Thesis Title
MicroRNAs and biomarkers of bone health in diabetes

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Thesis abstract

Diabetes is associated with an increase in fracture risk. Patients with type 1 diabetes experience a low bone mineral density, which explains their increase in fracture risk. In type 2 diabetes, despite the observation of normal or relatively high bone mineral density, these patients are also at an increased risk for fractures. This suggests that, especially in type 2 diabetes, the measurement of bone mineral density underestimates true fracture risk. This calls for additional measures for better fracture risk prediction in diabetes. In this context, microRNAs are of interest. Literature provides evidence for using microRNAs as novel non-invasive biomarkers for disease diagnosis, including diabetes and osteoporosis. At present, literature provides limited evidence on the role of miRNAs in diabetes and bone health and their association with markers of bone strength and bone metabolism. Rather, the literature provides substantial evidence on the role of microRNAs in type 1 and type 2 diabetes, osteoporosis, fractures, bone turnover and bone mineral density individually. Hence, I performed a systematic review of the literature to identify miRNAs that are dysregulated in diabetes (type 1 and type 2) and abnormal bone phenotype (fractures, bone mineral density and bone turnover). 64 such dysregulated microRNAs were identified. Further, a sensitivity analysis performed on these 64 microRNAs identified miR-21-5p, miR-335, miR-375, miR-320a-3p and miR-124-3p to be upregulated; and miR-17-5p, miR-155-5p, miR-25 and miR-223-3p to be downregulated in diabetes and abnormal bone phenotype, suggestive of these nine miRNAs to be promising biomarker candidates in diabetes and bone fragility. To validate my findings, I measured these miRNAs in clinical studies related to type 1 and type 2 diabetes. Our data was suggestive of miR-133a-3p as a potential biomarker candidate for type 1 diabetes and miR-148a-3p and miR-574-3p as potential biomarker candidates for type 2 diabetes and bone fragility.

Besides miRNAs, I provide evidence for no further decline in bone turnover, bone loss, and cortical porosity at the tibia in type 1 diabetes and neuropathy after a follow-up period of 4 years and 4 months. I also provide evidence for age, gender, and BMI-adjusted aBMD at the total hip to be low in T2D. Using the T-score bone marker plot, I show T2D as characteristic of low bone turnover and positive bone balance.

While measuring miRNAs in this thesis, I faced systemic difficulties related to miRNA variability. Due to the lack of knowledge in the literature on miRNA variability in diabetes and osteoporosis, I assessed the capability of a commercially available microRNA bone biomarker kit called the ‘OsteomiR kit’ to predict incident fractures in an independent and well-characterised study population of postmenopausal women. I observed the combined adjusted expression of miR-214-3p and miR-31-5p to support the prediction of incident vertebral

fractures and the combined adjusted expression of miR-19b-3p and miR-29b-3p to support the prediction of incident non-vertebral fractures.

Overall, this thesis suggests that additional information on miRNA variability is required before they can be used as suitable biomarkers for diagnosing and treating diabetic bone disease.

Acknowledgements

I want to thank Prof. Richard Eastell for giving me this wonderful and exciting opportunity of coming to Sheffield to work with him and his experienced team on bone research. These 4 years have been exceptional with plenty of learning. His guidance, teachings, and encouragement during my PhD has taught me a lot professionally, as well as personally. I would also like to thank Dr. Matthias Hackl for giving me the opportunity to be trained in miRNA measurements by his experienced team of scientists and for providing me with the opportunity to carry out all the miRNA-based experiments in his well-equipped laboratory at TAmiRNA. His guidance in all work related to miRNAs has been very helpful in shaping this thesis. I also extend my gratitude to Dr. Jennifer Walsh, my second supervisor and Prof. Morten Frost, for their valuable inputs through the course of my thesis. All of you have been very supportive, encouraging and taught me a lot.

I would like to extend a thank you to the senior members of the FIDELIO-ITN for their well-structured program, encouraging, motivating and collaborative environment, and extensive research network.

I would like to thank the AUBM team for being forever approachable, supportive and helpful. I am very grateful to Dr. Margaret Paggiosi, Dr. Tatiane Vilaca and Dr. Fatma Gossiel for their help in sorting out all sorts of applications, data management, and technical help related to my research and for their continuous encouragement. I would also like to thank the whole CRF team including Jill Thompson for accommodating me at the CRF to perform my research activities and for providing me with a joyous environment during my clinical research. I would also like to thank all other my colleagues at AUBM for making this journey a joyous experience. I extend my gratitude to all my friends and colleagues at FIDELIO for helping me build wonderful memories at training courses, conferences, and annual meetings. We shared doubts, complaints, fears, anxiety, and uncountable laughter. I will forever cherish these memories.

I would like to thank the University of Sheffield Biorepository for being supportive in my research activities. I extend a very huge thank you to all the volunteers who took part in my research study. Your time and contributions to my work has been invaluable.

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Contributions

MicroRNAs in diabetic bone disease: a systematic review (Chapter 3)

The protocol for the systematic review was designed and written by me under the guidance of Dr. Tatiane Vilaca and Prof. Richard Eastell.

The literature research and first round of paper selection (based on the title and abstract) was done by me and cross verified by Dr. Lisa Dowling. Dr. Lisa Dowling also helped with the selection of original studies at the full text level. I designed and piloted the data extraction form under the guidance of Dr. Tatiane Vilaca. Complete data extraction was done by me and cross verified by Dr. Lisa Dowling. I also designed and piloted the questionnaire for the sensitivity analysis under the guidance of Dr. Tatiane Vilaca. The sensitivity analysis scoring was done by me and cross verified by Dr. Lisa Dowling.

All the collected data were analyzed using bioinformatic tools by me under the guidance of Dr. Matthias Hackl.

The whole process was supervised by Prof. Richard Eastell.

