no complications have an increased risk of fracture and that no single diabetes related complication could explain the risk (62). Hypoglycemia may also increase the risk of fracture (63, 64). A study by *Vestergaard et al.* finds an increased risk of fracture in diabetes after adjustment for hypoglycemic events (65). Further, both the studies of *Bonds et al.*, *Napoli et al.* and *Schwartz et al.* adjust by self-reported falls and still find an increased risk of fractures in patients with diabetes (50, 66, 67). The recent study by *Lee et al.* (68) reports an increased risk of fall-related fractures in patients with diabetes. However, falls do not offer a full explanation for the

increased risk of fractures and suggests there may be additional underlying mechanisms.
T2D is associated with obesity, and the fat deposits may act as internal hip protectors (69). As external hip protectors decrease fracture risk in relation to falls (70, 71) this factor may - besides the increased BMD – offer an explanation for the decreased fracture risk in patients with T2D compared to patients with T1D. Although, falls may explain some but not all of the association between fracture and diabetes; fractures still are multi-factorial and rely on – among other factors - decreased bone quality. It is unknown if hypoglycemia may affect bone quality.

1.42 DIABETES DURATION

The duration of diabetes may influence fracture risk. After diagnosis of diabetes a rapid lowering of p-glucose levels may increase the risk of falls and thereby fractures. A longer duration of diabetes would mean a longer period of elevated p-glucose and increase the formation of AGE (72). Insulin, which is a bone anabolic factor (73), is outside normal range for a prolonged period in diabetes. Decreased exposure to insulin and formation of AGE are both factors which may relate to bone quality. Thus, the risk of fractures at the time of diabetes diagnosis could be due to an increased number of falls, whereas longer diabetes duration may relate to decreased bone quality. Patients with T1D have diabetes for the remainder of their lives and the fracture risk may be due to decreased bone quality caused by the long duration of diabetes. However they are also susceptible to hypoglycemia throughout life. Patients with T2D on average have shorter diabetes duration than patients with T1D. Hypoglycemia may be rarer than in patients with T1D; however bone quality may also be affected as the patients with T2D may have diabetes for years previous to diagnosis and a normal longevity (74).
A recent Spanish study reports an increased risk of fracture in newly diagnosed patients with T2D when adjusted for previous falls (75). Other studies report an association between increased diabetes duration and fracture risk (76, 77). Although these studies are conflicting, they may present the same mechanism as diabetes duration in patients with T2D is difficult to assess. The patients with T2D in the study by *Martinez-Laguna et al.* (75) may have had diabetes for a longer period previous to diagnosis (78).

1.43 GLUCOSE, HBA1C, AND DECREASED BONE QUALITY

Insulin resistance and β-cell dysfunction lead to increased p-glucose levels in patients with diabetes (47). Increasing glucose levels relate to an increased production of AGE (79). Enzymatic cross-linking plays an important role in the bone strength. However, by glycation and oxidation these crosslinks can become non-enzymatic AGEs (80). AGE crosslinks are less strong and may decrease bone quality and thereby bone strength. The mineralization of bone reflected in scanning techniques by BMD may be normal, whereas the tissue giving both strength and elasticity may be defect. Therefore, the mean glycemic burden (as reflected by HbA1c) may play a very important role in the pathogenesis of diabetic osteoporosis.

In a retrospective cohort, the risk of hip fracture is reported to be increased with HbA1c levels above 9 % compared to levels of 6-7 % (81). Another study report, that a 1 % increase in HbA1c level is associated with almost a two times increased risk of a prevalent fracture (82). In the same study, a pmol/ml increase of pentosidine, an AGE marker, increases the fracture risk by 1.02 (82). Similar results have previously been reported for HbA1c (83). However, a randomized controlled trial found no difference in falls or fracture between standard glycemic control (HbA1c of 7.5 %) and intensive glycemic control (HbA1c of 6.4 %), although the total number of fractures were limited (84).
Results are conflicting regarding the relationship between BMD and HbA1c. Poor glycemic control is associated with worse bone outcomes in women with T1D, which is reflected by BMD (85). *Vestergaard* finds no effect of HbA1c on BMD in patients with diabetes (11), whereas *Ma et al.* reports a positive effect of HbA1c on BMD at the spine and hip (86). A single HbA1c value may not be informative of the general glucose control in patients with diabetes as the values may rapidly improve or worse. The relation between yearlong averages of HbA1c and BMD may add to the understanding of the bone structure in diabetes.

1.5 DIABETES AND STRUCTURAL BONE SCANS

Table 1 presents studies that have investigated bone structure in diabetes by different scanning techniques. Dual energy x-ray absorptiometry (DXA) scan is used to measure BMD and thereby diagnose osteoporosis and predict fracture risk (9, 87). BMD is, as described above, increased in patients with T2D and slightly decreased in patients with T1D compared to non-diabetes subjects even though both have an elevated fracture risk (11). A recent meta-analysis report decreased BMD in patients with T1D compared to non-diabetes subjects at the spine and at the hip in women, but not in men (88). A further meta-analysis report increased BMD in patients with T2D (86). Few studies have examined differences in BMD between patients with T1D and T2D. Age and BMI adjusted hip and femoral neck BMD is lower in patients with T1D than patients with T2D (89, 90) and in women with T1D than women with T2D (91), however others find no difference in BMD (37, 89, 92, 93). BMD is as measure of the mineralized tissue and will not detect weakening or disorganization of the non-mineralized matrix including collagen, which limits the use in diseases involving collagen. Rats with diabetes presented with decreased hydroxyapatite crystal perfection and decreased calcium / phosphate ratio compared to controls (94), thus mineralization was altered in rats with diabetes with a decrease in bone strength which may not be detected by BMD. Furthermore, whether BMD differences between patients with T1D and T2D are related to bone turnover is unknown (95). BMD seems less useful in diabetes but other techniques may add to the diagnosis and fracture risk assessments in subjects with diabetes. Trabecular bone score (TBS) may improve the value of traditional BMD measures in DXA. TBS is suggested to predict fracture risk better than traditional DXA in women with diabetes (96). TBS is lower in the lumbar spine of women with diabetes even though BMD is increased compared to controls (97). Another study finds no differences in

TBS in women with diabetes at the femur and spine and in men with diabetes at the hip compared to controls (98). The TBS values are positively correlated to lower HbA1c linking the TBS score directly to diabetes (97, 98).
High Resolution peripheral Quantitative Computed Tomography (HRpQCT) scan is a new technique which creates high resolution images of the bone tissue at peripheral sites (tibia, radius) and enables a distinction between cortical and trabecular bone (99). In patients with T2D, patients with a fracture had greater cortical pore size and cortical porosity at the tibia and radius compared to patients without a fracture (100). The same is not observed in non-diabetes individuals (100). Basic parameters as volumetric BMD (vBMD) are found not to differ between patients with diabetes and non-diabetes subjects (101, 102), even in a population where hip BMD is increased (102). Further, cortical porosity and cortical pore size are not different between patients with diabetes and controls (100-102). A single study recently evaluated HRpQCT in patients with T1D compared to controls (103). This study report lower vBMD at the radius and higher total bone area in patients with T1D compared to controls (103). Furthermore, it reports that patients with T1D and microvascular disease have deficits in the cortical and trabecular microarchitecture compared to patients with T1D without microvascular disease (103). However, the study sizes are small with 55 individuals at most in each group (see Table 1), which may explain the lack of differences (100-103). Larger studies are needed to compare differences between patients with T1D and T2D and further relate the measures of bone strength and porosity to bone turnover markers and markers of bone regulating pathways. My study assesses differences in HRpQCT variables between patients with T1D and T2D in a larger material than previous reported. Quantitative Computed Tomography (QCT) is another technique that visualizes bone at central positions but not with high resolution as the HRpQCT. QCT discriminate non-diabetes individuals with a vertebral fracture at a better rate than DXA (104) and it is also superior to DXA in discriminating previous hip fractures (105). A single study supports these results in patients with diabetes (106), however, again the number of individuals in each group is small (n=20). Magnetic resonance imaging (MRI) scan can visualize bone. In diabetes, young women with T1D display lower bone volume and increased trabecular spacing, which is associated with microvascular complications in particular (107).

Reference	Design	Number of patients	Result
Studies comparing Dual energy x-ray absorptiometry between diabetes and non-diabetes subjects			
Dhaliwal et al. 2014 (97).	Cross-sectional.	57 women with T2D and 43 women without diabetes.	Lumbar spine BMD was higher and lumbar spine TBS lower in women with T2D compared to controls. TBS was associated with glycemic control.

Kim et al. 2015 (98).	Cross-sectional.	1,229 men (325 with diabetes) and 1,529 postmenopausal women (370 with diabetes).	Lumbar spine BMD was lower in men and women with diabetes compared to control. Lumbar spine TBS was lower in men and women compared to controls.
Leslie et al. 2013 (96).	Retrospective cohort.	29,407 women 50 years and older (2,356 with diabetes).	BMD was higher and lumbar spine TBS was lower in patients with diabetes. TBS was a BMD independent predictor of fracture in patients with diabetes.
Ma et al. 2012 (86).	Meta-analysis.	15 observational studies (3,437 patients with T2D and 19,139 controls).	BMD was higher in patients with diabetes at the femoral neck, hip, and spine, but not forearm compared to controls.
Pan et al. 2014 (88).	Meta-analysis.	25 observational studies (2715 females with 965 patients with T1D and 1230 males with 537 patients with T1D).	BMD was lower in men with T1D at the spine and femur compared to controls. BMD was lower in women with T1D at the hip, femur, and forearm compared to controls.
Vestergaard 2007 (11).	Meta-analysis of observational studies.	62 observational studies.	Spine Z-score was -0.22 and 0.41 and Hip Z-score was -0.37 and 0.27 in patients with T1D and T2D, respectively compared to controls.
Studies comparing Dual energy x-ray absorptiometry between patients with type 1 and type 2 diabetes			
Bridges et al. 2005 (92).	Cross-sectional.	35 men with T1D, 90 men with T2D, and 55 controls.	No difference in peripheral BMD between patients with T1D and T2D.
Jehle et al. 1998 (91).	Cross-sectional.	27 patients with T1D, 25 patients with T2D, and 100	BMD of the hip and spine was lower in T1D compared to T2D.

		controls.	
Leidig-Bruckner et al. 2014 (93).	Cross-sectional.	139 patients with T1D, 243 patients with T2D, and 504 controls.	BMD was not different between patients with T1D and T2D.
Rakic et al. 2006 (89).	Cross-sectional.	34 patients with T1D, 194 patients with T2D, and 228 controls.	Hip and femoral neck BMD was lower in patients with T1D compared to patients with T2D.
Tuominen et al. 1999 (90).	Cross-sectional.	56 patients with T1D, 68 patients with T2D, and 498 controls.	BMD at the femur was lower in patients with T1D compared to patients with T2D and controls.
High resolution peripheral quantitative computed tomography			
Farr et al. 2014 (102).	Cross-sectional	30 patients with T2D and 30 controls.	Increased cortical thickness and trabecular number at the radius in patients with T2D compared to controls. Increased cortical thickness at the radius.
Patsch et al. 2013 (100).	Cross-sectional.	20 patients with diabetes without fracture, 20 patients with diabetes with fracture 20 controls without fracture, 20 controls with fracture.	Greater cortical porosity in patients with diabetes with fracture than those without.
Shanbhogue et al. 2015 (103).	Cross-sectional.	55 patients with T1D and 55 controls.	Patients with T1D had higher total bone area, trabecular area and lower vBMD and cortical vBMD at the radius. Patients with T1D and microvascular disease had a lower vBMD at both radius and tibia compared to patients with T2D without microvascular disease.

Shu et al. 2012 (101).	Cross-sectional.	25 postmenopausal women with T2D and 25 controls.	No difference between patients with T2D and controls in HRpQCT parameters.
Quantitative computed tomography			
Heilmeier et al. 2015 (106).	Cross-sectional	80 postmenopausal women (20 patients with T2D and a prior fragility fracture, 20 patients with T2D without a fragility fracture, 40 controls).	Patients with T2D and fracture had lower femoral vBMD and thinner cortex compared to patients with T2D without fracture.
Magnetic resonance imaging			
Abdalrahaman et al. 2015 (107)	Cross-sectional.	30 women with T1D and 28 controls.	Bone marrow volume was lower and trabecular spacing was higher in women with T1D.

Table 1. *Studies that investigate patients with diabetes by DXA, HRpQCT, QCT, and MRI.*

Table 2 present the characteristics of the different scanning techniques. The most standardized scan, DXA, which also is the only one that predicts incident fractures, is of poor use in patients with diabetes. New visualizing bone scans as the HRpQCT, QCT, and MRI may be able to predict fractures in diabetes, however, the scans only visualize bone structure and not bone metabolism. Furthermore, besides MRI; DXA, CT, QCT, and HRpQCT all visualize the mineralized matrix, i.e. the calcium containing matrix, and in diabetes perhaps the non-mineralized matrix may also be affected. In general little is known about the effect of long term elevated p-glucose

in diabetes and microvascular complications on the structure of bone and the relation to bone turnover.

Scanning technique	Radiation	Sites measurable	Resolution	Measures	Fracture predictor
DXA	Low	Central and peripheral	Low	Bone mineral density	Yes
HRpQCT	Very low	Peripheral	High	Bone mineral density and bone micro architecture.	Maybe
QCT	High	Central and peripheral	Medium	Bone mineral density and bone micro architecture.	Maybe
MRI	None	Central and peripheral	High	Bone micro architecture	Maybe

Table 2. *Overview of pros and cons of the visualizing bone scans* (108, 109).

1.6 BONE TISSUE BIOPSIES

With unclear results of structural and density bone scans, the solution may be inside the bone tissue. Bone tissue biopsies may both provide structural, dynamic, and mechanical loading parameters. However, few and small bone biopsy studies are conducted on patients with diabetes. Two human studies find low bone turnover in diabetes (95, 110). In these studies bone biopsies are performed on 8 and 5 patients with diabetes, respectively (95, 110). However, another study in 18 patients with T1D does not show any differences in static or dynamic histomorphometric variables compared to non-diabetes subjects (111). Patients in this study were well controlled with a with a mean HbA1c of 6.8 % (111), which may influence the results as the fracture risk is highest in levels above 9 % (81). Evidence from bone biopsies is conflicting since no comparison between patients with T1D and T2D has been published. Bone biopsies may contain valuable information but are difficult to obtain.

Another invasive technique is microindentation, which allows a quantification of bone material strength (BMS) with no removal of bone (112, 113). A single study is performed in patients with T2D (102). It reveals decreased BMS in patients with T2D compared to controls, even though the HbA1c is decently controlled over the previous ten years (7.4 %) and the BMS is negatively correlated to it (102).

1.7 BIOCHEMICAL BONE MARKERS AND DIABETES

Bone metabolism may be measured by biochemical markers of bone turnover. The markers represent different processes in bone turnover from collagen synthesis (procollagen type 1 amino terminal propeptide (P1NP)) and degradation (C-terminal cross-linked telopeptide of type-I collagen (CTX)) to markers secreted by bone cells (osteocalcin (OC) and bone specific alkaline phosphatase (BAP)) (114). Thus bone markers may reflect different parts of bone turnover as well as different compartments. In paper 1 (115), paper 2 (114), and paper 3 (116) biochemical bone markers in diabetes are explained in detail. Both serum and plasma samples may have been used in the studies included in paper 1-3 and in the thesis these samples are described as serum samples (s-). Bone turnover markers include formative markers (OC, BAP, P1NP), resorptive markers (CTX and N-terminal cross-linked telopeptide of type-I collagen (NTX)), important players of bone turnover (RANKL, OPG, PTH, and sclerostin), and markers of other metabolic pathways (IGF-1, pentosidine, and adiponectin). The qualitative evaluation of previous studies in the narrative review of observational studies, paper 1 (115), revealed that s-CTX and s-OC seem to be lower in patients with T1D, while s-tartrate resistant acid phosphatase (TRAP) and s-OC seem lower and s-sclerostin and urine (u-) NTX higher in patients with T2D in comparison to subjects without diabetes. Furthermore, bone turnover markers seem stable over time in patients with T1D (117-119) and even after intensive insulin therapy (120), whereas the s-CTX as the only bone marker seems to increase over time in patients with T2D (117, 119). The apparently stable bone turnover suggests constant bone affection in diabetes. In patients with T2D, glycemic control may decrease bone resorption (121-124) and the metabolic status may thus affect bone turnover. The differences in biochemical bone markers between patients with diabetes and non-diabetes subjects are quantified in a meta-analysis based on observational studies, paper 2 (114). S-OC, s-CTX, and s-25 hydroxy vitamin D (25 OHD) levels are decreased, whereas s-phosphate is increased in patients with diabetes compared to non-diabetes subjects. It is unclear whether or not the increase in phosphate is related to an impaired FGF-23 secretion. When stratifying by diabetes type fewer studies are included, but s-OC levels are significantly decreased in patients with T1D and borderline significantly decreased in patients with T2D ($p=0.06$) compared to non-diabetes subjects. Furthermore, s-phosphate is only increased in patients with T2D and not in patients with T1D compared to non-diabetes subjects. Specific markers of bone turnover, s-CTX and s-OC, seem to be decreased in patients with diabetes, whereas other markers displayed no differences. Although CTX is decreased in diabetes patients compared to non-diabetes subjects, NTX, a marker reflecting resorption by degradation of the same molecule as CTX, is borderline increased in patients with diabetes ($p=0.06$). The discrepancy between CTX and NTX is a paradox, but may be explained by differences in patient characteristics in the evaluated studies and half-life of the bone turnover marker, although it previously has been concluded that no

difference is present between NTX and CTX independent of measurement in serum or urine (125) .
Bone turnover markers displayed large inter-study heterogeneity in the meta-analysis, which may be a result of the variety of assays as well as differences in the assessed populations. However, the studies compare diabetes and non-diabetes populations with the same assays and find the patients with diabetes and subjects without diabetes comparable. The heterogeneity may be explained by the aforementioned factors; however it is unclear whether specific diabetes related characteristic as medication use, glycemic level and so forth could be the real explanation of the heterogeneity.
The systematic review, paper 3 (116), updated the literature search and further examined the relation between bone markers and fracture and the influence of antidiabetics on the markers. Bone turnover markers still seem decreased in both patients with T1D and T2D; however, s-BAP tends to be increased, which may reflect a hypermineralization of bone. Further, decreased levels of s-OC (126), s-IGF-1 (126-128), increased levels of s-sclerostin (127, 129) and an imbalance in bone turnover by high s-CTX and low s-P1NP (130) are all associated with prevalent fractures. Recently, in an abstract presented at the European Association for the Study of Diabetes (EASD) 2014 in Vienna, s-IGF-1 seemed associated with incident fractures in postmenopausal women with T2D (131). These potential fracture predictive markers all relate to an impaired bone formation and low bone turnover. Antidiabetic treatment may also affect bone turnover: In randomized controlled trials glitazone treatment increased s-CTX (132-135) and increased s-sclerostin levels (134). Furthermore, dipeptidyl peptidase IV (DPP IV) inhibitors decreased the resorptive marker u-deoxypyridinoline (DPD) whereas metformin did not change u-DPD or s-OC (136). Further studies are needed to examine the effects of antidiabetic treatments on bone turnover markers as they may influence it.
It is unknown whether the apparent low bone turnover is caused by a specific pathway (RANKL/OPG, Wnt, and FGF-23) and if the bone turnover is affected differently in patients with T1D and T2D.

1.71 GLUCOSE AND BONE TURNOVER MARKERS

Bone turnover markers display large heterogeneity in diabetes (114), which may be caused by different p-glucose levels. An OGTT decreases both resorptive (s-CTX) and formative markers (s-P1NP and s-OC) within twenty minutes after ingestion in healthy young individuals (137). Similar effects are also seen by food ingestion (138). Furthermore, s-CTX decreases both in an intravenous glucose tolerance test (IVGTT) and in an OGTT in healthy women; however, the decrease is significantly greater after an OGTT (139). During an OGTT s-CTX decreased significantly in postmenopausal women with T2D, however the decrease was significantly smaller than in normoglycemic postmenopausal women and s-OPG levels were unaffected (140). The effects of OGTT on bone turnover markers are abolished by the somatostatin analogue, octreotide (137), reflecting a possible link to insulin secretion or gastrointestinal hormones. This link may be via the glucagon-like

peptide 2 (GLP-2), which when subcutaneously injected reduces bone resorption markers, whereas bone resorption is unaffected by GLP-1 injection (138). Another possibility is the gastric hormone ghrelin. However, whether the effect of glucose is direct or indirect through gastrointestinal hormones is yet unknown. In diabetes it is not known whether increasing p-glucose levels decrease bone turnover markers and if so by which bone regulatory pathway.
In vitro studies show effects of high glucose levels on osteoclasts and osteoblasts. In osteoclasts, high glucose levels inhibited RANKL-induced osteoclastogenesis (141) and decreased Tartrate resistant acid phosphatase (TRAP) activity and the pit resorption area for osteoclasts (142). Furthermore, high glucose levels increase mineralization at 12 mM levels and especially at 24 mM levels (Figure 6) and hyperosmotic stress induces over expression of Toll like receptors in osteoblasts, which are known to cause insulin resistance (143).

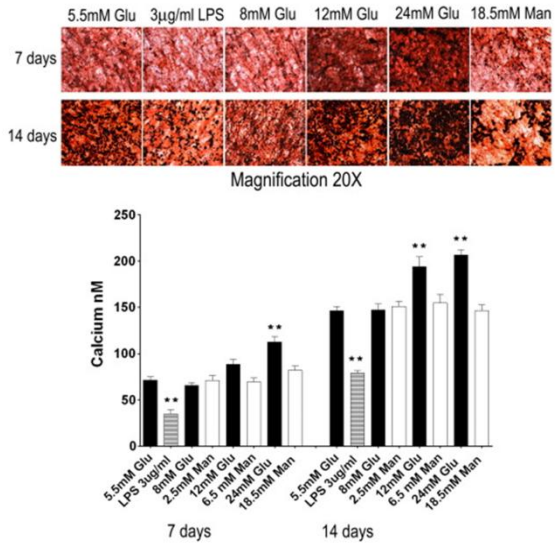

Figure 6. *High levels of glucose on the biomineralization of osteoblastic cells at 7 and 14 days of treatment (From Garcia-Hernandez with permission)* (143).

In contrast, another study showed that hyperglycemia and high osmotic pressure for 24 hours increased the production of collagen type I in osteoblasts and decreased the expression of BAP (144), thus the effects of glucose on mineralization are unclear. Osteocyte like cells exposed to high glucose levels decrease expression of sclerostin protein, but not RANKL (145) and osteocytes may thus also be affected. The in vitro studies highlight a direct effect of glucose on bone cells, which may support a change in biochemical properties brought about by the hyperglycemia per se. Further research is needed to determine the relationship and mechanisms.

1.72 OSTEOCALCIN AND GLUCOSE

OC, a product of bone formation, is in animal models shown to reverse insulin resistance, hyperglycemia, and obesity. Furthermore, the undercarboxylated form stimulates proliferation of beta-cells and increases insulin release and sensitivity. A forward loop may exist as insulin may decrease OPG secretion and enhance OC gene expression and thereby promote release of undercarboxylated OC (ucOC) during the acidic conditions under bone resorption, which again promotes insulin release and insulin sensitivity (Figure 7). In humans, s-OC is associated with insulin sensitivity, p-glucose levels, HbA1c, and diabetes (146, 147). However, evidence is still too limited in humans to determine an effect (148).

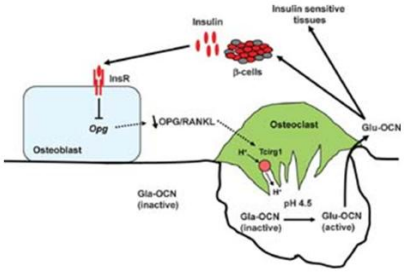

Figure 7. *The feedforward regulation of osteocalcin, bone resorption, and insulin (From Clemens et al. with permission)* (147).

1.8 ANTIDIABETICS AND FRACTURES

As hyperglycemia may both exert effects on bone turnover and increase fracture risk, p-glucose lowering may be the answer to prevent diabetic bone disease. The evidence is limited as few studies have examined the effect of antidiabetics on fractures. Glitazones are associated with an increased fracture risk in patients with T2D (149-151). Metformin and sulphonylureas are associated with a decreased risk of fractures, whereas insulin and other types of oral antidiabetics have neutral outcomes (65). However, other studies show neutral effects of metformin (152-154) and of sulphonylureas (76, 152). The more recent products as the dipeptidyl peptidase IV DPP-IV inhibitors have shown neutral outcomes (155, 156) and may even protect against fractures as reported in a meta-analysis of randomized controlled trials – however, the mean trial duration is as short as 35 weeks (157). Glucagon-like Peptide-1 receptor agonists (GLP-1 RA) have shown neutral effects in a meta-analysis of randomized controlled trials (158) and in an observational study (159). However, another meta-analysis of randomized controlled trials divides the GLP-1 RA treatment by liraglutide and exenatide and finds opposite effects; liraglutide decrease fracture risk and exenatide increase fracture risk (160). Again, the mean trial duration is short (158, 160).

The effects of antidiabetics may simply be caused by tighter glycemic regulation. However, besides the harm of glitazones it is unknown whether a specific treatment is optimal for bone health in diabetes.

1.9 SUMMARY

Figure 8 depicts the factors that may influence the increased fracture risk in diabetes patients. In summary diabetes is associated with an increased risk of fracture, which is not explained by BMD by DXA, FRAX, diabetes complications or falls although they may contribute to it. AGE products, TBS, increased cortical porosity, increased s-sclerostin levels, and HbA1c may all contribute to the increased fracture risk. However, at present only associations have been described and further studies are thus needed. Treatment with glitazones has a detrimental effect on bone in diabetes patients, whereas other antidiabetic treatments seem safe. Based on s-OC and s-CTX bone formation and resorption seem to be decreased in diabetes patients. However it is unknown why the turnover is decreased although effects of glucose on both osteoclasts and osteoblasts may be responsible.

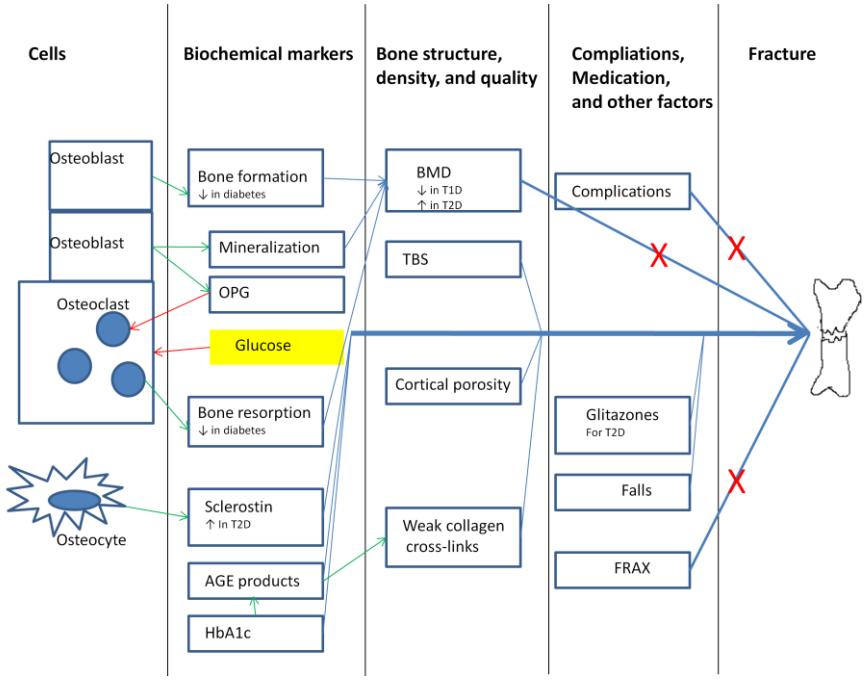

Figure 8. *Factors that may influence the risk of fracture in diabetes patients. OPG: Osteoprotegerin, AGE: Advanced Glycation End, TBS: Trabecular bone score, BMD: Bone mineral density, FRAX: Fracture Risk Assessment Tool. Blue arrows indicate relationships. Red arrows indicate inhibitory effects. Green arrows indicate a positive effect or secretion. X mark associations that do not explain the increased fracture risk in diabetes patients. ↓ marks a decrease compared to subjects without diabetes. ↑ marks an increase compared to subjects without diabetes.*

2. AIM AND HYPOTHESIS

Primary hypotheses:

I. Patients with type 1 and type 2 diabetes differ in bone density measured by BMD, where BMD is decreased in type 1 diabetes.

II. To explain the expected decreased in BMD in patients with type 1 diabetes they have higher bone turnover measured by circulating biochemical markers compared to patients with type 2 diabetes.

Secondary hypotheses

III. Bone turnover markers are decreased in patients with diabetes compared to non-diabetes controls.

IV. HbA1c levels predict fractures in patients with diabetes.

V. Increasing plasma glucose decreases circulating bone turnover markers.

The aims of the thesis were:

I. To examine differences in bone structure between patients with type 1 and type 2 diabetes by DXA and HRpQCT.

II. To examine differences in bone turnover between patients with type 1 and type 2 diabetes by biochemical markers.

III. To evaluate whether medication use and biochemical parameters are associated with fracture risk in patients with diabetes.

IV. To investigate differences in bone turnover markers between patients with diabetes and controls.

V. To examine the relationship between plasma glucose and bone turnover markers in patients with diabetes.

VI. To establish whether the effect of glucose on bone turnover markers is direct or mediated by gastrointestinal absorption of glucose.

3. PAPERS

This PhD resulted in seven papers.

Paper 1	Diabetes, biochemical markers of bone turnover, diabetes control, and bone Starup-Linde J Frontiers in Endocrinology, volume 4 March 2013 A literature study on the difference in bone turnover markers between patients with diabetes and controls.
Paper 2	Biochemical markers of bone turnover in diabetes patients – a meta-analysis, and a methodological study on the effects of glucose on bone markers Starup-Linde J, Eriksen SA, Lykkeboe S, Handberg A, Vestergaard P Osteoporosis International, Volume 25 June 2014 A meta-analysis on the difference in bone turnover markers between patients with diabetes and controls.
Paper 3	Biochemical bone turnover markers in diabetes mellitus – A systematic review Starup-Linde J, Vestergaard P Bone, Available online February 2015 A systematic literature study on biochemical bone turnover makers in diabetes.
Paper 4	Low-density lipoprotein cholesterol is associated with fracture risk in diabetes patients – a nested case-control study. Starup-Linde J, Gregersen S, Vestergaard P Manuscript A case control study examining possible fracture predictors in patients with diabetes.
Paper 5	Differences in biochemical bone markers by diabetes type and the impact of glucose Starup-Linde J, Lykkeboe S, Gregersen S, Hauge E-M, Langdahl BL, Handberg A, Vestergaard P Manuscript submitted A cross-sectional study examining differences in biochemical bone markers between patients with T1D and T2D and the influence of glucose.

Paper 6	Bone structure and predictors of fracture in type 1 and type 2 diabetes Starup-Linde J, Lykkeboe S, Gregersen S, Hauge E-M, Langdahl BL, Handberg A, Vestergaard P Manuscript submitted A cross-sectional study examining differences in bone structure between patients with T1D and T2D and possible fracture predictors.
Paper 7	The effect of glucose on bone – direct or indirect? Westberg-Rasmussen S, Starup-Linde J, Hermansen K, Vestergaard P, Gregersen S. Manuscript in preparation An interventional study comparing the bone turnover marker response by oral ingestion and intravenous infusion of glucose.

4. METHODS

4.1 LITERATURE SEARCH

Literature searches were performed for the reviews and meta-analysis (114-116).

4.11 THE NARRATIVE REVIEW (PAPER 1)

A literature search was first performed in August 2012 with the assistance of a research librarian. Results from this search were used in the narrative review, paper 1 (115). The databases searched were Medline at Pubmed, Embase, Cinahl, Svemed+, Cochrane Library, and Bibliotek.dk. If applicable the search was conducted using the thesaurus with the search terms: "Diabetes mellitus" or "Diabetes mellitus type 1" or "Insulin dependent diabetes mellitus"or "Diabetes mellitus type 2" or "Non insulin dependent diabetes mellitus" or "Hemoglobin A glycosylated" or "HbA1c and "Bone" or "Bone and Bones" or "Bone diseases" or "Bone turnover" ". Records were eligible if they were observational and examined biochemical markers of bone turnover in patients with diabetes. If records used the same population only the best study based on design, methodology, and number assessed markers, evaluated by J Starup-Linde, was included. Records assessing the effect of different medications were excluded. In total 1,188 records were retrieved after the removal of duplicates. All records were screened by title and abstract for the eligibility criteria and 1,113 records were removed. 75 records were assessed for inclusion by full text screening with the above described eligibility criteria. In total 43 records were included (32 of cross-sectional design and 11 of a prospective design).
If the patients with diabetes were grouped as insulin treated diabetes (ITD) or non-insulin treated diabetes (NITD) they were interpreted as patients with T1D and T2D, respectively. NITD cannot contain patients with T1D, however ITD may contain patients with T2D.

4.12 THE META-ANALYSIS (PAPER 2)

The same literature search profile used in paper 1 (115) was performed for paper 2 (114). The eligibility criteria for the meta-analysis were records comparing biochemical bone turnover markers between patients with diabetes and controls. Studies had to be observational and studies assessing different medications or reusing populations were excluded. Studies were also included in the meta-regression analyses if they evaluated patients with diabetes but did not compare to a control group. 1,118 records were screened by title and abstract resulting in assessment of 75 records. 22 studies were included in the meta-analysis and additional 18 studies were included in the meta-regression. The study selection is presented in Figure 9.

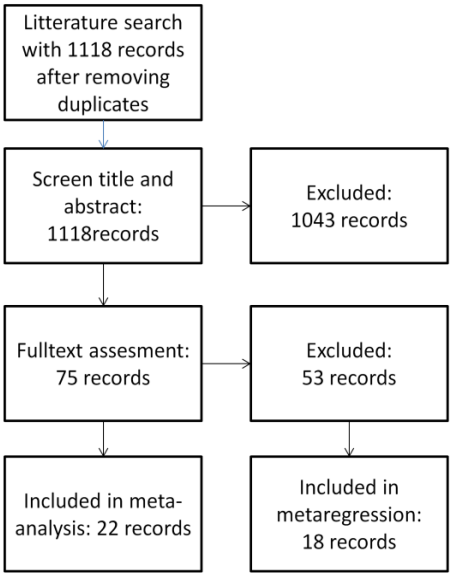

Figure 9. *The steps of selection- from Starup-Linde et al. 2014 with permission* (114)*. 18 records assessing bone turnover markers in patients with diabetes but with no comparison to a control group were included in the meta-regression.*

4.13 THE SYSTEMATIC REVIEW (PAPER 3)

For the systematic review (116) a systematic literature search was conducted in August 2014 using the databases Medline at Pubmed and Embase. Medline at Pubmed was searched by the MESH term "Diabetes Mellitus" and the free text term "bone turnover markers". Embase was searched by the thesaurus using the terms "Diabetes Mellitus" and "bone turnover" with the limitation of human studies. 611 records were retrieved. Figure 10 present the steps of selection. The difference in the number of publications included in section 4.11, 4.12 and 4.13 is due to differences in eligibility criteria. For this search they are described below.

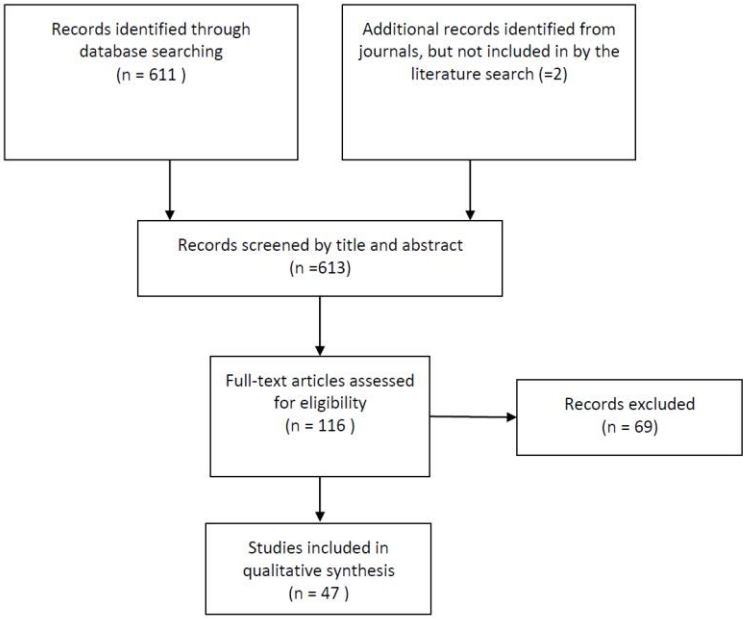

Figure 10. *The steps of selection –from Starup-Linde et al. 2015with permission* (116).