MicroRNAs in type 1 diabetes and the effect of prolonged duration of diabetes and neuropathy on type 1 diabetes and bone health (Chapter 4)

I wrote the study protocol and subsequent amendments under the guidance of Dr. Tatiane Vilaca and Prof. Richard Eastell. I obtained approval from Ethics, Research and Development and Feasibility team at the CRF. I was responsible for site file maintenance, submission of amendments and overall study management. I also wrote the participant information sheets, participant invitation letter, consent forms, participant study case report forms and other supporting documents. I was responsible for study recruitment and filling in the case report forms. Jill Thompson helped post out the participant documentation set (participant invitation letter and participant information sheet) which was followed by the participant appointment letter for those willing to take part in the study. I booked the participants in for their visit and obtained written consent from them. I was responsible for performing phlebotomy and the neurophysiologic function tests. I also helped sort out the participants travel expenses.

All the DXA and HR-pQCT scans, as well as anthropometric measurements were performed by Dr. Margaret Paggiosi. Dr. Margaret Paggiosi set up the scanning protocol, obtained approval from the Medical Imaging and Medical Physics team to conduct the scans part of the study. 3D registration of the scans was done by Dr. Danielle Whittier. Dr. Margaret Paggiosi

performed all the scan analysis and finite element analysis. Regular clinical tests (eGFR, HbA1c) were performed by the Clinical Chemistry and Hematology Departments at Sheffield Teaching hospitals, Sheffield. The research measurements (BTMs, miRNAs) were done by me. Data on miRNA expression was analyzed under the guidance of Dr. Matthias Hackl. Data entry from the case report forms and participants notes into an electronic version was done and maintained by me, as well as filing of all the paper-based documentation. All the statistical analysis part of this study was done by me.

I was guided by Prof. Richard Eastell throughout this study.

MicroRNAs and bone mineral density in type 2 diabetes: relationship to bone turnover and bone balance (Chapter 5)

I wrote the study protocol and subsequent amendments, as well as justification for the research study under the guidance of Prof. Richard Eastell. Dr. Lorenza Mattia provided input towards the study protocol. Dr. Margaret Paggiosi helped in creating a material transfer agreement between University of Sheffield and Southern Denmark University for a seamless transfer of clinical data and clinical samples. Dr. Margaret Paggiosi also helped in identifying the matched controls suitable to be used in this study. I obtained approval from the South Yorkshire North Derbyshire Musculoskeletal Biobank to use samples available in the biorepository for this research. Measurement of miRNAs, BTMs and other hormones known to regulate bone remodeling were done by me. Data on miRNA expression was analyzed under the guidance of Dr. Matthias Hackl. All the statistical analysis part of this study was also done by me.

I was guided by Prof. Richard Eastell throughout this study and received valuable input from Dr. Matthias Hackl and Prof. Morten Frost.

Capability of the ‘OsteomiR kit’ in predicting incident vertebral and non-vertebral fracture risk in the OPUS population (Chapter 6)

The overall study protocol and all aspects of study management was done by the respective study partners. The DXA measurements were done by the respective study site technicians. The study protocol related to measurement of miRNAs was written by Patryk Zarecki. He also helped obtain approval from the South Yorkshire North Derbyshire Musculoskeletal Biobank for the use of required samples in this research study. The miRNAs measurements were done

by Patryk Zarecki and David Carro Vazquez. The BTMs were measured by Dr. Fatma Gossiel. Dr. Margaret Paggiosi provided me with the clinical database for this study. Data on miRNA expression was analyzed under the guidance of Dr. Matthias Hackl. All the statistical analysis was done by me.

I was guided by Prof. Richard Eastell throughout this study.

Communications

Publications

Matthias Walle, **Ankita Duseja**, Danielle E Whittier, Tatiane Vilaca, Margaret Paggiosi, Richard Eastell, Ralph Müller, Caitlyn J Collins, Bone remodelling and responsiveness to mechanical stimuli in individuals with type 1 diabetes mellitus, *Journal of Bone and Mineral Research*, 2024;, zjad014, https://doi.org/10.1093/jbmr/zjad014.

Dowling L., Duseja A., Vilaca T., Walsh J. S., and Goljanek-Whysall K. (2022) MicroRNAs in obesity, sarcopenia, and commonalities for sarcopenic obesity: a systematic review, *Journal of Cachexia, Sarcopenia and Muscle*, 13, 68–85, https://doi.org/10.1002/jcsm.12878.

Oral Presentation

BMD in type 2 diabetes: relationship to overall bone turnover and bone balance

Duseja A, Gossiel F, Paggiosi M, Walsh J, Nielsen MF, Hermann P, Eastell R. aBMD in type 2 diabetes: relationship to overall bone turnover and bone balance. European Calcified Tissue Society Meeting 2023, Liverpool, UK.

ECTS Travel Award

Poster Presentations

Duseja A, Gossiel F, Paggiosi M, Walsh J, Nielsen MF, Hermann P, Eastell R. Bone turnover and bone balance in type 2 diabetes. American Society of Bone and Mineral Research 2022, Austin, USA.

Duseja A, Dowling L, Vilaca T, Hackl M, Eastell R. MicroRNAs in Diabetic Bone Disease: a Systematic Review. American Society of Bone and Mineral Research 2021, Virtual meeting.

Abbreviations

aBMD	Areal bone mineral density
AGEs	Advanced glycation end products
APCs	Antigen-presenting cell
AR	Aldose reductase
ATP	Adenosine triphosphate
AUC	Area under the ROC curve
BAP	Bone alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
BTMs	Bone turnover markers
BV/TV	Trabecular bone volume fraction
CKD	Chronic kidney disease
CML	Carboxymethy lysine
CRF	Clinical research facility
Ct.Po	Cortical porosity
Ct.Th	Cortical thickness
Ct.vBMD	Cortical vBMD
CTRL	Control
CTX	Cross-linked C-telopeptide
DAG	Diacylglycerol
DGCR8	DiGeorge critical region 8
DKK1	Dickkopf-1
DM	Diabetes mellitus
DMP1	Dentine matrix protein 1
DNA	Deoxyribonucleic acid
DPN	Diabetic peripheral neuropathy
DR	Diabetic retinopathy
DSPN	Distal symmetric polyneuropathy
DSPN	Distal symmetrical polyneuropathy
DXA	Dual energy X-ray absorptiometry
eGFR	Estimated glomerular filtration rate
ELISA	enzyme linked immunosorbent assay
Est.Fail.Load	Estimated failure load
EVs	Extracellular vesicles
F	Female
FAB	Fat and Bone
FN	Femoral neck
FPG	Fasting plasma glucose