The eligibility criteria for inclusion were assessment of bone turnover markers in either patients with T1D or T2D. If records used the same population the best study based on design, methodology, and number assessed markers, rated by J Starup-Linde, was included. No records were excluded on the basis of treatment by antidiabetics, antiresorptives, bone anabolic treatment or statins as opposed to the literature search described in section 4.11. The meta-analysis was included, thus records presented herein were not included (114). Data was extracted from the records, which include study design, randomization and intervention (if applicable), HbA1c, study duration, number of participants, diabetes type, diabetes assessment, bone turnover markers, fasting status at blood sample collection, and renal disease. Study quality was not assessed as both observational studies and intervention studies were included.
Two additional records were added as they were not included in the systematic literature search but found in their respective journal and were within in the scope of the systematic review. 613 records were screened for eligibility by title and abstract, which lead to 116 potential records. These were evaluated for eligibility by full text assessment leading to 47 included studies.

4.14 THE SYSTEMATIC LITERATURE SEARCH FOR THE THESIS

A systematic literature search was conducted for the thesis . Medline at Pubmed and Embase were searched in the period June 2014 till February 2015 on the search terms “diabetes” and “fracture” giving 229 records at Medline at Pubmed and 567

records at Embase. Furthermore, a search was performed to find previous studies assessing bone structure differences between diabetes types by the search terms "type 1 diabetes", "type 2 diabetes", and "bone mineral density" with no limitations leading to 44 records at Medline at Pubmed and 179 records at Embase. These results were pooled with the results of the searches described in sections 4.11 and 4.13 and give basis for the systematic literature search for the thesis.

4.2 THE META-ANALYSIS (PAPER 2)

Data from the 22 records for meta-analysis and additional 18 records for meta-regression were extracted and tabulated independently by J Starup-Linde and SA Eriksen. The data extracted included age, BMI, gender, diabetes type, study design, fasting status at the time of blood sample, follow up years (if applicable), HbA1c, OC, BAP, alkaline phosphatase (AP), collagen type 1C propeptide (CICP), CTX, NTX, DPD, calcium, phosphate, 25 hydroxy D vitamin (25 OHD vitamin), and PTH. Data was sought on other bone markers, but it was not possible to conduct a meaningful meta-analysis. ITD patients were grouped as patients with T1D and NITD as patients with T2D. The quality of the studies was ascertained by the Newcastle Ottawa Scale (NOS) for cohort studies and a modified scale for cross-sectional studies. J Starup-Linde and SA Eriksen scored the studies and P Vestergaard settled disagreements.
Mean values and 95 % confidence intervals of bone turnover markers were tabulated and by random effects modeling common weighted estimates were calculated. Pooled analyses were only performed if three study groups were available. Publication bias was assessed visually by funnel plot and also by the Eggers test. Heterogeneity between studies was calculated by I^2 test. Analyses were also stratified by diabetes type. Meta-regression analyses were performed using the variables; age, BMI, gender, HbA1c, diabetes, diabetes duration, fasting status, and creatinine levels.
Calculations were performed using The RevMan software program and STATA 8.

4.3 THE METHODOLOGICAL STUDY ON THE EFFECTS OF GLUCOSE ADDED TO SERUM ON THE MEASUREMENTS OF BONE TURNOVER MARKERS (PAPER 2)

A methodological study was conducted at the Department of Clinical Biochemistry, Aalborg University Hospital. From two healthy individuals a fasting blood sample was drawn and fasting glucose was measured (5mM in each sample). To each sample glucose was added to final glucose concentrations of 5 mM (reference), 10 mM, 15 mM, and 25 mM. The reference and glucose supplemented samples were incubated at 37 °C and s-CTX, s-P1NP, and s-OC were measured at time 0, after 1 hour, after 2 hours, and after 3 hours. Bone markers were measured on an automated Cobas analyzer (Roche Diagnostics, Mannheim Germany). The coefficients of variation for CTX, P1NP, and OC were less than 6 %, less than 4 %, and less than 2 %, respectively. Serum stability was 8 hours for CTX and 24 hours for P1NP and

OC, as provided by the manufacturer. Multiple linear regressions were performed using Microsoft Excel 2007 and SPSS 20.0.

4.4 THE CASE CONTROL STUDY (PAPER 4)

The STROBE guideline for reporting of case-control studies was followed (161). The study was approved by the Danish Data Protection Agency.

4.41 DESIGN

The study was designed as a nested case control study in a cohort of patients with diabetes. Patients with diabetes mellitus were sampled from a register, based on the diagnosis appearing in the period January 1 1977 to December 31 2011. Cases were patients with a fracture subsequent to the diagnosis of diabetes in the period 2008-2011 and controls were patients with diabetes without a fracture in the same time period.

4.42 DIABETES ASSESSMENT AND FRACTURE ASSESSMENT

From The Danish National Hospital Discharge Register in the time period 01-01-1977- 31-12-2011 all patients with diabetes were extracted using International Classification of Diseases (ICD)-10 (E10-E14) and ICD-8 codes (249-250). The Danish National Hospital Discharge Register covers all inpatient contacts from 1977 and from 1995 also all outpatient visits at hospitals, outpatient clinics, and emergency rooms. ICD 8 and ICD 10 codes were used to extract specific diagnoses. Cases were defined by ICD-10 fracture codes (ICD-10 codes: S02.0–S02.9, S07.0–S07.9, S12.0–S12.9, S22.0–S22.9, S32.0–S32.8, S42.0–S42.9, S52.0–S52.9, S62.0–S62.9, S72.0–S72.9, S82.0–S82.9, and S92.0–S92.9). The fracture codes cover both low and high energy fractures. Due to the unique social security number it was possible to link the diagnosis of diabetes and a diagnosis of fracture.

4.43 EXPOSURE VARIABLES

Anatomical Therapeutic Chemical Classification system (ATC) codes and Nomenclature for Properties and Units (NPU) codes were used to extract data on prescription reimbursement and measured biochemical parameters. NPU coding is an international classification system used in Clinical Laboratory Sciences. Information on exposure variables were obtained from The Danish National Hospital Discharge Register regarding comorbidities, the Central Region of Jutland, biochemical parameters (2008-2011) and medication use from the prescription registry (2008-2011). Only data on redeemed drugs on prescription was available and not over-the-counter drugs. The age of the patients was defined as the age at January 1, 2008. The duration of diabetes was defined as the time from diabetes diagnosis to the end of the prescription register, December 31, 2011. Data on biochemical markers were obtained from the hospitals in the Central Region of Denmark, thus the markers were for clinical use and not available for all patients. LDL-cholesterol was calculated by the Friedewald formula. Statin duration was

calculated as the time span between first redeemed prescription and to time of a fracture (for cases) or to the end of follow up, December 31, 2011 (for controls). Drug users of either nicotine substitution, vareniclin, bupropion, inhaled β-agonists, inhaled anticholinergics or inhaled corticosteroids were defined as smokers. Users of antihypertensive drugs were defined as having hypertension. Users of bisphosphonates, teriparatide, strontium ranelate, denosumab or hormone replacement therapy were grouped as one. An alcohol-related diagnosis was defined as an ICD-10 code F10 or ICD-8 code 303.

4.44 THE STUDY POPULATION

156,698 patients with diabetes were available from The Danish National Hospital Discharge Register. 29,929 of these had data on pharmaceutical use from the prescription registry. Individuals with nephropathy were excluded due to the detrimental effects on bone health. 2,627 patients with diabetes had information on selected biochemical markers. In total 2,627 patients with diabetes were available for analyses.

4.45 STATISTICAL ANALYSIS

The STATA 8 statistics package was used. Use of a pharmaceutical was categorized as (yes/no) and biochemical parameters were processed as numerical values. An unadjusted and an adjusted case control analysis were performed using logistic regression. Results are expressed as odds ratios (OR). The term risk will be used synonymously with OR as fracture is a rare outcome. Subgroup analyses were performed by diabetes type and by LDL-cholesterol levels grouped in eight quantiles. The division into eight quantiles was performed to investigate differences between the lowest and highest levels of LDL-cholesterol. Statin duration was included as a variable in separate analyses.

4.5 THE CLINICAL STUDY (PAPER 5 AND 6)

The STROBE guideline for reporting of cross-sectional studies was followed (161). The clinical study was registered at ClinicalTrials.gov (NCT01870557). The study was approved by the Danish Data Protection Agency and the Ethics Committee of the Central Denmark Region. The clinical study was conducted according to the Helsinki Declaration.

4.51 DESIGN, SETTING, AND PARTICIPANTS

A clinical study was performed to investigate differences in bone turnover markers and bone structural scans between patients with T1D and T2D. Also, data on fractures was collected to investigate potential fracture predictors. To evaluate this, a cross-sectional design was used. Furthermore, a case control design was used to analyze associations between fractures and bone markers. The study was a multicenter study conducted at two Danish University Hospitals. All patients were recruited from the outpatient clinics at the University Hospitals. From the

Department of Endocrinology and Internal Medicine, Aarhus University Hospital 121 patients were included and from the Department of Endocrinology, Aalborg University Hospital 76 patients were included. Electronic patient journals were screened for eligibility and exclusion criteria and patients were asked if they were interested in the study at an outpatient clinic visit. Possible participants were invited to give informed consent and were interviewed for eligibility in the study. Two patients were excluded due to use of hormone replacement therapy and two patients withdrew their informed consent shortly after enrolment. Patients were recruited and included consecutively. The eligibility criteria for the patients were that they had to be diagnosed by a physician with diabetes mellitus, to be at least 50 years old, to be in antidiabetic treatment (oral antidiabetics, insulin treatment or GLP-1-RA), and report a body mass index (BMI) between 19 kg/m^2 and 35 kg/m^2. BMI ranged from 18 kg/m^2 to 40 kg/m^2 at the examination. Patients were not excluded for this reason. All patients presented a recent HbA1c of at least 49 mmol/mol (6.6 %) and had a relatively stable HbA1c within the last six months (± 1% based on the DCCT scale). Furthermore, all patients had a recent estimated glomerular filtration rate (eGFR) as a minimum of 50 ml/minute.
Patients were excluded if diagnosed with diseases affecting bone including renal dysfunction, uncontrolled thyroid disease, NYHA class IV heart failure, and cancer. Patients with a metal implant at both ankles and wrists were excluded. Patients with a recent HbA1c above 85 mmol/mol were excluded. Individuals treated with antiepileptics, oral glucocorticoids, lithium, antiresorptive treatment, oral estrogen treatment, and bone anabolic treatment were excluded. One patient was treated with bisphosphonate a month before inclusion, but as bone turnover markers (p-CTX, p-P1NP, and p-OC) were not different from other patients; the individual remained in the study. Two of the female patients had ongoing menstrual cycles all others were postmenopausal. In general, patients were in stable antidiabetic treatment with no recent drug type or dosage changes.

4.52 BLOOD SAMPLE AND URINE SAMPLING

All patients had a non-fasting blood sample drawn and all but two delivered a 24 hour urinary sample. Blood samples were generally drawn in the morning, obtaining plasma samples for the conventional biochemical tests: Glucose, glycated hemoglobin (HbA1c), LDL-cholesterol, high-density lipoprotein (HDL)-cholesterol, total-cholesterol, triglycerides, 25 OH vitamin D, creatinine, sodium, and potassium. These parameters were measured at the Department of Clinical Biochemistry at the respective University Hospital. The remaining samples were divided into aliquots and stored at −80°C.
Freshly thawed samples blinded to clinical status for the technician was used for measuring bone turnover markers. All bone turnover markers were analyzed on EDTA-plasma, except for RANKL and OPG where serum was used. P-CTX, p-OC, and p-P1NP were measured as single determinations on an automated Cobas analyzer from Roche Diagnostics (Mannheim, Germany). P-.ucOC (Takara Bio Inc, Otsu, Japan), p-FGF-23 (Immutopics, San Clemente, USA), p-sclerostin/s-OPG

(Biomedica, Vienna, Austria) and free non-OPG-bound s-RANKL (Biomedica, Vienna, Austria) were measured as ELISA double determinations. The analytical coefficients of variation were according to the manufacturers as follows: CTX < 6%, P1NP < 4%, OC < 2%, ucOC < 10%, FGF-23 < 5%, Sclerostin < 10%, RANKL < 3%, OPG < 5%. Measurements were performed in clinical biochemical laboratories accredited according to ISO 15189.

4.53 DXA (PAPER 6)

Measurements of areal BMD (aBMD) were performed using DXA scans. aBMD is a common used predictor of fracture (9). All patients were scanned at the spine, left hip, and right radius on the DXA scan. All scans were performed by trained and experienced staff at a clinical department. A single patient experienced extreme dizziness during the scan and it was aborted. At the Aarhus University Hospital center Hologic scans were performed using two different Hologic Discovery scanners and at the Aalborg University Hospital Center scans were performed using two different Lunar Prodigy scanners. To obtain information on precision coefficient of variation (CV) and difference in measured values a Hologic Discovery phantom was scanned ten times at each scanner. The precision CVs were 1 % for both Hologic Discovery and Lunar Prodigy scanners. One of the Hologic Discovery scanner selected as reference (based on the number of patients, n = 108). The Lunar Prodigy scanners measured BMD 14 % and 15 % significantly higher than the reference and the other Hologic Discovery scanner measured 2 % significantly higher. BMD was calculated based on the conversion factors reported above. T-scores and Z-scores were calculated based on the paper of *Kelly et al.* (162). The calculated T-scores were -0.23, -0.40 lower at the hip and femur, respectively compared with 21 randomly selected values from the discovery machine. Spine T-scores did not differ between calculated values and values directly from the scanners, whereas the difference in radius T-score was 0.47. The Z-scores differed by -0.04 at the hip, -0.15 at the femur, 0.39 at the spine, and 2.52 at the radius. Due to the large variation between calculated and directly measured T- and Z-scores at the radius, BMD is given instead. Reported hip and femur T-scores are corrected by the factors -0.23 and -0.40, respectively.

4.54 HRPQCT (PAPER 6)

The HRpQCT scan (Xtreme CT, Scanco Medical, Switzerland) volumetric BMD (vBMD) and the finite element analysis provides functional parameters of the tissue (163). Patients included at Aarhus University Hospital were scanned using HRpQCT at the right radius and right tibia. If a prior fracture was present at either of these sites or pain made it impossible to scan, the left side was scanned. Two individuals had too large feet for the HRpQCT carbon fiber shell and were not scanned at the tibia. Four individuals had either too large wrists for the carbon fiber shell or the scan was of poor quality at the wrist and data were excluded from analysis. For each scan, a scout view was performed to define the measurement region using a threshold of 9.5 mm and 22.5 mm for the radius and tibia, respectively. At each

skeletal site 110 images were obtained. All images were graded based on suggestions from the Manufacturer from 1 to 5 (1 = best, 5 = worst) and all images graded 4 or 5 required a rescan. All scans were analyzed with software provided by Scanco Medical (Switzerland). The analyses performed were standard evaluation providing structural and density parameters, finite element analysis with providing stiffness, maximum load, and deformation parameters, and cortical evaluation which provides cortical porosity parameters.
All scans and all evaluations were performed by J Starup-Linde. 18 scans were performed in duplicates (17 at the radius and 16 at the tibia) to assess the CV of the scan. The first scan was performed and afterwards the carbon fiber shell was removed and repositioned. The CV was 0.7 % and 1 % for the tibia and radius scans, respectively.

4.55 EVALUATION OF VERTEBRAL FRACTURE (PAPER 6)

Vertebral fractures were evaluated by Vertebral Fracture Assessment (VFA), an analysis from the Hologic Discovery DXA scan on patients from the center at Aarhus University Hospital and by x-ray of thoracic and lumbar spine at the Aalborg University Hospital center (164). Fractures were defined as a 20 % reduction in vertebral height and graded by the Genant Classification (165). All x-rays were analyzed by a radiologist. If the VFA was suspected of a fracture an x-ray was performed to confirm the diagnosis.

4.56 REGISTRY DATA (PAPER 6)

From the Danish National Hospital Discharge Register all diagnoses of the 197 patients with diabetes were extracted in the time period 01-01 1977- 10-03 2015. We obtained data on fractures in the period 1977-2015 the ICD-10 codes (ICD-10 codes: S02.0–S02.9, S07.0–S07.9, S12.0–S12.9, S22.0–S22.9, S32.0–S32.8, S42.0–S42.9, S52.0–S52.9, S62.0–S62.9, S72.0–S72.9, S82.0–S82.9, S92.0–S92.9) and the ICD 8 codes (800–808.09, 808.11–808.19, 808.91–816.09, 816.19, 816.99–820.12, 820.18–820.92, 820.98–821.22, 821.28–821.32, 821.38–821.92, 821.98–824.03, 824.08–824.13, 824.18–824.93, 824.98–825.99, 826.01–826.19, 826.99–829.99). Fractures were grouped into those prior to the examination and incident fractures subsequent to the examination. Furthermore, major osteoporotic fractures were defined as fracture at hip, spine, shoulder or forearm. Charlson comorbidity index was obtained using codes previously described in detail by *Christensen et al*. (166). Previous hypoglycemic events, and falls were investigated using the ICD 10 codes E160, E161, and E162 and ICD 8 codes 25101 and 25100 and W codes, respectively. Previous measured biochemical parameters were obtained from the hospitals in Central Region of Denmark (2008-) and the Northern Region of Denmark (2006-). The previously measured biochemical parameters were evaluated in a clinical context; therefore some specific parameters are only available for a subgroup of patients.

4.57 POWER CALCULATION

The study size was determined on a power calculation based on BMD measurements from the previous studies by *Shu et al* . (101) and *Vestergaard.* (11). *Vestergaard.* found a difference in Z-score of 0.63 between patients with T1D and T2D and *Shu et al.* reported a standard deviation of 1.4, therefore we used an SD of 1. Setting 2α to 5 % and β to 10 % we estimated that 51 patients were needed in each group to find differences in BMD and therefore we rounded up to 100 participants in each.

4.58 STATISTICS

Descriptive statistics was used to investigate associations between structural parameters, biochemical parameters, and patient characteristics. STATA 8 was used to perform the statistics. Mean values of previously measured biochemical parameters and the time span between these parameters were calculated. Unpaired t-test was performed to test for differences between patients with T1D and T2D. Linear regression solely adjusted by diabetes type was performed to determine associations between characteristics, biochemical parameters, and structural scans. To assess differences based on binary variables diabetes type stratified unpaired t-tests were performed. Multiple linear regression models were performed stratified by diabetes type. Assumptions were checked before performing the linear regressions. A composite fracture endpoint combining vertebral fractures and incident fractures was constructed. To assess the association between the composite fracture endpoint and structural and biochemical markers of bone, unadjusted and adjusted unconditional logistic regression stratified by diabetes type was performed.

4.6 THE INTERVENTIONAL STUDY (PAPER 7)

The interventional study was registered at ClinicalTrials.gov (NCT02213276). The study was approved by the Danish Data Protection Agency and the Ethics Committee of the Central Denmark Region. The clinical study was conducted according to the Helsinki declaration.

4.61 DESIGN AND PARTICIPANTS

The study was an experimental cross over trial, in which participants underwent an OGTT and an isoglycemic intravenous glucose infusion (IIGI) on separate days divided by at least one week. Twelve healthy Caucasian males were recruited by postings at Aarhus University and online at forsøgsperson.dk. Participants gave informed voluntary consent to participation in the study. To be eligible for inclusion the participants had to be between 20 and 50 years of age, be otherwise healthy, and receive no pharmaceutical products. Before each experimental day, participants were asked to refrain from exercise, smoking, and taking vitamin supplements for one day. They were given a standardized meal between 17 and 23 o'clock. Hereafter they were asked to fast (water allowed) from 23 o'clock until they arrived for the experiment the next morning. They were asked to arrive by car or bus. At arrival participants filled in questionnaires about eating and exercise habits.

4.62 ORAL GLUCOSE TOLERANCE TEST

An OGTT was performed on the first experimental day. The participants consumed an oral glucose solution consisting of 82.5 g of glucose monohydrate (equal to 75 g of glucose), 225 ml of water and 225 mg of benzoic acid. Upon arrival, a peripheral intravenous catheter was placed a cubital vein. Blood samples were collected at -15, -10, 0, 15, 30, 60, 120, and 180 minutes from ingestion of the glucose solution. P-glucose was measured at -15, -10 minutes from ingestion and every 5 minutes for the first 2 hours and every 15 minutes for the last hour using an Accu-Chek inform II apparatus (Roche Diagnostics, Basel, Switzerland).

4.63 ISOGLYCEMIC INTRAVENOUS GLUCOSE INFUSION

An IIGI was performed on the second experimental day. A 20% glucose solution was intravenously infused. The infusion rate was adjusted according to the p-glucose levels measured on the first experimental day to reproduce the glucose curve observed at the OGTT. Upon arrival, a peripheral intravenous catheter was placed in each of the participant's cubital veins; one was used for glucose solution infusion and one was used for collecting blood samples. Blood samples and p-glucose were sampled at the same time points as for the OGTT.

4.64 BLOOD SAMPLES

A fasting blood sample was drawn at the first experimental day obtaining plasma samples for HbA1c, TSH, and 25 OHD vitamin, calcium, PTH, triglyceride, total cholesterol, LDL-cholesterol and HDL-cholesterol. Other samples were divided into aliquots and stored at -80 °C.
S-CTX and s-P1NP were measured by immunometric sandwich assays using the COBAS 6000 E at the time points 0, 60, 120, and 180 minutes. The coefficients of variation for the analyses were 5 % and 3.7 %. Analyses were carried out at the Department of Clinical Biochemistry at Aarhus University Hospital, an ISO 15189 certified laboratory.

4.65 STATISTICAL ANALYSIS

Statistical analysis was carried out in a STATA 13 package. Repeated measures ANOVA was used for analysis. Normality of data was checked by q-q-plots. The compound symmetry assumption was not violated when examining the pooled within-subject covariance. To validate the results three conservative F-tests were performed.

4.7 METHODOLOGICAL CONSIDERATIONS

4.71 METHODLOGICAL CONSIDERATIONS IN PAPER 1-3

The papers 1, 2, and 3 are based on literature searches. To conduct a literature search it is important to have described a clear aim, which leads to a selection of search terms and use of eligibility criteria for the inclusion of records. The quality of the review, meta-analysis, and systematic review is determined by the quality of the

literature search. The quality of the literature search is based on the search terms used. The search terms may neither be too narrow (missing relevant papers) or too broad (too many papers resulting in a huge workload). In the papers 1-3 the literature searches conducted was quite broad resulting in many hits of which relatively few were relevant, however this minimizes the chance of missing important records. Furthermore, when deciding whether a record is eligible, papers may be excluded wrongfully. One reviewer J. Starup-Linde decided for the eligibility of the records which gives consistency between the papers 1-3, however it increases the risk of a systematic error. Under optimal circumstances two reviewers should have decided whether the records were eligible for inclusion. Furthermore, when conducting a literature search, using indexed search terms, there is a risk of missing relevant papers due to incorrect indexing or papers so recent, that they have not been indexed. For the papers 1 and 2 only observational records were included. Due to the cross-sectional design of most of the records both the narrative review and the meta-analysis cannot conclude on causality and only find associations. For the paper 3 both observational records and randomized controlled trial records were included. However, when examining bone turnover markers in patients with diabetes versus subjects without diabetes it is impossible to randomize to diabetes status. To evaluate on the effects of antidiabetic medication on bone turnover markers as in paper 3, records of a double blinded randomized controlled design is optimal.
A study (167) in the systematic review, paper 3, reported on patients with T2D in hemodialysis and controls in hemodialysis and reported no association between fracture and PTH and BMD. It is a limitation, however it did not change the general interpretation or conclusions of the paper.

4.711 THE META-ANALYSIS (PAPER 2)

The data extraction from the records is an important step in conducting the meta-analysis. If the data is incorrectly extracted it will lead to errors in the results. To avoid this, data extraction was performed independently by two reviewers J. Starup-Linde and S.A. Eriksen. The strengths of the meta-analysis were the large number of studies included and that the comparison between patients with diabetes and controls was made in the individual study. This generates a high statistical power compared to a single study. A drawback to the meta-analysis is the lack of information on antidiabetic treatment, statin use, and antiosteoporotic treatment and inconsistencies between studies. As patients with diabetes may differ in characteristics as well as renal function, fasting status at the sample collection, 25 OH vitamin D and PTH status it can be difficult to pool the studies, however this should not limit the value of results as the comparison to controls was performed in each study. Neither renal function nor fasting status is likely to affect the results, as fasting status did not determine the levels of bone turnover markers in the meta-regression and only one study reported results from patients with renal disease (168).

4.712 THE METHODOLOGICAL STUDY ON THE EFFECTS OF GLUCOSE ADDED TO SERUM ON THE MEASUREMENTS OF BONE TURNOVER MARKERS (PAPER 2)

This investigation was conducted on serum samples from healthy individuals and thereby it cannot conclude on in vivo conditions or on patients with diabetes. The study is limited to examining the analysis of the bone turnover markers and resolve whether it changes by the addition of glucose. Using a control without glucose addition and following the bone turnover markers over time are strengths to the investigation.

4.72 METHODLOGICAL CONSIDERATIONS IN PAPER 4

The case control study in paper 4 was a retrospective case-control study and thus causality cannot be assessed. The study relies on the validity of the diagnoses of diabetes and fracture and the registration of the diagnoses. In general the validity of the diagnoses is high in the Danish registries. The Danish registers cover the entire Danish population; however the study was restricted to inpatient and outpatient diagnoses from the hospitals and therefore not patients treated only by the general practitioner. Furthermore, it was not possible to examine whether medication was actually taken although registered medication has been bought. However, non-compliance may only be a minor problem as most diabetes associated therapies are taken on a regular basis. In an attempt to adjust for falls; fall diagnoses were collected but only 5 of the 2,627 patients had a prior diagnosis of tendency to falls, and none of these experienced a fracture and it was not included in the analysis. The study may be restricted by the limited number of subjects which biochemical markers and pharmaceutical use were available for; this may lead to selection bias as it is unknown whether the total population of patients with diabetes shares the same characteristics as these 2,627 patients with diabetes. It was not possible to adjust for important life style factors; BMI data was missing for most patients, but was previously shown not to be significantly associated with LDL-cholesterol (169) and smoking and alcohol use was based on proxy variables.
The strength of a case control design is that few outcomes are needed to determine an association as the selection is made on the outcome unlike cohort studies where the selection is based on the exposure. Furthermore, the case control study is hypothesis generating for future studies.

4.73 METHODOLOGICAL CONSIDERATIONS IN PAPER 5 AND 6

4.731 THE DESIGN

The strengths of the cross-sectional study (paper 5 and 6) was the large sample size of well characterized relatively dysregulated diabetes patient, the number of fractures, and the option to adjust the finding for relevant factors that may influence results. In general a cross-sectional study may define many research questions and collect large amounts of information and be hypothesis generating for future studies. Another advantage is that the information can be collected within a short time frame

with multiple outcomes. However, the cross-sectional study was restricted by its own design as it was not able to determine causality. Another weakness to the cross-sectional study is that if the trait under investigation is rare in the general population a large sample is needed; however diabetes is a prevalent disease and the patients were recruited from outpatient clinics at the University Hospitals that have a large patient flow. The recruitment from outpatient clinics may lead to selection bias as these represent a group of diabetes patients unlike those followed solely at the general practitioners. Furthermore, the cross-sectional study is sensitive to recall bias and misclassification. All patients were interviewed for medical history, medication use by a trained MD, J. Starup-Linde, and secondly by the extracting medical files which increase the reliability of the collected data.

4.732 BIOCHEMICAL BONE MARKERS

All biochemical bone markers were analyzed by trained staff at the same laboratory (Department of Clinical Biochemistry, Aalborg University Hospital). A limitation was that the blood samples were taken under non-fasting conditions. It is known that feeding decrease s-CTX, whereas s-OC and p-FGF-23 are known not to be affected (139, 170), thus the interpretation of p-CTX may be difficult. However, the same conditions applied to all participants and p-CTX was associated with p-glucose in the same manner as p-P1NP and p-OC. Furthermore, fasting status did not relate to measured s-CTX or s-OC values in the meta-analysis (114). It is difficult to obtain a fasting condition in diabetes as it is characterized by an increased fasting p-glucose (47). A limitation to the bone turnover markers is the missing s-PTH, s-BAP, s-calcium, and s-phosphate values. These may further explain the bone status in patients with diabetes.
The comparison between bone markers measured in the cross-sectional study and reference ranges should be interpreted with caution as we cannot be sure that the laboratories measure the same levels of bone turnover markers. The manufacturer are not obliged to provide information on possible new measurement levels in the kits used for bone turnover markers, thus it may be difficult to compare to previous reference ranges.

4.733 DXA

DXA images were obtained by trained technicians. Quality control of all DXA-scanners was performed on a daily basis using phantom scans. Due to the usage of four different DXA-scanners that were significantly different the BMD measures were recalculated based on the quality control scans of a phantom (described in section 4.53) so the DXA results were comparable between the DXA-scanners. When vertebral fractures were observed the fractured vertebrae were not included in the BMD as it may lead to overestimation. As patients with diabetes may have more aortic calcification it may overestimate the lumbar spine BMD.

4.734 HRPQCT

To ensure comparability all HRpQCT-scans were performed and all images evaluated by J. Starup-Linde. Patients with diabetes have heterogeneous bones; where some have large bones and other small bones and in few cases it was not possible to fit the cast to the extremity. The accuracy and reproducibility of the scans depend on patient positioning. The precision CV performed for a subgroup is acceptable (described in section 4.54). Motion artifacts could not be avoided completely although the scanner include a motion detector and the scan could be repeated. To avoid drift of the X-ray source routine quality control scans were performed on routine basis.

4.735 VERTEBRAL FRACTURES

All VFA and X-ray images were obtained by trained staff. To ensure the diagnosis of a fracture it had to be confirmed by a radiologist. The collection of vertebral fracture may be limited by the two different methods; VFA and X-ray. VFA detected a vertebral fracture in 2 % of the patients whereas X-ray detected a fracture in 28 % of the patients. Studies have concluded that X-ray and VFA are comparable (171, 172), although VFA had a reduced sensitivity for mild fractures (based on the Genant classification) and could not asses all vertebral levels (171, 172).

4.734 REGISTRY DATA

Information on diagnoses was collected from the Danish National Hospital Discharge Register. This gives the opportunity to both validate previous diagnoses and to do a longitudinal follow up on specific endpoints- in this case fracture. The Danish registries are an easy method to collect hard endpoints, however they are susceptible to misclassification and confounding.

4.74 METHODOLOGICAL CONSIDERATIONS IN PAPER 7

The experimental study examined bone turnover markers in the same individuals at OGTT and IIGI and thus avoided confounding by differences in characteristics. It may only be generalized to healthy young men as it was the group examined. The examination was standardized and all individuals were asked to refrain from exercise, smoking, and intake of vitamin supplements the day before and were given a standardized meal the night before, thus individual lifestyle habits are unlikely to influence the study. It is, however, limited by the lack of a control examination where the decrease of s-CTX is investigated over time. Furthermore, it is uncertain which gastro-intestinal hormones, may affect bone turnover as these are not examined.

5. SUMMARY OF RESULTS

5.1 PAPER 1: "DIABETES, BIOCHEMICAL MARKERS OF BONE TURNOVER, DIABETES CONTROL, AND BONE."

A qualitative analysis investigating bone turnover in patients with T1D and T2D in comparison with normal controls was performed (115). Section 1.7 describes the bone turnover markers in further details, while this section and sections 5.2 and 5.3 summarize the most relevant information on bone turnover markers for patients with diabetes. Figure 11 summarize the analysis. In general s-OC and s-CTX seemed to be lower in both patients with T1D and T2D compared to controls, whereas s-sclerostin and u-NTX seemed to be increased and s-P1NP and s-TRAP seemed to be decreased in patients with T2D. S-IGF-1 and s-OPG seemed to be decreased in patients with T1D compared to controls. In comparison to controls; patients with T1D seemed to have unchanged s-calcium, u-calcium, s-PTH, s-alkaline phosphatase, s-BAP, s-TRAP, s-NTX, and u-crosslaps. In comparison to controls patients with T2D seemed to have unchanged s-calcium, u-calcium, s-PTH, s-alkaline phosphatase, s-BAP, s-procollagen type 1 carboxyl terminal propeptide (P1CP), and s-CICP. Bone turnover markers did not seem to differ between the diabetes types. From fig. 10 it could be speculated that a difference in s-sclerostin was present between patients with T1D and T2D as s-sclerostin was not different between patients with T1D and controls in the study by *Gennari et al.* (37). A need for further investigations into sclerostin was this present and addressed in my studies. The narrative review pointed at heterogeneity in the biochemical variables, which is explored further below in a formal meta-analysis.

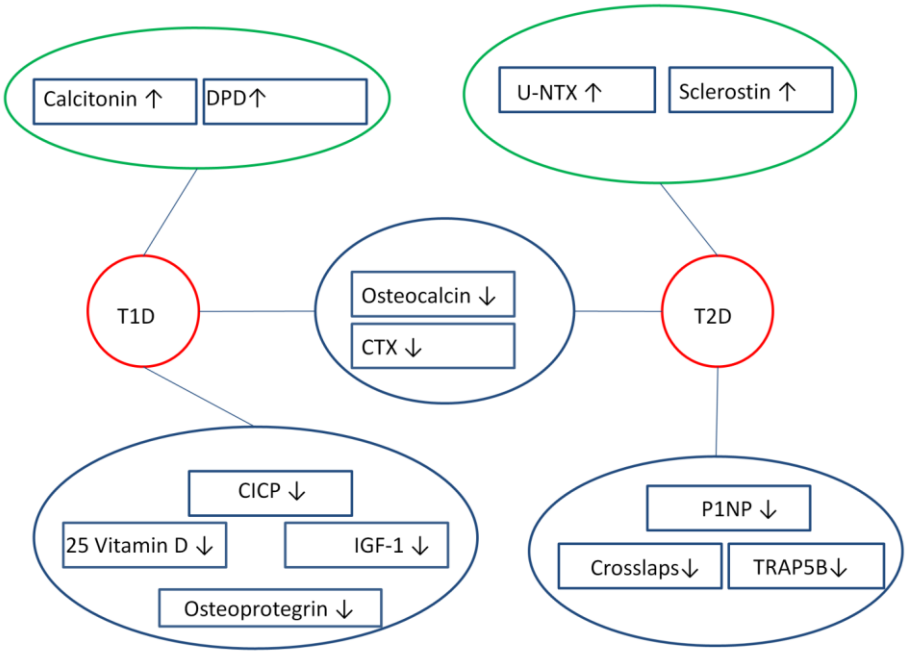

Figure 11. *Adapted from Starup-Linde et al. 2013 with permission* (115). *Overview of the bone turnover markers which are likely to differ between patients with diabetes and controls. ↑indicate raised marker ↓ indicate lowered marker. Blue and green circles indicate markers that appear decreased and increased in patients with diabetes, respectively.*

5.2 PAPER 2: ”BIOCHEMICAL MARKERS OF BONE TURNOVER IN DIABETES PATIENTS- A META-ANALYSIS, AND A METHODOLOGICAL STUDY ON THE EFFECTS OG GLUCOSE ON BONE MARKERS.”

5.21 THE META-ANALYSIS

A quantitative meta-analysis of bone turnover markers in patients with diabetes compared to non-diabetes subjects was performed. 22 studies were included in the meta-analysis and 18 studies also in the meta-regression. The NOS score ranged between 5 and 9 and thus the quality of the studies was fair. Heterogeneity was present in participant characteristics and use of methods between the studies. The study sizes were also heterogeneous with diabetes populations as small as 25 and at the most 890. The control group sizes varied in the same manner from 15 to 1,058. Twelve different methods were used to determine OC and five different methods to determine CTX. A description of the studies included in the pooled analysis is available in paper 2 (114).

The evaluated bone markers displayed heterogeneity among the studies; however no publication bias was present. In the pooled analysis s-25 OH vitamin D was lower in patients with diabetes compared to controls (-11.14 nmol/l 95% CI: -20.13 ; -2.15), whereas s-phosphate was increased in patients with diabetes (0.13 mg/dl 95% CI: 0.11 ; 0.16). Both s-OC (-1.15 ng/ml 95% CI: -1.78 ; -0.52) and s-CTX (-0.14 ng/ml 95% CI: -0.22 ; -0.05) was lower among patients with diabetes and u-NTX borderline significantly increased (15.44 nM/mM creatinine 95% CI: -0.74 ; 31.62), whereas no difference was present regarding s-PTH, s-calcium, s-BAP, s-CICP, and s-DPD. The results of s-OC and s-CTX are displayed in Figure 12 and 13. Subgroup analysis by patients with T1D and T2D revealed decreased s-25 OH vitamin D and s-OC in patients with T1D and increased s-phosphate in patients with T2D compared to controls. S-OC was borderline significantly lower in patients with T2D ($P = 0.06$). S-OC was decreased independent of gender and menopausal status, whereas s-CTX was decreased in male and premenopausal patients with diabetes, but not in postmenopausal women with diabetes.