FRAX	Fracture risk assessment tool
HbA1c	Glycated hemoglobin A1c
HC	Healthy controls
HLA	Human leukocyte antigen
HR-pQCT	High resolution peripheral quantitative computed tomography
IGF-1	Insulin like growth factor 1
IL	Interleukin
IOF	International Osteoporosis Foundation
IR	Insulin resistance
IRAS	Integrated research application system
IRS	Insulin receptor substrate
LRP	Low-density lipoprotein receptor
LS	Lumbar spine
M	Male
M-CSF	Macrophage colony-stimulating factor
MetS	Metabolic syndrome
miRNAs	MicroRNAs
MVD	Microvascular complications
NDR	Non-diabetic retinopathy
NGH	Northern general hospital
NGS	Next generation sequencing
NGT	Normal glucose tolerance
NOS	Newcastle-Ottawa scale
NPDR	Non-proliferative diabetic retinopathy
OC	Osteocalcin
OGTT	Oral glucose tolerance test
OP	Osteoporosis
OPG	Osteoprotegerin
OPUS	Osteoporosis and Ultrasound Study
OUH	Odense university hospital
PBMC	Peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDR	Proliferative diabetic retinopathy
PINP	Procollagen type 1 N-propeptide
PKC	Protein kinase C
PM	Post-menopausal
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RISC	RNA induced silencing complex
RNA	Ribonucleic acid

RNA-Seq	RNA sequencing
RR	Risk ratio
RT-PCR	Real time polymerase chain reaction
SD	Standard deviation
SNPs	Single nucleotide polymorphisms
SYNDMB	South Yorkshire North Derbyshire musculoskeletal biobank
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
Tb.vBMD	Trabecular vBMD
TCNS	Toronto clinical neuropathy score
TGF-β	Transforming growth factor beta
TH	Total hip
TNF-α	Tumor necrosis factor alpha
TRAcP 5b	Tartrate-resistant acid phosphatase
TRBP	TAR RNA binding protein
UTR	Untranslated region
vBMD	Volumetric total BMD
VNTRs	Variable number tandem repeats
Wnt	Wingless related integration site

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Chapter 1 - Introduction

1.1 Diabetes mellitus

Diabetes mellitus is a metabolic disorder characterised by hyperglycaemia due to a lack of insulin secretion by pancreatic β-cells, defects in insulin secretion and action, or both [1], [2]. As of 2021, one in every 15 people in the United Kingdom is affected by diabetes, accounting for a total diabetic population of approximately 4.9 million. Additionally, 13.6 million people are at an increased risk for type 2 diabetes in the United Kingdom [3]. Huge increases in social, economic and medical burden are associated with diabetes [1], [3].

Diabetes diagnosis

Diagnosis of diabetes is done based on plasma glucose criteria. According to the American Diabetes Association (ADA), diabetes is diagnosed based on having fasting plasma glucose concentration (FPG) ≥ 126 mg/dL (7.0 mmol/L) (OR) 2-hour plasma glucose concentration (PG) ≥ 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT) (OR) glycated haemoglobin (HbA1c) ≥ 6.5% (48 mmol/mol) [4]. The two most common types of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D). 5-10% of the total diabetic population is diagnosed with T1D, while the remaining 90% accounts for people with T2D [1]. It has been recognised that this large population of T2D remain asymptomatic during the initial years of disease onset [2]. The prolonged duration of diabetes leads to microvascular complications, mainly neuropathy, nephropathy and retinopathy. Recent evidence suggests the increased risk of fractures to be a significant complication associated with increased duration of diabetes [5].

1.1.1 Types of diabetes

1.1.1.1 Type 1 diabetes

Type 1 diabetes is an autoimmune disease that is characterised by the destruction of the pancreatic β-cell islets, leading to metabolic failure. The destruction in β-cell islets by the immune system acts via a T-cell mediated inflammatory response and B-cell mediated humoral response, leading to a lack of insulin production. This leads to the presence of auto-antibodies in circulation, which is a hallmark of T1D. These patients with T1D are dependent on lifelong insulin treatment [1], [6].

1.1.1.2 Type 2 diabetes

Type 2 diabetes is characterised by insulin resistance and relative insulin deficiency (Guthrie, 2004; Mahler & Adler, 1999). Insulin resistance causes an increase in the target cells' demand for insulin. In response, the pancreatic β-cells produce more insulin to maintain normal blood

glucose levels and homeostasis [1]. When the demand for insulin by the target cells is not met, insulin resistance increases, overworking the β-cells and eventually leading to their gradual destruction [2], [7].

This form of diabetes is more common in the elderly population (> 40 years). However, it is becoming increasingly common in the younger generation due to lifestyle changes such as lack of exercise, obesity and unhealthy eating habits [2].

1.1.2 Pathogenesis of diabetes

1.1.2.1 Pathogenesis of type 1 diabetes

The pathogenesis of T1D and the mechanism by which T cells help the B cells produce insulin autoantibodies is described in Figure 1.1 and Figure 1.2.

There is evidence for mutations in the human leukocyte antigens (HLA DR and DQ) important for T-cell antigen presentation to predispose a person to T1D and variable number tandem repeats (VNTRs) and single nucleotide polymorphisms (SNPs) to contribute to the lack of insulin. Along with genetic predisposition to T1D, viral infections and environmental factors have also been linked to disease onset [8], [9].

This form of diabetes is more common in adolescents and young adults. The symptoms of T1D include polyuria, polyphagia, polydipsia, lack of energy, extreme tiredness, slow wound healing, recurring infections, sudden weight loss and ketoacidosis [1], [2].