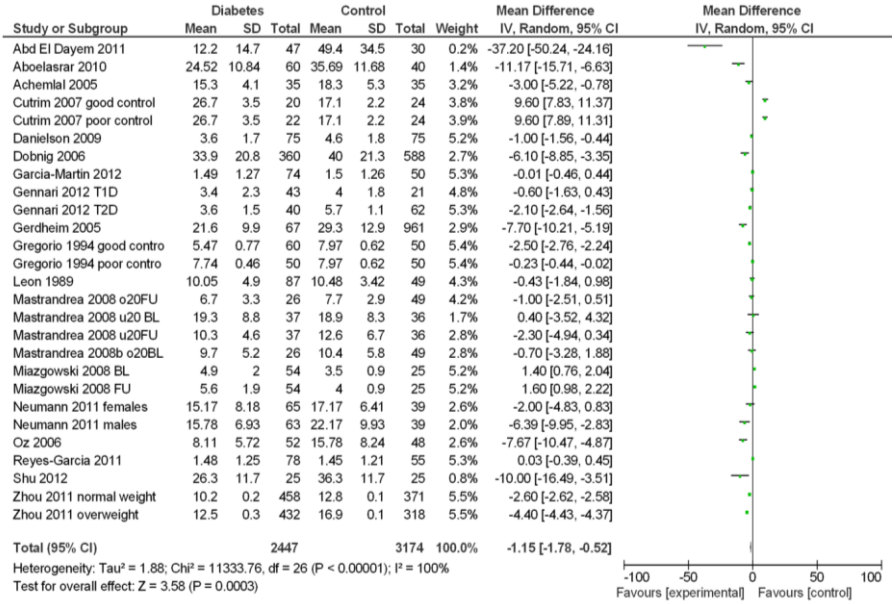

Figure 12. *Pooled analysis of differences in osteocalcin levels between patients with diabetes and controls. Analysis performed by random effects model. With permission from Starup-Linde et al.* (114).

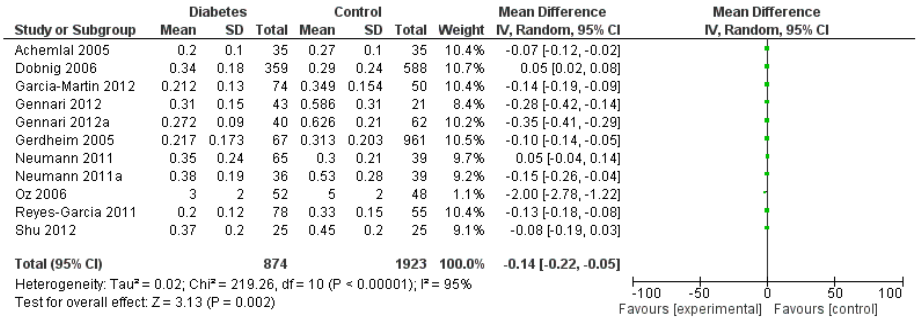

Figure 13. *Pooled analysis of differences in CTX levels between patients with diabetes and controls. Analysis performed by random effects model. With permission from Starup-Linde et al.* (114).

5.211 META-REGRESSION

A meta-regression on possible determinants of bone turnover markers was performed as a sensitivity analysis to the meta-analysis. The meta-regression analyses was restricted to patients with diabetes and revealed that neither, age, gender, BMI, diabetes type, diabetes duration, HbA1c, fasting status nor creatinine levels determined s-CTX or s-OC levels. However, when stratified by diabetes type age and diabetes duration was positive determinants of s-OC in patients with T1D. Also, HbA1c was positively associated with s-BAP levels.

5.22 THE METHODOLOTGICAL STUDY ON THE EFFECTS OF GLUCOSE ADDED TO SERUM ON THE MEASUREMENTS OF BONE TURNOVER MARKERS

The narrative review and meta-analysis raised the question of glucose per se could interfere with the measurements of say P1NP and CTX e.g. through glycation and thus altered presentation of the epitopes, perhaps leading to these failing to be recognized by the assay. This was explored further in a methodological study. Addition of glucose to serum did not change the bone turnover markers s-P1NP, s-OC, and s-CTX neither by the dose of glucose or the increasing incubation period. The results are presented in Table 3.

Regression Coefficient	P1NP	Osteocalcin	CTX
Time	-.416 (0.432)	-,386 (0.348)	-0.006 (0.013)
Addition of glucose	0.000 (0.065)	0.006 (0.053)	0.000 (0.002)
R^2	0.031	0.041	0.007

Table 3. *Multiple linear regression analysis by time and dose of added glucose for P1NP, osteocalcin, and CTX.*
Values are regression coefficients (standard error). Bold indicates significance (P<0.05). R^2 prediction power for the model including time and addition of glucose.

5.3 PAPER 3: "BIOCHEMICAL BONE TURNOVER MARKERS IN DIABETES MELLITUS – A SYSTEMATIC REVIEW."

From the studies above it was concluded that methodological issues related to the glucose levels could not explain the decreases in s-OC and s-CTX, and that large methodological variations were present and that bone turnover markers were likely to be lower in diabetes than in controls. However, the decrease varied between the markers and even within markers from the same molecule (CTX and NTX) variations were seen. This was further explored and related to among other fracture risk in a systematic review.

The forty seven studies included were and comprised 1 meta-analysis, 29 cross-sectional studies, 4 longitudinal studies, and 13 randomized controlled trials. The study sizes were heterogeneous and varied from 24 (173) to 1,605 (132).
The included studies are presented in paper 3. The diabetes ascertainment used by the studies was in general valid and the studies reported fasting bone turnover marker values with the exception of five studies. Individuals with kidney disease were in general excluded, although a single study reported on hemodialysis patients (167). Based on the studies bone markers still seemed to be decreased in both patients with T1D and T2D, however, s-BAP tends to be increased. Potential fracture predictors were evaluated and decreased levels of s-OC (126), s-IGF-1 (126-128), increased levels of s-sclerostin (127, 129) and an imbalance in bone turnover by high s-CTX and low s-P1NP (130) were all associated with prevalent fractures.

5.4 PAPER 4: "LOW-DENSITY LIPOPROTEIN CHOLESTEROL IS ASSOCIATED WITH FRACTURE RISK IN DIABETES PATIENTS – A NESTED CASE-CONTROL STUDY."

5.4.1 CHARACTERISTICS

Prior studies on fracture risk in diabetes have not explored systematically the full range of potential confounders for fracture risk. Some potential confounders such as atherosclerosis and hypertension may be associated with diabetes, and smoking, which is a risk factor for atherosclerosis is also a risk factor for fractures. Atherosclerosis may be treated with statins, which per se have also been associated with a decreased risk of fractures. I therefore conducted a nested case-control study in order to investigate fracture risk in diabetes and the potential impact of confounders.
2,627 diabetes mellitus patients were included in the study. 175 had a fracture after diabetes diagnosis. Table 4 displays patient characteristics. Patients with a fracture were significantly older, had significantly longer diabetes duration, and were more prone to have a history of previous fracture, and an alcohol-related diagnosis. Gender and diabetes complications were not significantly different between cases and controls.
The unadjusted and the adjusted analysis are displayed in Table 5. Age (1.02 ; 95CI: 1.01-1.03), diabetes duration (1.04 ; 95CI: 1.01-1.07), previous fracture (2.05 ; 95CI: 1.48-2.82), an alcohol related diagnosis (3.04 ; 95CI: 1.95-4.73), and neuropathy (1.92 ; 95CI: 1.11-3.32) increased risk of fracture in the unadjusted analysis. In the adjusted analysis age (1.02 ; 95CI: 1.01-1.04), diabetes duration (1.06 ; 95CI: 1.02-1.09), history of a previous fracture (2.19 ; 95CI: 1.55-3.10), and an alcohol related diagnosis (2.97 ; 95CI: 1.78-4.98) remained significant. Furthermore, total cholesterol increased the risk (2.53 ; 95CI: 1.22-5.25) and LDL-cholesterol (0.34 ; 95CI: 0.16-0.74) decreased risk of fracture significantly. LDL-cholesterol was still significantly associated with a decrease in fracture risk in the specific analysis for patients with T2D and significance levels did not change.

	Fracture after diabetes diagnosis (n = 175) (95% CI)	No fracture after diabetes diagnosis (n = 2,452) (95% CI)
Characteristic		
Age /years *****	65.9 (63.8 ; 68.0)	62.5 (61.9 ; 63.0)
Diabetes duration /years *****†	6.7 (6.1 ; 7.3)	5.8 (5.6 ; 6.0)
Sex male %	48.6 (41.1 ; 56.0)	54.2 (52.2 ; 56.2)
Previous fracture % *****†	36.6 (29.4 ; 43.8)	22.0 (20.3 ; 23.6)
Alcohol-related diagnosis % *****†	15.4 (10.0 ; 20.8)	5.7 (4.8 ; 6.6)
T2D % †	85.2 (78.6 ; 91.8)	89.7 (88.3 ; 91.1)
Neuropathy % †	9.1 (4.8 ; 13.5)	5.0 (4.1 ; 5.8)
Retinopathy %	4.0 (1.1 ; 6.9)	3.3 (2.6 ; 4.1)
Vascular complications %	11.4 (6.7 ; 16.2)	10.8 (9.6 ; 12.8)
Biochemical parameters (mean ± SE)		
Creatinine µmol/l †	85.2 ± 2.4	84.5 ± 0.7
HbA1c mmol/mol	53	55
Total cholesterol mmol/L †	4.4 ± 0.8	4.4 ± 0.2
HDL mmol/L *****†	1.4 ± 0.4	1.3 ± 0.1
LDL mmol/L †	2.2 ± 0.1	2.3 ± 0.1

Table 4. *Baseline characteristics of the 2,627 patients with diabetes.*
** P < 0.05 for difference between patients with diabetes with incident fractures and patients with diabetes without incident fractures.*
† T-test performed with unequal variances, due to estimation by Bartlett's test

Characteristic	Unadjusted odds ratio (95 % CI)	Adjusted odds Ratio (95 % CI) [α]
Age (years)	1.02 (1.01-1.03)*	1.02 (1.01 ; 1.04)*
Diabetes duration (years)	1.04 (1.01-1.07)*	1.06 (1.02 ; 1.09)*
Sex male	0.80 (0.59 ;1.08)	0.74 (0.52 ; 1.06)
Sex male n= 1,663[‡]	0.70 (0.46	0.61 (0.37 ; 1.00)
Smoker proxy variable	0.75 (0.46 ; 1.23)	0.69 (0.40 ; 1.19)
Previous fracture	2.05 (1.48 ; 2.82)*	2.19 (1.55 ; 3.10)*
Alcohol-related diagnosis	3.04 (1.95 ; 4.73)*	2.97 (1.78 ; 4.98)*
Neuropathy	1.92 (1.11 ; 3.32)*	1.69 (0.93 ; 3.07)
Retinopathy	1.20 (0.55 ; 2.65)	0.95 (0.41 ; 2.20)
Peripheral artery disease	1.06 (0.65 ; 1.72)	0.73 (0.43 ; 1.24)
Biochemical parameters		
Creatinine µmol/l	1.00 (1.00-1.00)	1.00 (0.99 ; 1.00)
HbA1c %	0.89 (0.77-1.02)	0.93 (0.78 ; 1.10)
Total cholesterol mmol/L	1.10 (0.92-1.30)	2.53 (1.22 ; 5.25)*
Total cholesterol mmol/L n= 1,663[‡]	1.19 (0.96 ; 1.49)	3.68 (1.27 ; 10.65)*
HDL mmol/L	1.54 (1.09-2.17)*	0.56 (0.25 ; 1.24)
LDL mmol/L	0.88 (0.70-1.10)	0.34 (0.16 ; 0.74)*
LDL mmol/L n= 1,663[‡]	0.99 (0.75 ; 1.34)	0.27 (0.09 ; 0.80)*
Triglycerides mmol/L	1.03 (0.90-1.18)	0.83 (0.60 ; 1.14)

Table 5. *Unadjusted and adjusted odds ratio for fracture after diagnosis of diabetes n = 2,627. Nephropathy cases were excluded from the analysis.*
** P < 0.05, [α]Adjusted by potassium levels, sodium levels, alanine transaminase levels, alkaline phosphatase levels, antipsychotic use, antiepileptic use, glucocorticoid use, antihypertensive use, antidepressant use, statin use, previous heart failure, antidiabetic use (stratified by type), drugs affecting bone turnover, previous acute myocardial infarction, in addition to all variables in the table.*
[‡] Analysis performed after excluding all patients with type 1 diabetes (n=1,663)

5.42 LDL AND FRACTURE RISK

The LDL-cholesterol level was divided by 8-quantiles in patients with T2D. The lowest quintile (0-1.53 mmol/l) had a significantly higher risk compared to the other quintiles. Compared to the reference of 2.23-2.44 mmol/l higher LDL-cholesterol levels had lower fracture risk. The analysis was adjusted by statin use as it may influence results. Figure 14 presents the relationship between LDL-cholesterol levels and fracture risk in patients with T2D.

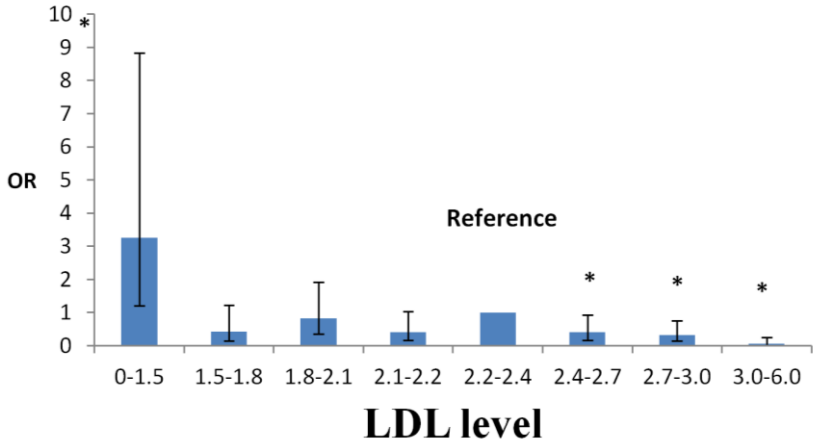

Figure 14. *Adjusted Odds Ratio with 95 % confidence intervals for fractures in patients with type 2 diabetes by LDL-cholesterol level (n=1,663).* * P < 0.05 compared to the reference**.** *Adjusted by age, diabetes duration, sex, smoker proxy variable, previous fracture, alcohol diagnosis, retinopathy, neuropathy, peripheral artery disease, AMI, heart failure, alat, alkaline phosphatase, creatinine, HbA1c, total cholesterol, HDL cholesterol, triglycerides, sodium, potassium, insulin, biguanides, β-cell stimulants, GLP-1 receptor agonists, statins, antihypertensive, glucocorticoids, bone affecting drugs, antidepressants, antipsychotics and antiepileptics.*

5.5 PAPER 5: " DIFFERENCES IN BIOCHEMICAL BONE MARKERS BY DIABETES TYPE AND THE IMPACT OF GLUCOSE."

5.51 CHARACTERISTICS

In order to study the impact of turnover markers on bone structural parameters and fracture risk, a cross-sectional study was performed. The cross-sectional study is covered by paper 5 and paper 6. Paper 5 examines differences in bone turnover markers between patients with T1D and T2D. 197 patients with diabetes were included distributed with 101 patients with T1D and 96 patients with T2D. Patient characteristics are given in Table 6. Patients with T2D had a significantly higher BMI and age and lower diabetes duration compared to patients with T1D. Gender,

smoking, alcohol consumption, and microvascular complications did not differ between the diabetes types. The patients were relatively dysregulated with a mean HbA1c of 8.0 % (64.0 mmol/mol) and a mean non-fasting p-glucose of 10.8 mmol/l. HbA1c, creatinine, and p-glucose levels were not different between patients with T1D and T2D.

5.52 BIOCHEMICAL MARKERS OF BONE TURNOVER

The biochemical markers of bone turnover and the differences between patients with T1D and T2D are presented in Table 6. P-P1NP and p-OC were significantly higher in patients with T1D (41.2 ng/ml vs. 35.7 ng/ml and 19.5 ng/ml vs. 15.5 ng/ml, respectively), whereas p-CTX and p-ucOC were not different between diabetes types. S-RANKL was significantly higher in patients with T1D (62.1 pmol/ml vs. 44.9 pmol/ml) whereas s-OPG was significantly lower (4.65 pmol/l vs. 5.32 pmol/l) in patients with T1D when comparing with patients with T2D. P-FGF-23 (97.3 RU/ml vs. 113 RU/ml) and p-sclerostin (68.1 pmol/l vs. 73.5 pmol/L) were not different between diabetes types.

Variable	T1D mean (95%CI) (n=101)	T2D mean (95%CI) (n=96)	P-value
Characteristics			
Age (years)	60.7 (59.2 ; 62.2)	65.2 (63.6 ; 66.7)	**<0.001**
Gender (% male)	59.4	64.6	0.457
BMI (kg/m^2)	25.8 (25.1 ; 26.6)	30.7 (29.9 ; 31.6) (n=95)	**<0.001**
Diabetes duration (years)	24.4 (21.9 ; 26.8)	14.6 (13.1 ; 16.2)	**<0.001**†
Microvascular complications (%)	46.5	45.8	0.922
Macrovascular complications (%)	3.96	15.6	**0.006**†
Current smoker (%)	18.8	29.2	0.089
Alcohol use (units/week)	6.86 (5.36 ; 8.36)	4.85 (3.51 ; 6.20)	0.050
Statin use (%)	60.4	79.2	**0.0041**
Biochemical parameters			
CTX (ng/l)	217 (197 ; 236)	189 (166 ; 211)	0.062
Osteocalcin (ng/ml)	19.5 (18.3 ; 20.7)	15.5 (14.3 ; 16.8)	**<0.001**
Undercarboxylated osteocalcin (ng/ml)	4.99 (3.08 ; 6.90)	6.38 (3.50 ; 9.2)	0.426
P1NP (ng/ml)	41.2 (38.3 ; 44.1)	35.7 (32.7 ; 38.7)	**0.010**
FGF-23 (RU/ml)	97.3 (78.6 ; 116)	113 (99.3 ; 126)	0.185†
Sclerostin (pmol/l)	68.1 (62.1 ; 74.2)	73.5 (66.0 ; 81.0)	0.269
RANKL (pmol/l)	0.0621 (0.0489 ; 0.0753)	0.0449 (0.0342 ; 0.0557)	**0.0471**†
OPG (pmol/l)	4.65 (4.22 ; 5.08)	5.32 (4.91 ; 5.73)	**0.0277**
Creatinine (mmol/l)	74.9 (71.7 ; 78.1)	76.6 (72.8 ; 80.4)	0.493
25 OHD vitamin (nmol/l)	75.1 (68.6 ; 81.6)	64.5 (58.3 ; 70.6)	**0.019**
HbA1c (mmol/mol)	64.5 (62.7 ; 66.4)	63.4 (61.4 ; 65.3)	0.378
Mean HbA1c of previous 5.9 years (mmol/mol)	66.3 (64.7 ; 68.0)	64.4 (62.8 ; 66.0)	0.102
Glucose (mmol/l)	10.7 (9.74 ; 11.6)	10.9 (10.1 ; 11.6)	0.744$^{\beta}$

Table 6. *Characteristics of the patients with diabetes divided into diabetes types (n = 197).*
T1D: Type 1 diabetes, T2D: Type 2 diabetes. P-values for differences between diabetes types in unpaired t-test, ***Bold indicates statistical significance,*** †*unequal variance from Bartletts test and t-test performed with unequal variance*

5.53 LINEAR REGRESSION

To examine association between bone turnover markers and factors that may influence bone turnover linear regression analyses were performed. In simple linear regression analyses adjusted by diabetes type; non-fasting p-glucose levels were related to a significant decrease in p-CTX (β = -4.23), p-P1NP (β = -0.651), p-OC (β = -0.244), and p-ucOC (β = -4.23) and an increase in s-OPG (β = 0.135). These

associations are presented in Figure 15. HbA1c was significantly associated with p-OC (β = -0.112), but not with p-P1NP (P =0.058), p-CTX (P = 0.361), p-ucOC (P = 0.458) or s-OPG (P = 0.507). The associations between HbA1c and bone turnover markers are illustrated in Figure 16. In unadjusted analysis LDL-cholesterol levels were significantly associated with p-P1NP (β = 5.77) and p-OC (β = 1.75), however this was not present when adjusting by diabetes type.

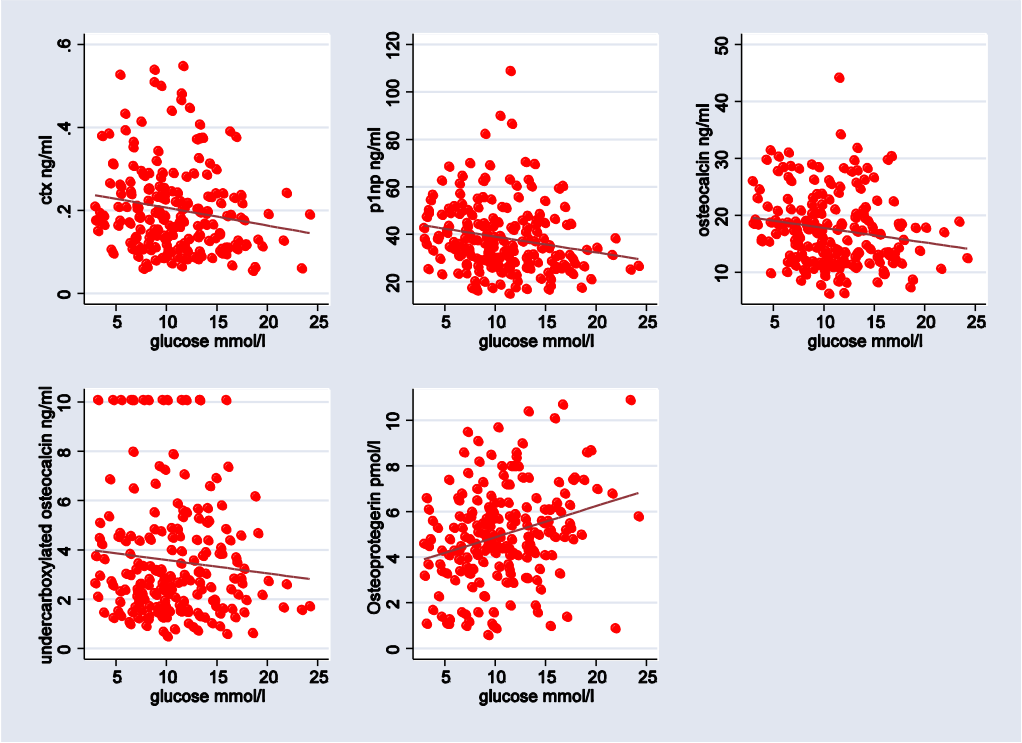

Figure 15. *The unadjusted association between non-fasting glucose levels and bone turnover marker levels in patients with diabetes (n = 197).*

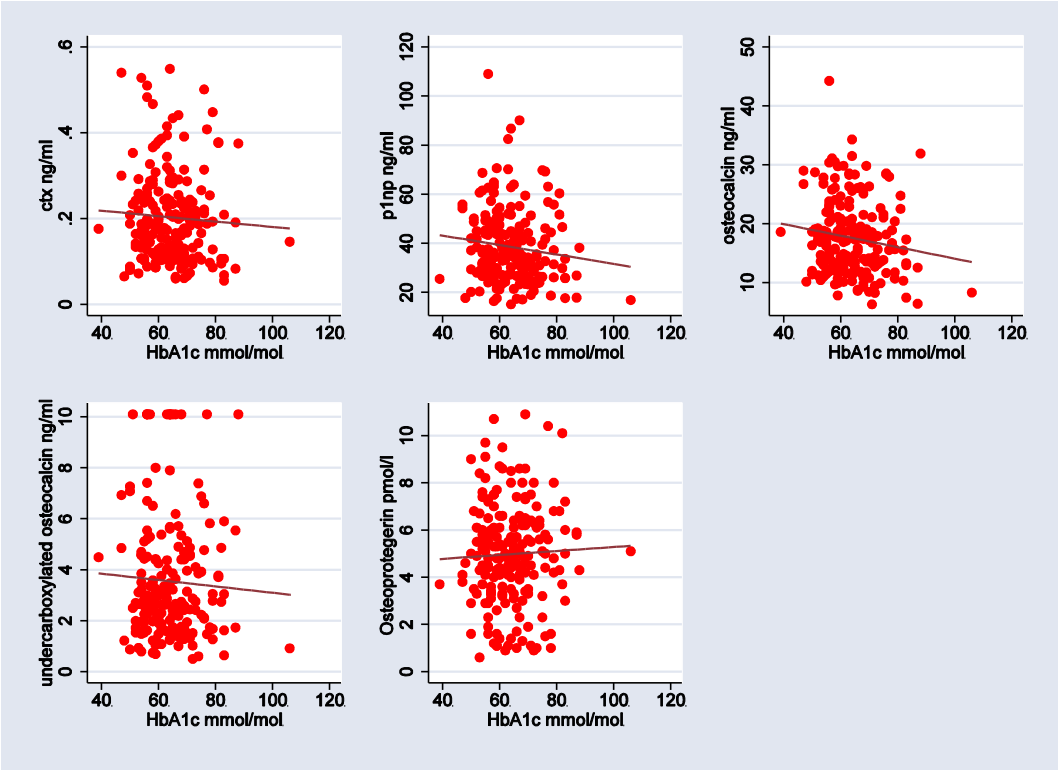

Figure 16*. Unadjusted association between HbA1c and bone turnover marker levels in patients with diabetes (n = 197).*

5.54 MULTIPLE LINEAR REGRESSION

Table 7 present the adjusted association between biochemical markers of bone and non-fasting p-glucose. In the adjusted multiple linear regression non-fasting p-glucose was significantly negatively associated with p-CTX (β = -4.31), p-P1NP (β = -0.660), p-OC (β = -0.227), and p-ucOC (β = -0.246) and positively associated with s-OPG (β = 0.136). HbA1c was still significantly associated with p-OC in the multiple linear regression (β = -0.105). When stratifying by diabetes type non-fasting p-glucose was significantly negatively associated with p-CTX (β = -5.49) and p-P1NP (β = -0.738) and significantly positively associated with s-OPG (β = 0.117) in patients with T1D. In patients with T2D non-fasting p-glucose was significantly associated with s-OPG (β = 0.159).

Variable	Glucose (mmol/l) (95 % CI)
CTX (ng/l) *	**-4.31 (-7.55 ; -1.07)**
P1NP (ng/ml) †	**-0.660 (-1.11 ; -0.208)**
Osteocalcin (ng/ml) †	**-0.227 (-0.405 ; -0.0489)**
Undercarboxylated osteocalcin (ng/ml)‡	**-0.426 (-0.825 ; -0.0264)**
OPG (pmol/l) ¥	**0.136 (0.0725 ; 0.199)**

Table 7. *Multiple linear regression of bone turnover markers and the association with non-fasting glucose (n=197).*
Bold indicates statistical significance
** Adjusted by age, gender, BMI, diabetes type, microvascular disease, alcohol use, HDL-cholesterol, and creatinine.*
† Adjusted by age, gender, BMI, diabetes type, alcohol use, HDL-cholesterol, and creatinine.
‡ Adjusted by age, gender, BMI, diabetes type, microvascular disease, and creatinine.
¥Adjusted by age, gender, BMI, and diabetes type.

5.6 PAPER 6: "BONE STRUCTURE AND PREDICTORS OF FRACTURE IN TYPE 1 AND TYPE 2 DIABETES."

5.61 CHARACTERISTICS

Paper 6 investigated differences in bone structure between patients with T1D and T2D and associations with fractures. 101 patients with T1D and 96 patients with T2D were included. Table 6 presents characteristics of the included patients. The Charlson Comorbidity Index was lower in patients with T1D (0.317 points vs. 0.875 points) compared to patients with T2D; however more patients with T1D had experienced a previous hypoglycemic event (31.7 % vs. 2.1 %). No difference was observed between patients with T1D and T2D when considering the number of previous fractures, previous major osteoporotic fractures, prevalent vertebral fractures assessed by VFA or x-ray and incident fracture. The characteristics of the population are further described in section 5.51.

5.62 STRUCTURAL PARAMETERS

Table 8 presents data on the structural parameters by diabetes type. DXA T-scores at the spine, hip, and femur (-0.580 vs. 0.123, -0.925 vs. -0.261, and -1.07 vs. -0.669) were lower in patients with T1D compared to patients with T2D. No difference was present at the forearm BMD. Structural and density parameters and cortical porosity measured by HRpQCT was not different between patients with T1D and T2D. The tibia and radius cortical pore size and the standard deviation of the pore size were increased in patients with T1D compared to patients with T2D. Furthermore, cortical pore volume at the radius was decreased in patients with T1D compared to patients with T2D. The finite element analysis revealed increased tissue stiffness in patients with T2D at the tibia. In multiple linear regressions the hip T-score and tibia

DXA parameters	T1D mean (95%CI) (n=101)	T2D mean (95%CI) (n=96)	P-value
Hip T-score*	-0.925 (-1.15 ; -0.703)	-0.261 (-0.505 ; -0.0164)	**<0.001**
Femur T-score*	-1.07 (-1.29 ; -0.839)	-0.669 (-0.931 ; -0.407)	**0.024**
Spine T-score	-0.580 (-0.863 ; -0.297)	0.123 (-0.232 ; 0.478)	**0.002**
Total forearm BMD	0.556 (0.536 ; 0.575)	0.578 (0.560 ; 0.596)	0.102
HRpQCT Tibia			
vBMD (mg HA/cm^3)	287 (272 ; 302)	298 (285 ; 309)	0.253
Cortical porosity (%)	7.29 (6.57 ; 8.01)	7.02 (6.50 ; 7.55)	0.540
Tissue Stifness (kN/mm)	236 (218 ; 254)	261 (244 ; 278)	**0.046**
Failure load (N·10^7)$^{\pm}$	3.52 ± 3.45	2.71 ± 2.90	0.437

Table 8. *Differences in structural bone measurements by diabetes type (n=196). T1D: Type 1 diabetes, T2D: Type 2 diabetes P-value for difference between T1D and T2D in unpaired t-test, Bold indicate statistical significance, †unequal variance from Bartletts test and t-test performed with unequal variance * Two patients with type 2 diabetes were not scanned at the hip and femur due to prosthesis.*

tissue stiffness remained significantly increased in patients with T2D compared to patients with T1D whereas no other structural parameters differed. Mean HbA1c of the previous 5.9 years was not associated with aBMD at the hip or spine and vBMD of the tibia and radius in either patients with T1D or T2D.

5.63 VERTEBRAL FRACTURES

11 patients experienced an incident fracture subsequent to inclusion in the study (5 forearm fractures, 2 tibia
fractures, 2 fractures of the humerus, 1 fracture of a metacarpal bone, and 1 with multiple fractures of spine, clavicle, and rib). 6 fractures were in patients with T1D and 5 fractures were in patients with T2D. 15 patients with T1D and 8 patients with T2D presented with a vertebral fracture at examination. The unadjusted logistic regression revealed that fracture was associated with diabetes duration (OR=1.04), previous major osteoporotic fracture (OR = 4.47), increasing hip T-score (OR=0.498), p-25 OHD vitamin (OR= 1.02), vitamin D supplementation (OR= 4.08), p-sclerostin (OR= 0.974), and p-sclerostin divided by tertiles (OR= 0.471) in patients with T1D. P-sclerostin was also significantly associated with fracture risk in the multivariate analysis (OR = 0.413) in patients with T1D. Figure 17 presents the association of p-sclerostin and fracture in patients with T1D. The strongest association was observed within the third tertile (OR = 0.188) of p-sclerostin

compared to the first tertile. No factors were associated with fracture in patients with T2D.

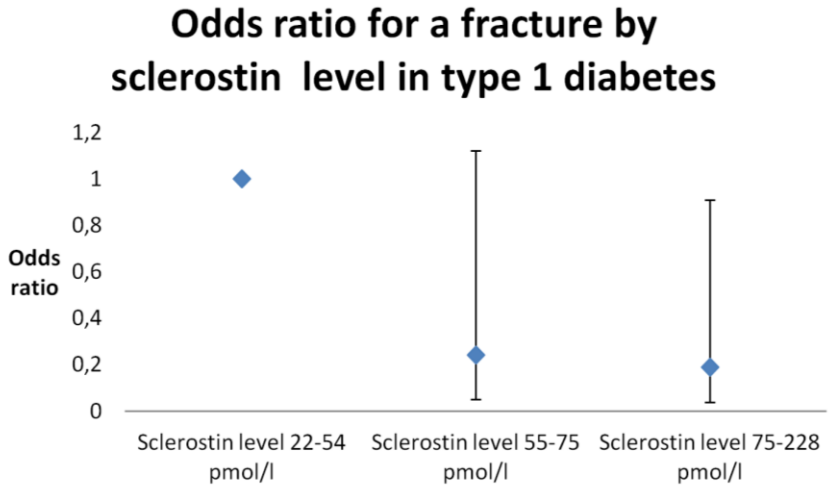

Figure 17*. Fracture risk by sclerostin levels in patients with type 1 diabetes. * Statistical significantly different from the reference. Adjusted by age, BMI, gender, diabetes duration, previous major osteoporotic fracture, Hip T-score, and 25 OHD vitamin level.*

5.7 PAPER 7: "THE EFFECT OF GLUCOSE ON BONE – DIRECT OR INDIRECT?"

As the differences in turnover markers in diabetes were not spurious brought about by assay problems and as turnover was associated more to actual glucose levels than to HbA1C, a methodological study was performed to further investigate if it was the glucose load per se or the mode of ingestion (oral or i.v.) that affected turnover. An effect of oral ingestion and not i.v. infusion would perhaps point at an effect of factors from the gut.

Table 9 presents characteristics of the healthy men. On average they were 30.6 years old and normal weight. None had bone metabolic disease or diabetes based on fasting p-glucose, HbA1c or two hour OGTT values.

Variables	Mean	Range
Age (years)	30.6	25–49
Alcohol (units pr. week)	9.6	0.5-20
BMI (kg/m^2)	24.2	21.0-28.8
HbA1c	33.2	29-39
Mean glucose	5.6	5.0-6.5
PTH	4.4	3.0-6.9
Vitamin D	59.2	42-75
Calcium	1.24	1.17-1.28
s-CTX	0.63	0.23-0.93
s-P1NP	73.5	31.8-138.5

Table 9 *Characteristics of the included subjects (n=12).*

Figure 18 presents data on p-glucose levels by time and intervention. The p-glucose levels made a rapid increase and reached its maximum after 35 minutes with a secondary peak around 90 minutes. The p-glucose levels dropped and reached starting values after 150 minutes and made an additional drop for the remaining 30 minutes. The OGTT and IIGI glucose values did not differ at any time point and are thus comparable.

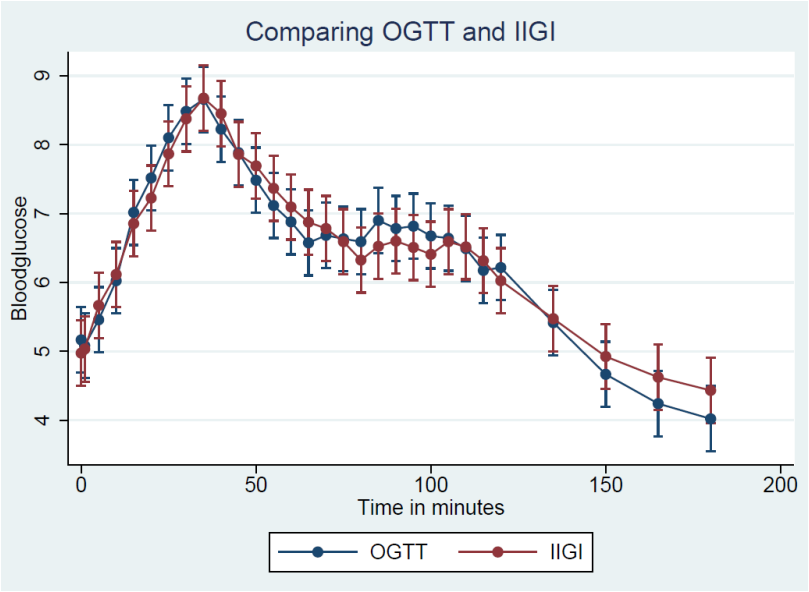

Figure 18. *Glucose levels by time and intervention.*

The repeated measurements ANOVA revealed a significant decrease in s-CTX over time and a significant interaction between time and intervention (OGTT or IIGI). S-P1NP did not change by time or intervention as illustrated by Figure 19.