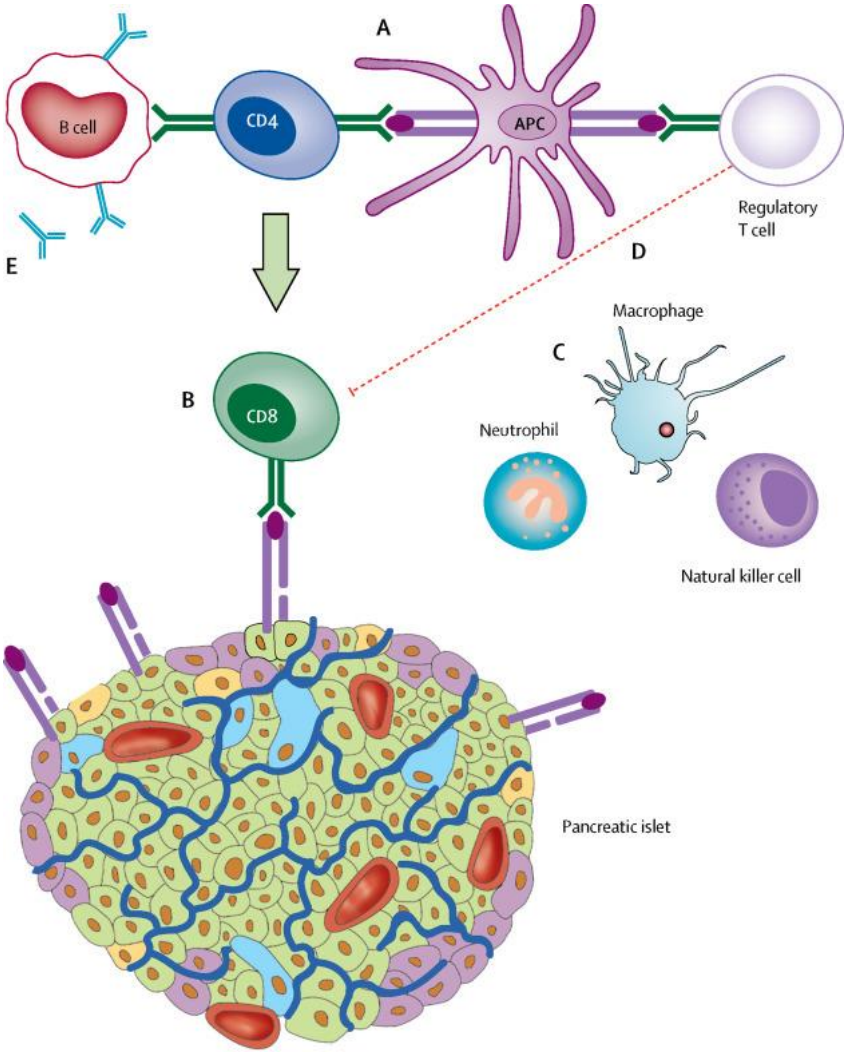

Figure 1-1. Pathogenesis of type 1 diabetes.

Onset of type 1 diabetes is initiated by the presentation of β-cell peptides by antigen-presenting cells (APCs). APCs bearing these autoantigens migrate to the pancreatic lymph nodes and interact with autoreactive CD4+ T lymphocytes, which in turn mediate the activation of autoreactive CD8+T cells (A). These activated CD8+ T cells return to the islet, lyse β-cells and express immunogenic self-antigens on major histocompatibility complex class I surface molecules (B). β-cell destruction is exacerbated by the release of proinflammatory cytokines and reactive oxygen species from innate immune cells (macrophages, natural killer cells, and neutrophils; C). This entire process is amplified by defects in regulatory T lymphocytes, which do not effectively suppress autoimmunity (D). Activated T cells within the pancreatic lymph nodes also stimulate B lymphocytes to produce autoantibodies against β-cell proteins. These autoantibodies are measured in circulation and are considered a defining biomarker of type 1 diabetes (E). In the pancreatic islet: green cells - beta cells; purple cells – alpha cells; blue cells - delta cells; red - dots capillaries.

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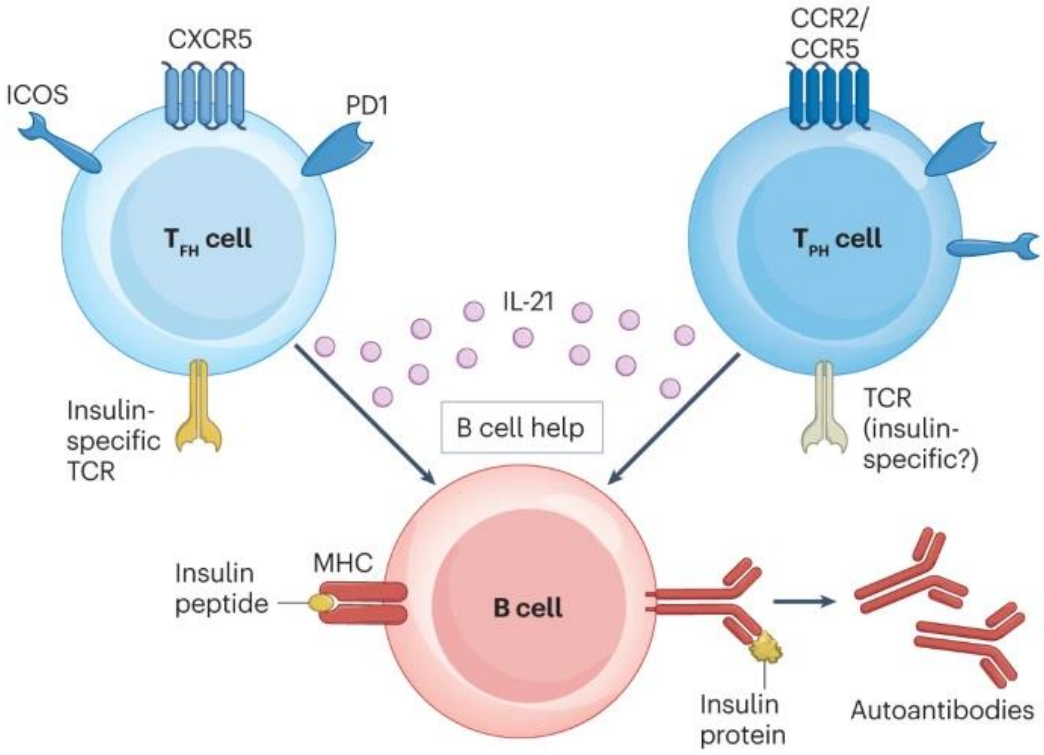

Figure 1-2. T cells along with B cells are linked to the pathogenesis of type 1 diabetes.