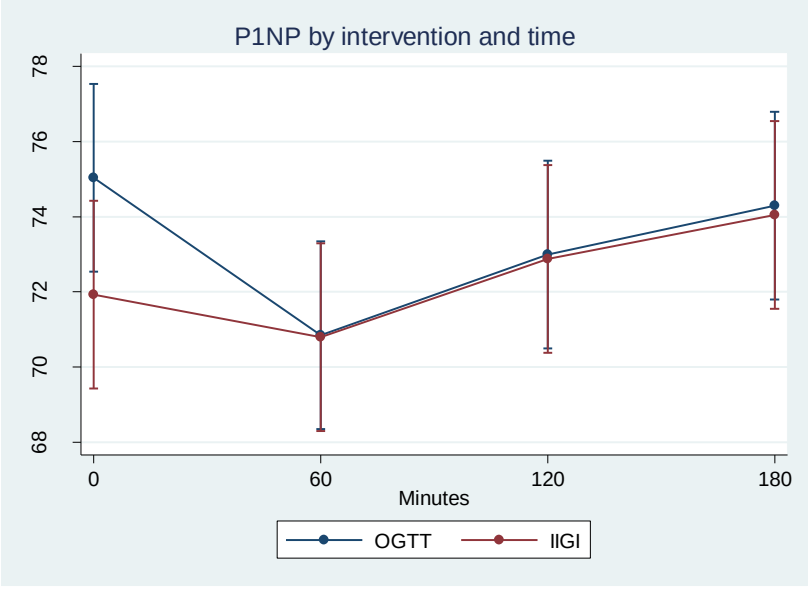

Figure 19. *P1NP levels by time and intervention.*

At the start of both interventions s-CTX was similar of 0.63 µg/l. Figure 20 presents s-CTX levels by time and intervention. S-CTX levels were significantly decreased at both interventions after one hour and the nadir of decrease was after two hours for the OGTT and after three hours for the IIGI. At all time points s-CTX decreased significantly more at the OGTT than the IIGI.

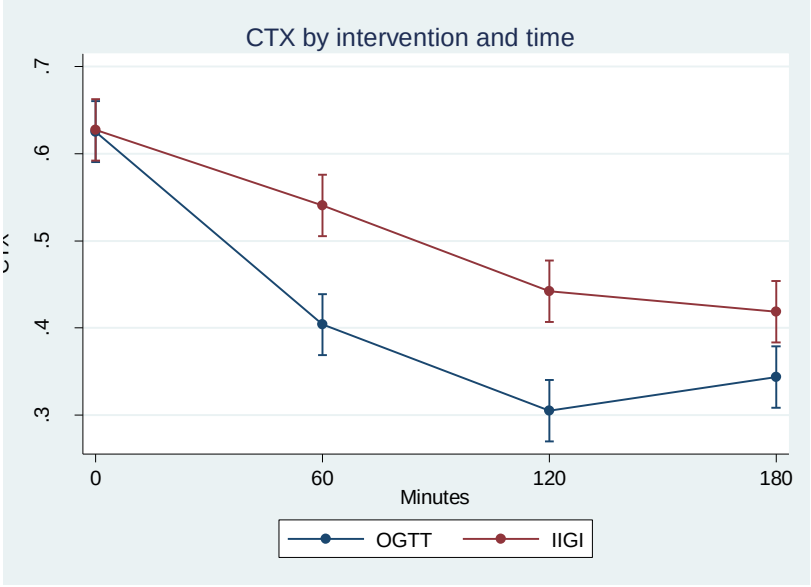

Figure 20. *CTX levels by time and intervention.*

6. DISCUSSION

6.1 SUMMARY OF RESULTS

- Bone turnover markers were altered in patients with diabetes with lower s-OC and s-CTX levels compared to controls. S-BAP, s-PTH, and s-calcium were not different between diabetes and non-diabetes subjects. The differences may be due to alterations in sclerostin and OPG. (Paper 1,2, and 3).
- Patients with T1D had increased p-P1NP, p-OC, and s-RANKL whereas s-OPG was lower compared to patients with T2D. P-sclerostin and p-FGF-23 were not different between patients with T1D and T2D. (Paper 5).
- Thus, bone turnover markers are different in patients with diabetes compared to non-diabetes subjects, but also differently between patients with T1D and T2D. (Paper 1, 2, 3, and 5).
- Acute changes of bone turnover may appear after a glucose load either given intravenously or orally. Thus, s- CTX decreased significantly after ingestion and infusion of glucose and also more with the OGTT than the IIGI. However, s-P1NP was not different between OGTT and IIGI and did not change significantly over time. (Paper 7).
- The effect of glucose added to serum samples did not change bone marker levels and thus the effect of glucose on bone turnover markers is not due not due to an immunochemical masking effect by bone marker glycation. (Paper 2).
- In patients with T1D and T2D p-glucose was positively associated with s-OPG levels and in patients with T1D inversely associated with markers of bone formation and resorption. HbA1c was only significantly associated with p-OC. (Paper 5)
- Hip T-score and tibia tissue stiffness was significantly higher in patients with T2D than patients with T1D, whereas differences in spine and femur T-score were abolished in the adjusted models.
- Possible fracture predictors were examined and LDL-cholesterol levels were associated with risk of fracture in patients with T2D with lower risk at higher levels. HbA1c was not associated with the risk of fracture. (Paper 4).
- P-sclerostin was, independently of hip T-score, associated with the risk of fracture in patients with T1D with lower risk at higher levels. (Paper 6).

6.2 BIOCHEMICAL BONE TURNOVER MARKERS

6.21 DIFFERENCES IN BONE TURNOVER MARKERS BETWEEN TYPE 1 AND TYPE 2 DIABETES

We hypothesized that patients with T1D have higher bone turnover measured by bone turnover markers compared to patients with T2D. The hypothesis was based on previous results of low BMD in patients with T1D, increased BMD in patients with T2D (11), and that bone loss relates to bone turnover (95). In paper 5 we found increased bone formative markers (p-OC and p-P1NP) in patients with T1D compared to patients with T2D, whereas p-CTX was not statistically significant but with a trend towards increased levels in patients with T1D. Unbound s-RANKL levels were increased in patients with T1D compared to patients with T2D, which may be explained by a higher level of s-OPG in patients with T2D. This difference in free active s-RANKL may explain the differences in p-OC, p-P1NP, and p-CTX as RANKL by binding to RANK promotes bone resorption that is tightly coupled to bone formation (25). S-RANKL has been reported not to differ between patients with T1D and T2D (28-30), which is in contrast to our results. However, two of the three studies reporting s-RANKL values measure it in the OPG bound form (28, 29) while the third does not provide information on whether s-RANKL is measured OPG bound or unbound (30). Neither p-FGF-23 nor p-sclerostin differed between patients with T1D and T2D. P-25 OH vitamin D was higher in patients with T1D; however for both patients with T1D and T2D the means were within normal range and thus should not affect our results. In the meta-analysis s-25 OH vitamin D was decreased and s-PTH unchanged in patients with diabetes compared to controls (114), this may be due to a blunted PTH response which previously has been observed in patients with insulin treated diabetes exposed to hypocalcemia (174). Other studies have assessed bone turnover marker differences between patients with T1D and T2D, however these studies were smaller with group sizes from 21 to 73 (37, 91, 91, 175, 176). These studies report no difference in bone turnover markers between patients with T1D and T2D. S-sclerostin was reported lower in patients with T2D compared to patients with T1D, which may be explained by an age difference of twenty years (37). We found evidence of increased bone formation and also a possible increased bone resorption in patients with T1D compared to patients with T2D. This difference may be driven by the RANKL/OPG system.

6.22 DIFFERENCES IN BONE TURNOVER MARKERS BETWEEN PATIENTS WITH DIABETES AND NON-DIABETS SUBJECTS

We hypothesized that bone turnover was different between patients with diabetes and controls. This was examined in the meta-analysis (114). Levels of s-OC, s-CTX, and s-25 OH vitamin D were lower in patients with diabetes, whereas u-NTX was borderline increased. Bone markers were very heterogeneous between studies; however the differences are reliable due to the comparison being made within each study. A lower bone turnover measured by s-CTX and s-OC is supported by the systematic review although studies also report no difference between subjects with

and without diabetes (116). Although the differences were evaluated quantitatively, the studies displayed heterogeneity. Factors as age, diabetes type, and diabetes duration may affect the results and explain the heterogeneity and should be further investigated.
The difference between the direction of s-CTX and u-NTX gives a complex picture of bone turnover in patients with diabetes. It is an odd finding as a review stated that no difference is present between CTX and NTX independent of measurement in serum or urine (125). The review (115) also concluded that bone turnover may be dissociated in diabetes where only selected markers decrease (s-CTX, s-TRAP and s-OC) and others do not. However, the results are restricted as the data are provided from several heterogeneous populations and thus diversity in patient characteristics may explain the difference.
In paper 5 the reported levels of p-FGF-23, p-sclerostin, s-RANKL, and s-OPG were different from previously reported intervals. P-FGF-23 was above the normal range (19.1 – 93.2 RU/ml) proposed from the analysis of 170 healthy individuals (170). Furthermore, the c-terminal FGF-23 (analyzed in paper 5) was stable during the day and not affected by food intake or physical activity (170). Thus, patients with diabetes seem to have increased p-FGF-23 levels. This may be due to increased s-phosphate levels, which were found in the meta-analysis (114). P-sclerostin levels for patients with T1D and T2D reported in paper 5 were increased compared to previous population based values in men and women (177). This is in line with the results of paper 1 were s-sclerostin levels seemed increased in patients with T2D compared to controls. Increased sclerostin levels will result in an inhibition of the Wnt pathway and a decreased bone formation, which may explain a decrease in bone strength in these individuals. Compared to the manufactures reference range s-RANKL levels were increased. This is supported by the internal healthy control, which was close to the mean of the manufacturer's range (from the Department of Clinical Biochemistry, Aalborg University Hospital). S-OPG levels matched the manufacturer's reference range. Thus, p-sclerostin and s-RANKL seemed to be altered in patients with diabetes compared to non-diabetes subjects. Theoretically an increased p-sclerostin and decreased s-RANKL would decrease both bone resorption and formation, which is in line with the decreased s-OC and s-CTX in patients with diabetes observed in the meta-analysis (114). As described in section 1.6 the evidence from bone biopsies was conflicting and limited and gives no clear indication of whether diabetes may have decreased bone turnover (95, 110, 111). CTX, P1NP, and OC reflect the bone turnover. Compared to the proposed reference ranges the levels of p-CTX, p-P1NP, and p-OC observed in paper 5 was not different. However, when comparing the results to a population of similar age both p-OC and p-CTX seem to be lower in patients with diabetes (178, 179).

6.23 BONE TURNOVER MARKERS AND FRACTURE

In non-diabetes subjects bone turnover markers predicted fragility fractures and related to deterioration of bone (180) and thus bone turnover may be a marker of fracture. In patients with diabetes s-PTH, s-BAP, s-OC, u-NTX, and s-CTX did not

predict fractures (82, 128, 153, 167, 181-183). However, the combination of low s-P1NP and high s-CTX was associated fracture in patients with diabetes (130) and increased s-sclerostin in patients with T2D was associated with fractures (127, 129). In the clinical study p-sclerostin levels had same direction as proposed above in patients with T2D, however results were not significant. In patients with T1D the upper tertile of p-sclerostin was associated with an 81 % decreased fracture risk compared to the lowest tertile independent of BMD. The role of sclerostin may thus be very different between patients with T1D and T2D. In patients with T1D a decrease in bone formation may be beneficial, whereas it may be harmful in patients with T2D. This may be explained by the bone formation which is higher in patients with T1D. Longer follow up time is needed to examine the effects on hip fracture, which may be the main fracture preventive goal in diabetes. Another possible fracture predictor may be IGF-1 as described in section 1.7. An abstract presented at the European Association for the Study of Diabetes (EASD) 2014 in Vienna reported that IGF-1 was associated with incident fractures in postmenopausal patients with T2D (131). IGF-1 and sclerostin are therefore potential fracture predictors in patients with diabetes.
The large heterogeneity of bone turnover markers in the meta-analysis limit the use of standard bone turnover markers (CTX, P1NP, OC, BAP) as fracture predictors in patients with diabetes, as they relate to patient characteristics (age, sex, diabetes duration) and method used to analyze the marker (114). Furthermore, the p-glucose level at measurement may affect the result. To help the interpretation of bone turnover markers and an effort to standardize the measurement in diabetes; measurements should always be accompanied with a p-glucose and a thorough description of the patients with diabetes (age, sex, diabetes duration, smokers, medication use, diabetes related complications, p-creatinine).

6.3 GLUCOSE AND BONE TURNOVER

It was hypothesized that glucose decreases bone turnover markers. Glucose may thus explain the large heterogeneity of bone turnover markers between studies by the lack of a normal fasting state in patients with diabetes (47). This is also present in the study by *Gennari et al.*, that reported decreased fasting s-CTX in patients with T1D and T2D compared to controls; however both patients with T1D and T2D have elevated fasting p-glucose (7.3 mmol/l and 7.8 mmol/l, respectively). To determine whether a possible relationship is due to immunochemical masking effect by bone marker glycation of bone turnover markers we examined the effect of in vitro added glucose to serum. The addition of glucose to serum did not change bone turnover markers, thus glucose does not interact with the analysis of the bone turnover markers. In the clinical study we found evidence of an association between bone turnover markers and p-glucose in patients with diabetes; p-CTX, p-P1NP, and p-OC, were negatively associated with and s-OPG positively associated with p-glucose levels in patients with T1D in the clinical study. S-OPG was associated with p-glucose in patients with T2D and p-CTX, p-P1NP, and p-OC trended towards lower values with increased p-glucose levels. Furthermore, ingestion of glucose

orally or intravenously decreases s-CTX levels in healthy men. However, the decrease was most pronounced in the OGTT. S-P1NP levels were unaffected of the way of administration of glucose. Although, glucose may affect bone turnover the BMD values reported in paper 6 were not associated with long term HbA1c values and thus density does not seem to relate to glycemic control.
In diabetes the effect of glucose on bone turnover markers may be through a direct stimulation of the OPG production in osteoblasts. *Cunha et al.* showed that 24 hours of hyperglycemia increased OPG 15 times more than RANKL in osteoblast-like cells (144); however *Garcia-Hernandez et al.* found the opposite after 7 and 14 days (143). Other studies reported decreased s-OPG after glucose infusion: OGTT did not change s-OPG levels in postmenopausal women with T2D after 2 hours, however s-OPG was decreased in non-diabetes individuals (140) and p-OPG decreased after 4 hours of hyperglycemic clamp in healthy individuals and p-OPG was negatively associated with serum insulin (184). The findings of *Chailurkut et al.* support an alteration of s-OPG under hyperglycemia in patients with diabetes as hyperglycemia lowered s-OPG in healthy individuals, but not in patients with T2D (140). Our finding of an association between p-glucose and s-OPG in patients with diabetes may be both due to prolonged or acute hyperglycemia. The association between s-OPG and p-glucose may also be due to very low serum insulin levels, which is very likely to be present in hyperglycemic patients with T1D and the relatively low insulin levels in relation to hyperglycemia in patients with T2D.
As described in the introduction; OGTT decreased s-CTX, s-P1NP, and s-OC in healthy individuals (137). This effect may be due to glucose itself or factors released during gastro-intestinal absorption. Somatostatin abolished the effect of an OGTT and injection with GLP-2 decrease s-CTX which both suggest an indirect effect of glucose through gastro-intestinal hormones (137, 138). We observed a decrease in s-CTX at both OGTT and IIGI whereas p-glucose levels did not differ. However, s-CTX decreased significantly more at the OGTT. These results suggest that the effect of glucose is mediated by the gastro-intestinal hormones, but it may mediate via a promotion of glucose itself as the IIGI also decreased s-CTX. The decrease of s-CTX may also be through insulin as the insulin response is amplified by the incretins. In vitro insulin added to the hyperglycemic condition partially prevented the OPG response (144). *Chailurkit et al.* observed a smaller decrease in s-CTX at an OGTT in patients with T2D compared to normal glycemic individuals after two hours (140). This may be due to an impaired insulin or gastro-intestinal response or due to slower uptake of glucose in patients with T2D. A study support the association of p-glucose and bone turnover markers in young patients with T1D; s-OC, s-CTX, and s-TRAP were lower at the diabetes onset but normalized after three months, but also with a corresponding drop in HbA1c from 11.3 to 5.9 % (185) .
The differential association between p-glucose and bone turnover markers may be due to the higher level of bone turnover markers in patients with T1D, which may be more susceptible to increased p-glucose levels than patients with T2D.

6.4 BONE STRUCTURE IN DIABETES

It was hypothesized that patients with T1D and T2D differ in bone density measured by BMD, where BMD is decreased in patients with T1D. In paper 6 DXA T-score, reflecting BMD, was lower in patients with T1D at the hip, femur, and spine compared to patients with T2D. Only hip T-score remained significantly different in the multiple adjusted analyses. Hip BMD may thus explain some of the difference in hip fractures reported. Our measures of BMD in patients with T1D and T2D are comparable to the reported Z-scores reported by *Vestergaard* (11). Conversion of the T-score to the age and gender matched Z-score revealed a value of -0.28 and +0.48 by in patients with T1D and T2D, respectively. *Vestergaard* reported similar values of -0.37 and +0.22 for patients with T1D and T2D, respectively and concluded that it could not explain the increased fracture risk (11). In paper 6 the T-score of hip, femur, and spine were not predictive of fracture in the adjusted analyses. Although we are not able to demonstrate a relation between BMD and hip fracture it adds to the evidence of relatively high BMD levels compared to the increased fracture risk in patients with diabetes.

Neither density, structural parameters nor failure load measured by HRpQCT were different between patients with T1D and T2D. As described in the introduction a study found deficits in trabecular and cortical microarchitecture in patients with T1D and microvascular disease compared with patients with T1D without microvascular disease (103). No association was present between microvascular disease and trabecular or cortical microarchitecture in paper 5 in neither patients with T1D nor T2D. The differences between the studies may be due to a lower number of patients or lower age among patients with T1D (mean age 46 years) in the study by *Shanbhogue et al.* (103). Low power may increase the risk of a type 1 error whereas age differences reflect differences in patient characteristics between T1D and T2D. However, age per se may also affect bone composition and biomechanical properties. Bone tissue stiffness at the tibia was lower in patients with T1D also in the adjusted analysis. Cortical pore diameter and the standard deviation of cortical pore diameter were higher in patients with T1D at both the radius and tibia compared to patients with T2D. None of the porosity parameters differed in the adjusted analyses. Thus, cortical microstructure, density, and structure were affected in the same manner in patients with T1D and T2D. Measures of cortical porosity have discriminated fracture in patients with T2D (100) and if it predicts fracture it is likely to do so in both diabetes types. Bone tissue stiffness is a measurement of bone mechanical competence and not solely a bone density parameter as the hip T-score. The peripheral bone tissue stiffness strongly correlated with the central tissue stiffness at the lumbar spine and femur (186). Tissue stiffness has proved as a fracture predictor in non-diabetes subjects (187, 188) and may also do this in patients with diabetes. Thus, the tissue stiffness may be able to elucidate the discrepant fracture rates in patients with T1D and T2D. No deficiency in bone mineral accumulation is present in the patients with diabetes based on the T-score, however as bone is composed of mineral and matrix in a tight structure any

deficiency may affect bone mechanical competence and we were not able to determine the collagen structure or matrix by the structural bone scans.