Increased levels of T cells with a follicular helper phenotype (TFH) and increased interleukin - 21 (IL-21) production by the T cells occurs in type 1 diabetes. This increases the response of insulin-specific antibodies. T cells with a peripheral helper phenotype (TPH) are also increased during the onset of T1D. Like TFH cells, TPH cells express an inducible T cell costimulator (ICOS) and another cytokine called Programmed death 1 (PD1) but lack the expression of CXCR5. They instead express chemokine receptors (CCR2 and CCR5) that are associated with migration to inflammatory sites. Together, the TFH and TPH cells mediate the presentation of insulin auto antibodies by B cells. Whether the T cell receptor (TCR) on TPH cells recognize T1D-associated antigens such as insulin remains unclear.

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1.1.2.2 Pathogenesis of type 2 diabetes

Genetic and environmental factors are involved in the onset of T2D. Dysfunction of the peroxisome proliferator-activated receptor – gamma (PPAR-γ) responsible for the regulation of inflammation and insulin sensitivity in the target cells, Akt and protein kinase C (PKC) signalling and insulin receptor substrate (IRS)-dependent phosphorylation is associated mainly with insulin resistance and T2D [2], [10]. As a result, glucose and fatty acids infiltrate the pancreas and cause damage to the pancreatic islets [1], [7]. Figure 1.3 details the signalling pathways involved in insulin secretion and the mechanisms that lead to its dysfunction.

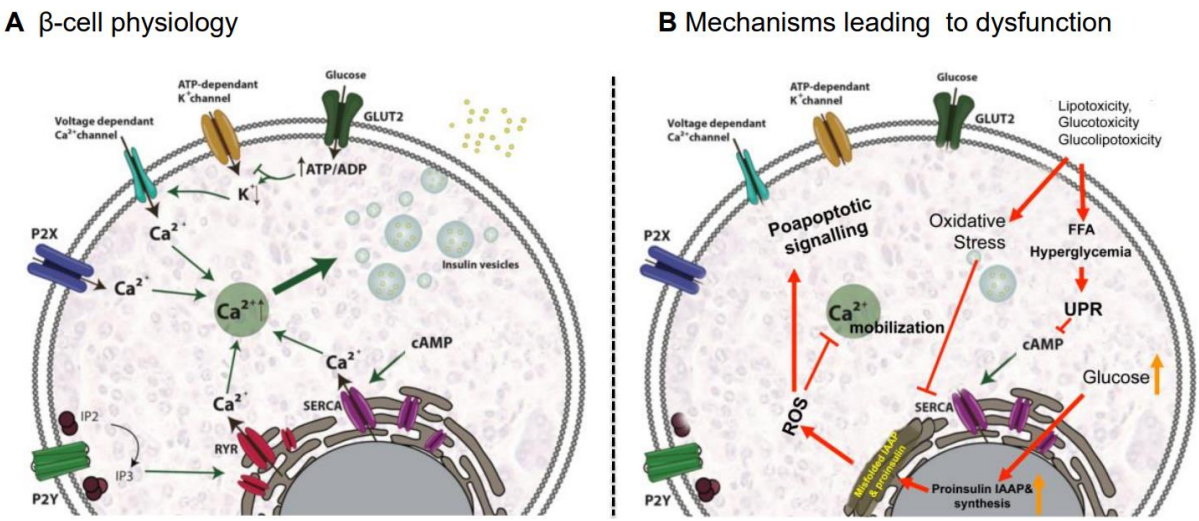

Figure 1-3. Signalling pathways involved in insulin secretion by β-cells.

Under healthy conditions (A) and mechanisms leading to its dysfunction in type 2 diabetes (B).

Insulin release is triggered by response to high glucose. Glucose is internalized mainly through the GLUT2 transporter. Glucose catabolism increases ATP/ADP ratio, closure of ATP-dependant potassium channels leading to membrane depolarization and opening of the voltage dependant Ca2+ channels. The latter enables Ca2+ influx triggering insulin exocytosis. Additional Ca2+ channels as P2X, P2Y, SERCA and RYR that contribute to Ca2+ mobilization and insulin secretion. (B) Hyperglycaemia and hyperlipidaemia increase oxidative stress leading to ROS generation inhibiting Ca2+ mobilization and activating pro-apoptotic signals. Increase in hyperglycaemia lead to the activation of the apoptotic unfolded protein response pathways and generation of ER stress. Sustained high glucose levels increase proinsulin and IAAP biosynthesis, which generate ROS. GLUT2: glucose transporter 2, ATP: Adenosine triphosphate, ADP: Adenosine diphosphate, Ca2+: Calcium, P2X: purinergic receptor X; P2Y: purinergic receptor Y; IP2: inositol 1,3-bisphosphate; IP3: inositol 1,4,5-trisphosphate; RYR: ryanodine receptor channel; SERCA: sarco-endoplasmic reticulum Ca2+-ATPase; FFA: free fatty acid, ROS: reactive oxygen species; UPR: unfolded protein response, cAMP: Cyclic adenosine monophosphate, IAAP: Islet amyloid polypeptide, ER: Endoplasmic reticulum.

Open access, reprinted from the International Journal of Molecular Sciences [12].

1.2 Microvascular complications due to diabetes

Although T1D and T2D have distinct disease pathophysiology, hyperglycaemia is a common hallmark. Tissue exposure to chronic hyperglycaemia is the central driving factor for microvascular complications in diabetes. Microvascular complications usually occur in tissues where glucose uptake is independent of insulin activity (e.g. kidneys, retina, and vascular endothelium). As a result, the prevailing state of hyperglycaemia results in endothelial damage due to oxidative stress, overproduction of superoxide, production of advanced glycation end products and production of sorbitol, leading to microvascular complications. Due to this, there is altered blood flow and pressure, changes in endothelial permeability and extracellular protein deposition and coagulation in blood vessels leading to organ dysfunction. Evidence in previous literature demonstrates a relationship between chronic hyperglycaemia and microvascular complications such as retinopathy, neuropathy and nephropathy [11]–[13].

This thesis involved patients with T1D neuropathy in a clinical study. Hence, neuropathy is discussed in detail in the below section.

1.3 Diabetic neuropathy

Diabetic neuropathy occurs due to damage to the nerves in the autonomic and peripheral nervous system and is presented in the form of sensory abnormalities. Distal symmetric polyneuropathy (DSPN) is the most common form of diabetic neuropathy. DSPN has a 'stocking and glove' distribution, meaning the hands and lower limbs are the most affected (Figure 1.4a). According to the Toronto Consensus Panel on Diabetic Neuropathy, DSPN is defined as a symmetrical, length-dependent sensorimotor polyneuropathy attributable to metabolic and microvessel alterations as a result of exposure to chronic hyperglycaemia and cardiovascular risk covariates [14]. Other forms of neuropathy are also common but usually occur secondary to diabetes. These other forms of neuropathy mainly affect the autonomic nervous system and lead to cardiac autonomic neuropathy, gastrointestinal dysmotility, diabetic cystopathy and impotence. Another form of neuropathy called focal neuropathy is less common and causes dysfunction of individual peripheral nerves and nerve roots, causing isolated mononeuropathy and radiculopathy [15].

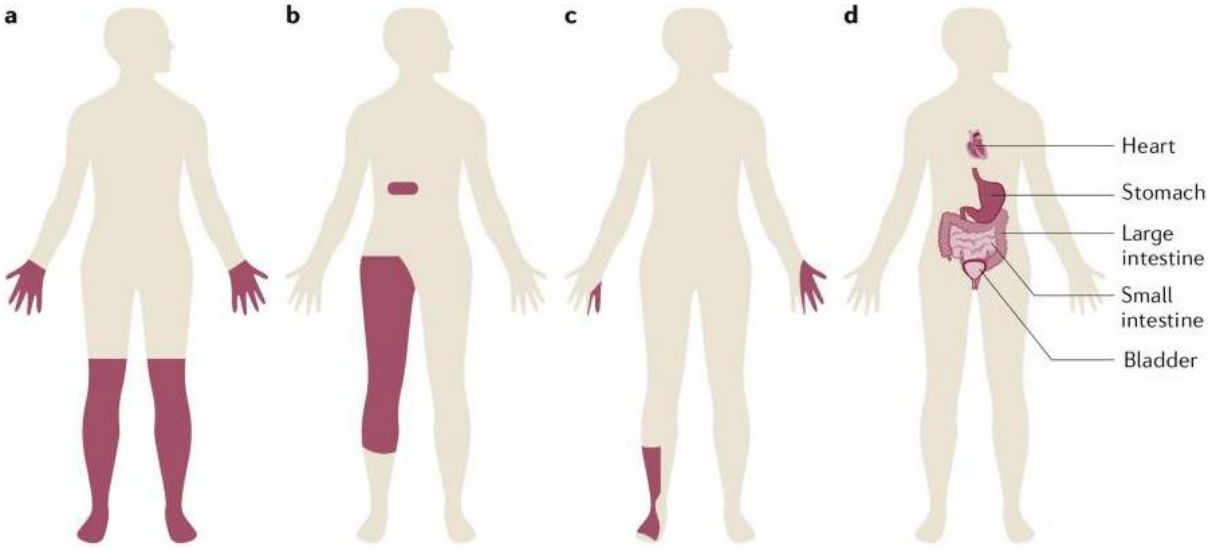

Figure 1-4. Patterns of nerve damage in diabetic neuropathy.

(a) Distal symmetric polyneuropathy (DSPN), (b) radiculopathy, (c) mononeuropathy and (d) autonomic neuropathy.

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1.4 Epidemiology of diabetic neuropathy

Diabetic neuropathy is a common diabetic complication that affects patients by increasing falls, causing pain and reducing their quality of life [15]. In the Rochester Diabetic Neuropathy cohort, neuropathy was reported in 59% of patients with T1D and 66% of patients with T2D. In this cohort, DSPN was the most common form of neuropathy reported in 50% of the entire cohort [16]. The common symptoms of neuropathy are sharp pain and loss of sensation to touch, temperature and vibration. Most of the patients grade the pain to be higher at night and describe the pain as an electric shock [17].

1.5 Mechanisms of action of diabetic neuropathy

In diabetic neuropathy, the peripheral nervous system is characteristic of degeneration in the distal and sensory nerve fibres, axonal loss and endoneurial microangiopathy [18]. Figure 1.5 shows an example of nerve fibre innervation into the epidermis in a healthy and diabetic patient.

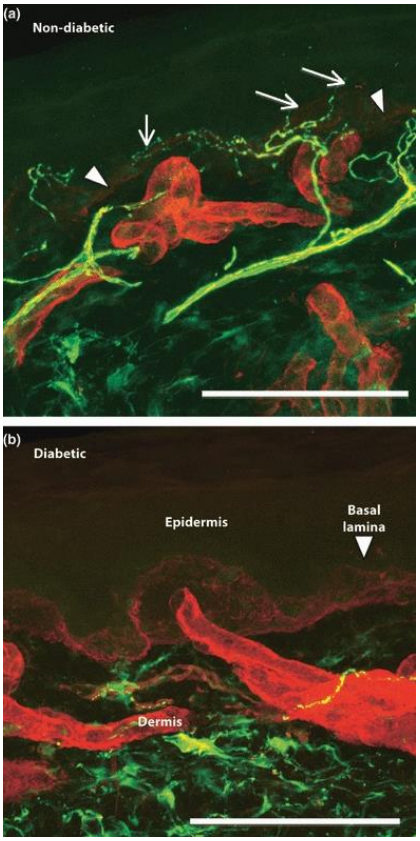

Figure 1-5. Epidermal nerve innervation in diabetes shown by immunostaining with protein gene product 9.5 (PGP9.5).

(a) In a normal subject (32-year-old man), small branching fibres (arrows) penetrate the basal lamina (arrowhead) derived from dermis and distribute diffusely ending in the surface of the epidermis of the skin. (b) In a diabetic subject (52-year-old woman with diabetes for 15 years) with symptomatic neuropathy, fibres in the epidermis are completely lost. Only a few fibres are sparsely left in the dermis and vascular systems also develop in the upper dermis (red colour of tortuous structure). Bar, 100 mm.

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The main biological pathways that are affected leading to the development of neuropathy are (i) polyol pathway, (ii) production of advanced glycation end products (AGEs) and (iii) activation of protein kinase C (PKC).