6.5 FRACTURE PREDICTORS IN DIABETES

It was hypothesized that HbA1c levels predict fractures in patients with diabetes. In paper 4 no association between HbA1c and fracture in patients with diabetes was present. Neither falls nor hypoglycemic events were associated with fracture risk in neither paper 4 or 6. Instead LDL-cholesterol was associated with fractures for both the whole cohort of patients with diabetes as well as limited to patients with T2D in paper 4. None of the antidiabetics was associated with fracture risk. To avoid the effects of renal disease nephropathy cases were excluded. LDL-cholesterol levels of 0-1.5 mmol/L were associated with increased risk of fracture whereas levels of more than 2.4 mmol/l was beneficial in terms of fracture risk compared to the reference level of 2.2-2.4 mmol/l. Current ADA guidelines recommend LDL-cholesterol < 1.8 mmol/l for subjects with prior cardiovascular disease and < 2.5 mmol/l for others (189). Although LDL-cholesterol lowering was beneficial a review concluded that the optimal target value was not firmly established and further research is needed (190). The results of paper 4 indicate that the optimal level might not be as low as possible with respect to fracture risk. LDL-cholesterol may affect bone turnover; in vitro LDL-depletion of osteoclasts-like cells inhibited the TRAP production which was abrogated with the addition of LDL (191). Also LDL prolonged the survival of osteoclast-like cells, whereas HDL induced apoptosis (192). Oxidized LDL may stimulate an osteoclast-associated receptor, which is a co-stimulator of osteoclast differentiation (193). Thus osteoclasts function may depend on LDL. Osteoblasts are found to have a LDL-receptor (194) and LDL in native and oxidized form killed osteoblasts like cells, although the level was not determined (195). LDL may also associate with the LRP-5 and 6 receptors, which are positive regulators of the Wnt pathway and stimulate osteoblast differentiation and bone formation (196, 197). In paper 5 an association between bone formation markers and LDL-cholesterol was observed which may suggest that LDL-cholesterol possibly through LRP-5 and LRP-6 may influence bone turnover. Loss of function mutations of the LRP-5 gene presents with osteoporosis, diabetes, impaired glucose tolerance, and altered lipid metabolism (198). LDL may stimulate the Wnt pathway and increase bone formation, but at possibly supra-physiological levels LDL may kill osteoblasts. Animal models are needed to translate the in vitro evidence of LDL and bone. Statins have been proposed to decrease fracture risk by HMG CoA-reductase activity (199). The association of LDL-cholesterol with fracture was independent of statins and statins itself did not associate with fractures. The JUPITER trial, a randomized controlled trial, report no beneficial effect of statins and even present a trend of more fractures in the treated group (200). Thus, statins may be a double edged sword in relation to bone with a beneficial effect through the mevalonate pathway (199) and a negative through LDL-cholesterol and Wnt (196).

6.6 VERTEBRAL FRACTURE

The previous meta-analyses especially reported an increased risk of hip fracture in patients with diabetes, whereas vertebral fractures (11, 48) were not increased. In the clinical trial 11.6 % of the patients with diabetes were diagnosed with a vertebral fracture and when limiting it to the patients examined by X-ray 28 % of the patients were diagnosed with a vertebral fracture. Most of these were Genant classification 1 and thus only likely to be diagnosed by the spine X-ray examination and not by a clinical diagnosis. In comparison to vertebral fractures, hip fractures are rarely undetected. The risk of vertebral fractures may thus also be increased in patients with diabetes, but due to rare use of the spinal X-ray examination it may not be diagnosed and registered as often as hip fractures. Furthermore it may be important to determine the vertebral fractures by spinal X-ray and not VFA as they detected vertebral fracture in 28 % and 2 %, respectively. The discrepancies between spinal X-ray and VFA may be due to a large amount of mild fractures in the x-ray evaluated population (80 % of the fractures); however it may be a major problem as fracture predictors are sparse in patients with diabetes and a mild vertebral fracture may be the first sign of ongoing diabetic bone disease.

6.7 THE DIABETIC BONE

In diabetes the paradox of increased fracture risk and apparent normal bone mineral remains unsolved. BAP is a marker of the mineralization process and while other turnover markers were decreased in diabetes s-BAP remained normal (110, 114, 116, 201, 202). Thus mineralization may continue at normal rate, whereas collagen and matrix remodeling is suppressed. This may lead to hypermineralized fragile bone as in some osteopetrotic subjects (116). It is also supported by bone biopsies from the study of Manavalan et al. (110); the biopsies from patients with T2D present reduced bone formation rate and mineralizing surface, however the adjusted apposition rate was not lower in patients with T2D reflecting that the decreased mineralizing surface is due to a decreased bone turnover. The hypermineralization may be caused by increased glucose levels (Figure 6) (143) although evidence is conflicting (144). *Cunha et al.* (144) found indices of decreased mineralization during 24 hours of hyperglycemia, whereas the hypermineralization observed by *Garcia-Hernandez et al.* was for 7 and 14 day periods (143). The discrepancy between the in vitro studies may simply be due to the differences in time intervals so a short period of hyperglycemia decrease bone mineralization, whereas a longer period as in patients with diabetes increase bone mineralization and hypermineralize bone. In spite of the hypermineralization observed BAP levels decreased at the highest level of glucose (24 mmol/l) (143), however these levels are supra-physiological for a longer period even in patients with diabetes. In a model using T1D mice osteoblasts, both hyperglycemia and hyperosmolality increased peroxisome proliferator-activated receptor γ (PPAR-γ) (203). Glitazones function through the PPAR- γ pathway by increasing insulin sensitivity, but also increase bone resorption and lead to preferential differentiation of mesenchymal stem cells into adipocytes instead of osteoblasts (135, 204). Also, based on cell cultures

hyperglycemia has been suggested to divide osteoblastic precursor cells into an adipogenic pathway causing inflammation in the bone (205). Thus, long term hyperglycemia may have detrimental effects to the bone by activating the PPAR- γ pathway. The meta-regression revealed that increasing HbA1c levels in patients with diabetes was a positive determinant of s-BAP (114). The associations between p-glucose and bone turnover markers in diabetes individuals suggest that patients with diabetes are in a state of constant cycling of decreasing and increasing bone turnover due to fluctuations in p-glucose. The association may be through an up regulation of OPG expression in either osteocytes or osteoblasts with the latter most likely. Furthermore, the effects of insulin and GLP-2 are unclear as they may be mediators of p-glucose or promoters of the response. Another factor causing hypermineralization in patients with diabetes may be a blunted PTH response, which previously have been reported and is supported by the findings of the meta-analysis (114, 174). Patients with hypoparathyroidism, which are characterized by a lack of PTH or a lacking function of PTH have very high BMD levels (206). Other factors than glucose, may thus cause hypermineralization in patients with diabetes. Figure 21 presents the hypermineralization hypothesis.

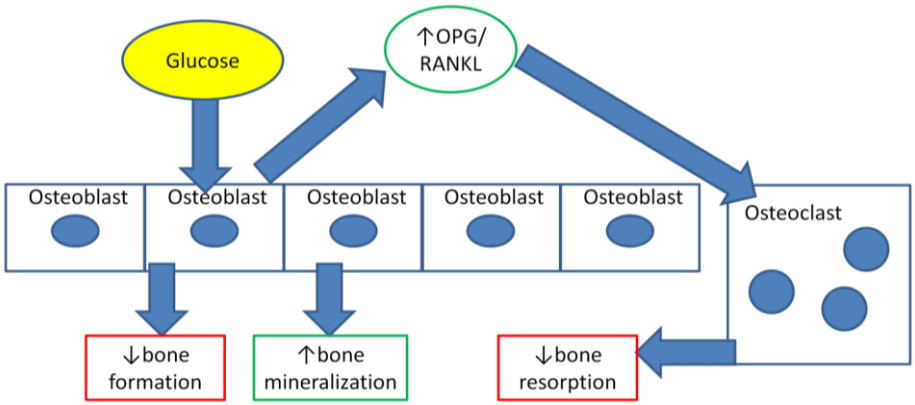

Figure 21. *The hypermineralization hypothesis.*

In the hypermineralization hypothesis the osteoblast is central. In patients with diabetes, the osteoblast may not produce sufficient amounts of collagen to balance the increased bone resorption in diabetes patients as reflected by decreased s-CTX and s-OC levels (114), however s-BAP and the mineralization process is unaffected (116). This imbalance between collagen production and mineralization may turn the bone fragile with a decreased calcium phosphate ratio of the mineral; resulting in low quality mineral (143). In an attempt to alter this ratio the bone react with a blunted PTH response and an increase in FGF-23, thus trying to maintain the calcium in the bone and remove phosphate, however this may have adverse effects and further hypermineralize the bone as in hypophosphatemic rachitis (207). Furthermore, the hyperglycemic conditions may increase the production of OPG by the osteoblast and thereby inhibit the osteoclast and the bone resorption. This

inhibition of the osteoclast and a blunted PTH response with lesser osteoclast activity causes the mineral accumulate in the bone as it is not removed in the normal rhythm. The hypermineralization may be regulated by fluctuations in p-glucose or accompanied effects of the gastro-intestinal hormones (as GLP-2), where hyperglycemia decrease bone turnover while mineralization is unaffected. The fragility of the hypermineralized bone may be further enhanced if the activity Wnt/LRP-5 pathway is reduced by low LDL-cholesterol levels.

6.8 EXPLANATIONS OF FRACTURE IN PATIENTS WITH DIABETES

Figure 22 depicts the factors that may influence the increased fracture risk in diabetes patients and to adds to Figure 8 in section 1.9. The association between diabetes and fracture is not fully explained by BMD, FRAX, diabetes complications or falls. However, each factor may contribute to it. As described above, hyperglycemia may decrease bone formation and hypermineralize the bone, which leave the bone weaker than what bone density explains. The bone fragility may be enhanced by a lack of LDL-cholesterol, which may activate bone formation by the Wnt pathway. Sclerostin which is antagonist of the Wnt pathway may display different actions in patients with T1D and T2D as increasing levels decrease fracture risk in patients with T1D and increase fracture risk in patients with T2D. This difference may be due to the lower bone formation overt in patients with T2D compared to patients with T1D, which also may be reflected by the BMD differences. Thus, a high p-sclerostin level in patients with T2D may with harmful effect further decrease the bone formation, whereas a decrease in bone turnover may be beneficial in T1D patients, which theoretically may be caused by a lower accumulation of weak collagen cross-links. Furthermore, bone tissue stiffness may contribute to the fracture risk difference between T1D and T2D as it differed between the patient groups. Several factors may contribute to the bone fragility observed in diabetes patients and so far no one factor has been able to explain the fracture risk fully.

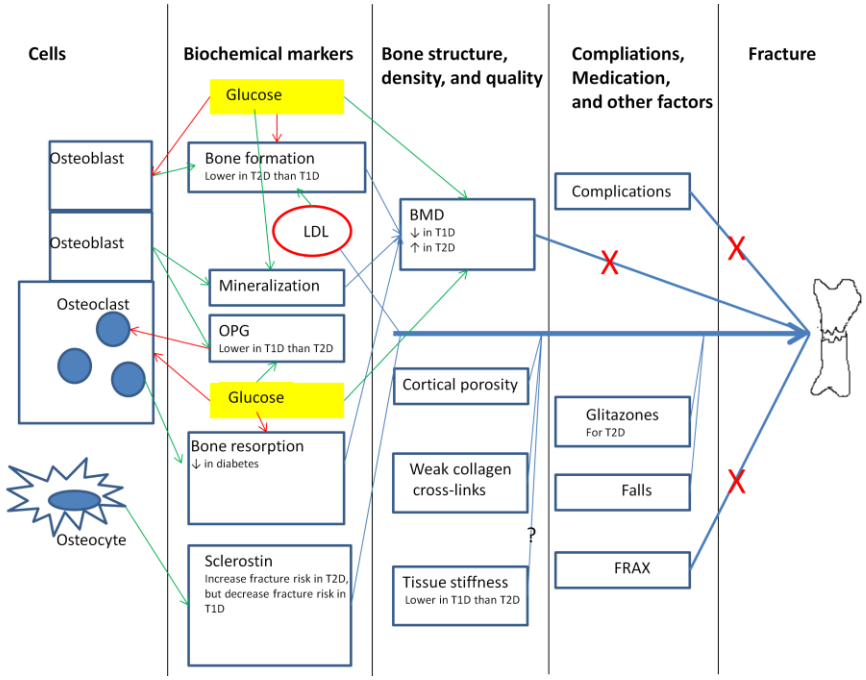

Figure 22. *Factors that may influence the risk of fracture in diabetes patients. LDL: LDL-cholesterol, OPG: Osteoprotegerin, AGE: Advanced Glycation End, TBS: Trabecular bone score, BMD: Bone mineral density, FRAX:* Fracture Risk Assessment Tool. *Blue arrows indicate relationships. Red arrows indicate inhibitory effects. Green arrows indicate a positive effect or secretion. X mark associations that do not explain the increased fracture risk in diabetes patients. ↓ marks a decrease compared to subjects without diabetes. ↑ marks an increase compared to subjects without diabetes.* ***?*** *marks if a relationship is unknown.*

6.9 GENERALISABILITY

The results presented in this thesis from paper 1-6 are generalisable to patients with diabetes. The association of LDL-cholesterol and fracture was only estimated specifically in patients with T2D, whereas the cross-sectional results is provided from a relatively dysregulated diabetes population. As the meta-analysis does not discriminate on patient characteristics; the results may apply to all patients with diabetes. The paper 7 is a basic physiological study, but the results are not generalisable to patients with diabetes, only healthy young men.

7. CONCLUSION

The initial hypotheses were:
Primary hypotheses:

I. Patients with type 1 and type 2 diabetes differ in bone density measured by BMD, where BMD is decreased in type 1 diabetes.
II. To explain the expected decreased in BMD in patients with type 1 diabetes they have higher bone turnover measured by circulating biochemical markers compared to patients with type 2 diabetes.

Secondary hypotheses

III. Bone turnover markers are decreased in patients with diabetes compared to non-diabetes controls.
IV. HbA1c levels predict fractures in patients with diabetes.
V. Increasing plasma glucose decreases circulating bone turnover markers.

Conclusions on initial hypothesis are:

I. Patients with type 1 diabetes had lower BMD at the hip compared to patients with type 2 diabetes independent of the patient characteristics.
II. Patients with type 1 diabetes had higher bone formation markers compared to patients with type 2 diabetes.
III. Patients with diabetes had decreased markers of resorption and formation compared to non-diabetes controls.
IV. HbA1c did not predict fractures in patients with diabetes; however LDL-cholesterol and p-sclerostin may be useful markers.
V. P-glucose was associated with lower bone turnover markers in patients with diabetes, which may be caused by increased s-OPG. In healthy males both oral and intravenously administered glucose decrease bone resorption, but not formation.

In conclusion, we observed indices of lower bone turnover and increased bone density and tissue stiffness in patients with T2D compared to patients with T1D. The bone turnover in patients with diabetes seemed to be lower than in non-diabetes controls. P-glucose levels were associated with increased s-OPG and decreased bone turnover, thus the diabetic bone disease may be due to p-glucose fluctuations altering the normal bone remodeling. Our results point at possible beneficial effects of glycemic control on bone health in patients with diabetes. Furthermore, we found indices of the usage of p-sclerostin and LDL-cholesterol as fracture predictors in patients with diabetes.

8. PERSPECTIVE

The presented study provides valuable information on bone status in patients with diabetes. Clinicians should be aware of the association between diabetes and osteoporosis, which is not reflected by the BMD. If osteoporosis is suspected, the DXA scan may be accompanied with an x-ray of the spine, as vertebral fractures are prevalent in patients with diabetes.

Diabetic bone disease may be caused by the insulin deficiency in patients with T1D and the relative insulin deficiency compared to glycemia in patients with T2D causing a hyperglycemic state, which decrease bone turnover, but also cause hypermineralization. Thus, diabetic bone disease may be prevented by strict glycemic control. Future investigations should apply the advanced techniques of continuous glucose monitoring to map the circadian rhythm of both p-glucose and bone turnover in patients with diabetes, which may be very different from what is seen in non-diabetes subjects. Furthermore, more evidence is needed on the effect of glucose on osteoblasts, osteoclasts, and osteocytes and if the hypermineralization caused by hyperglycemia may be translated into animal models.

Evidence of different bone structure between patients with T1D and T2D was found. The difference in hip BMD, may explain some of the discrepancy in fracture rates between patients with T1D and T2D, but still does not explain the increase compared to non-diabetes subjects. Tissue stiffness evaluated with the HRpQCT, which use very low x-ray doses, may further explain the differences in fracture as it is not only a bone density parameter. Future investigations should evaluate the association between HRpQCT parameters and fracture. Furthermore, a comparison between bone quality estimated by bone tissue biopsies and structural parameters is needed to determine if tissue stiffness or porosity may be a marker of decreased bone biochemical competence.

Finally we found associations between p-sclerostin levels and fracture in patients with T1D and LDL-cholesterol levels and fracture in patients with T2D. These results should be used for hypothesis generation and should be confirmed by other observational studies.

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SUMMARY

Diabetes mellitus is associated with an increased risk of fracture with and current fracture predictors underestimate fracture risk in both type 1 and type 2 diabetes. Thus, further understanding of the underlying causes of diabetic bone disease may lead to better fracture predictors and preventive measures in patients with diabetes.

This PhD thesis reports the results of two systematic reviews and a meta-analysis, a state-of-the-art intervention study, a clinical cross-sectional study and a registry-based study all examining the relationship between diabetes, glucose, and bone.

Patients with type 2 diabetes had lower bone turnover markers compared to patients with type 1 diabetes and bone mineral density and tissue stiffness were increased in patients with type 2 diabetes. The bone turnover markers were inversely associated with blood glucose in patients with diabetes and both an oral glucose tolerance test and an intravenous test decreased bone resorption markers in healthy men. In the registry based study; fracture risk in patients with type 2 diabetes were associated with low LDL-cholesterol levels.

In patients with diabetes, bone turnover and bone strength are associated with clinically relevant measures such as cholesterols and fluctuate on the basis of blood glucose level alterations.

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