1.5.1 Polyol pathway

The increase in polyol flux dysregulated by the activation of aldose reductase (AR) is a metabolic cascade that contributes to the development of neuropathy. The mechanism of how the polyol pathway affects neuropathy is still not completely understood. Some studies propose the increase in polyol flux to cause intracellular hyperosmolarity by the accumulation of impermeable sorbitol in the cytoplasm, causing expansion of cells and cell death [19], [20]. Greene et al. suggest that the poor use of energy due to accumulation of sorbitol causes depletion of other osmolytes of myo-inositol, taurine and adenosine in the cytoplasm. This causes a poor production of adenosine triphosphate (ATP), leading to reduced sodium and potassium (Na,K)-ATPase activity and protein kinase C (PKC) activity, leading to damage in nerve fibres [21]. The polyol metabolic cascade is described in Figure 1.6.

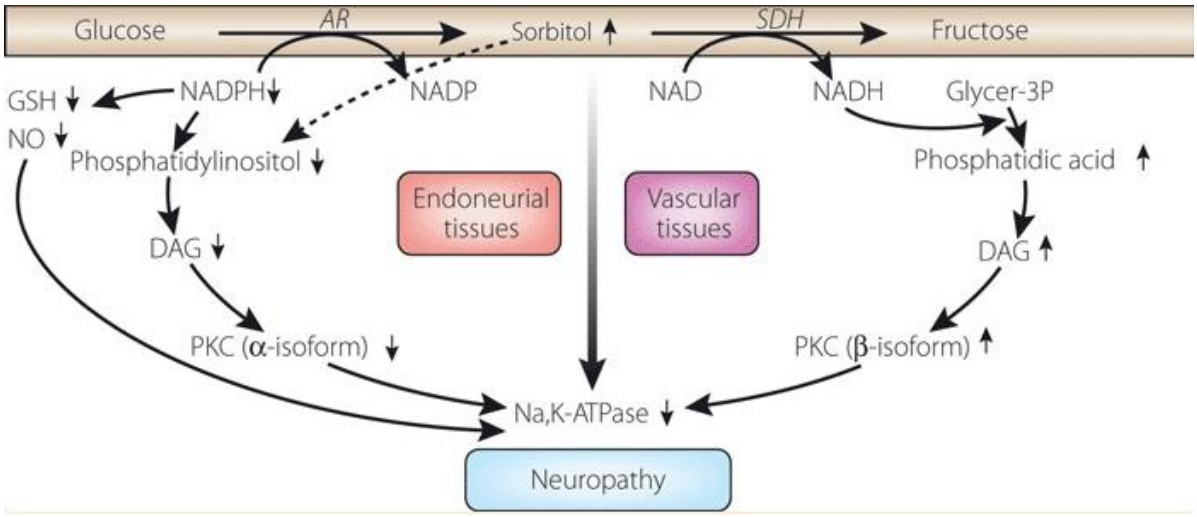

Figure 1-6. Polyol pathway and its metabolic cascade leading to diabetic neuropathy.

Hyperglycaemia in nerve tissues cause the conversion of glucose to sorbitol by aldose reductase (AR) and cause the depletion of reduced glutathione (GSH) and nitric oxide (NO) consequent from the overconsumption of nicotinamide adenine di-nucleotide phosphate (NADPH). As a result, intracellular myo-inositol is depleted to cause phosphatidylinositol (PI) depletion, which further suppresses diacylglycerol (DAG) production and protein kinase C (PKC) activity. As a result, Na,K-ATPase activity reduces resulting in functional and structural changes of neuropathy. In contrast, the second portion of the polyol pathway regulated by sorbitol dehydrogenase (SDH) is activated in the vascular wall in hyperglycaemic conditions. As a result of redox changes of NAD/NADH, conversion from glyceraldehyde-3-phosphate (Glycer-3P) to phosphatidic acid is promoted. The resulting enhanced synthesis of DAG results increases PKC activity resulting in structural changes of neuropathy. ATP: Adenosine triphosphate, Na,K ATPase: Sodium-Potassium-ATPase, NAD/NADH: Nicotinamide adenine dinucleotide/coenzyme of NAD,

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1.5.2 Production of advanced glycation end products

Advanced glycation end products (AGEs) are oxidative protein or lipid molecules which become glycated due to exposure to glucose and other sugars. This occurs in a non-enzymatic manner and can influence normal protein function. These AGEs deposit along nerve tissues and cause glycation of the basement membrane collagen and fibronectin and cause impairment of nerves [18]. The AGEs bind to their receptor for AGE (RAGE) in nerve tissues and cause diabetic neuropathy (Figure 1.7).

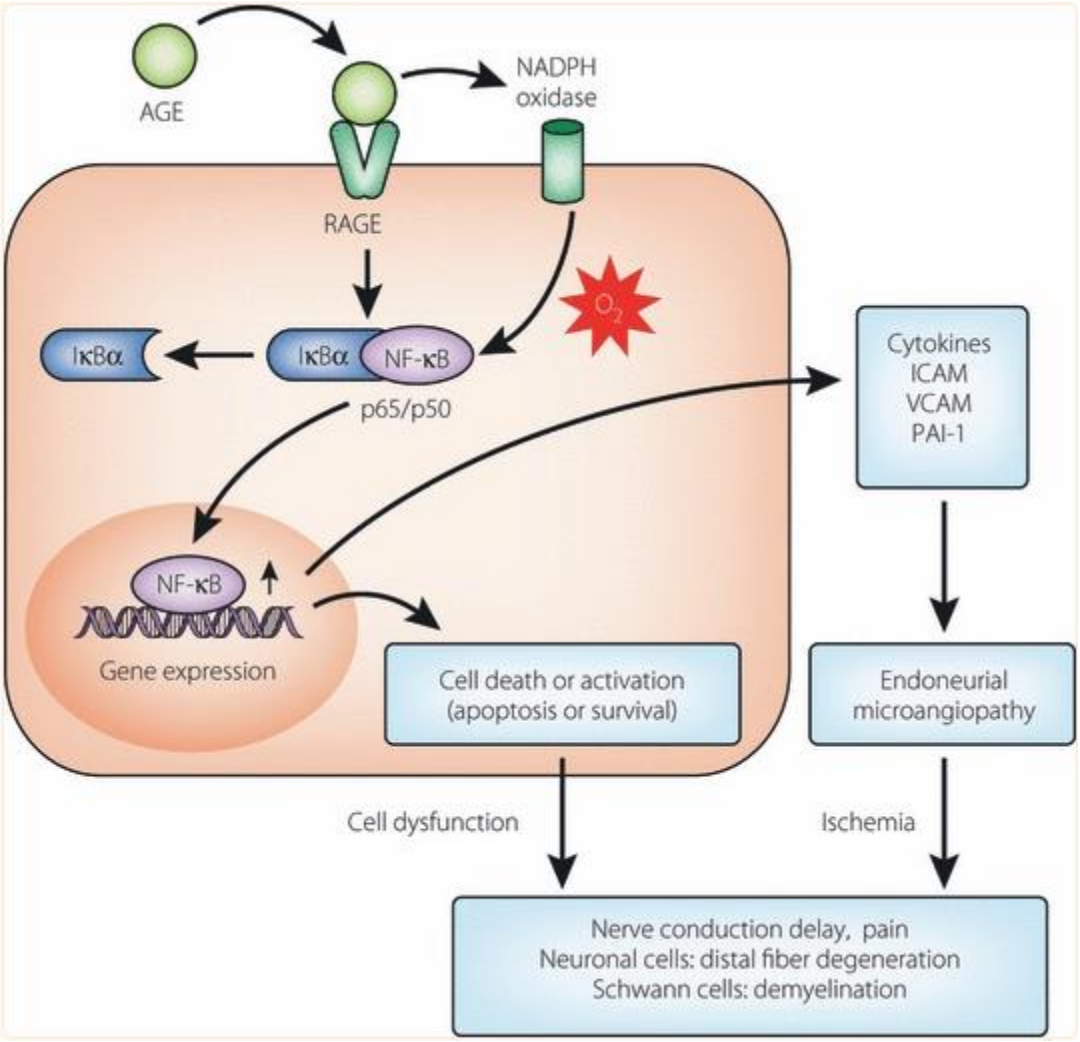

Figure 1-7. Advanced glycation end products (AGEs) and the receptor of AGE (RAGE) react together in the pathogenesis of diabetic neuropathy.

Nerve tissues, such as Schwann cells, nerve fibres and endothelial cells of vasa nervosum express RAGE. When AGEs bind to RAGE, oxidative stress is created through the activation of NADPH oxidase. Complexes of IκBα-nuclear factor (NF)-κB separate into fractions of IκBα and NFκB. NFκB translocates into the nucleus to activate genes related to cell death or survival. This results in microangiopathy and neural dysfunction. IκBα – Protein of the NFκB pathway, NADPH: Reduced form of Nicotinamide adenine dinucleotide phosphate, ICAM: Intercellular adhesion molecule, VCAM: Vascular cell adhesion molecule, PAI-1: Plasminogen activator inhibitor-1.

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1.5.3 Protein kinase C activation

Under high glucose conditions, Protein kinase C (PKC) activation occurs due to the increase in production of DAG (diacylglycerol) and an increase in oxidative stress (Figure 1.8). This leads to imbalances in the expression of certain signalling proteins, cell cycle factors, transcription factors, cytokines and functional enzymes (Figure 1.8) [18] causing complications including neuropathy. PKC is essential for normal nerve function. PKC contributes to neuropathy by altering blood flow and conduction velocity in the nerves [22].

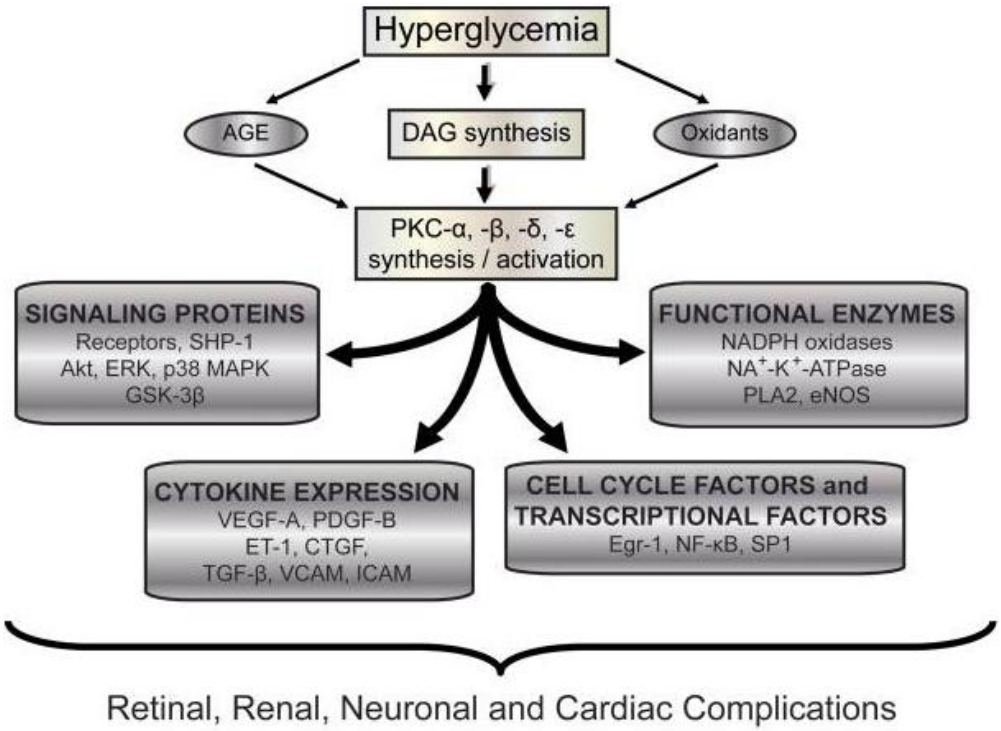

Figure 1-8. Representation of biological targets of protein kinase C isoform synthesis and activation due to hyperglycemia.

AGE: Advanced glycation end product, DAG: diglycerol, PKC: Protein kinase C, SHP-1: tyrosine phosphatase 1, Akt: Protein kinase B, ERK: extracellular signal-regulated kinases, MAPK: Mitogen-activated protein kinase, GSK-3: Glycogen synthase kinase 3, NADPH: Nicotinamide adenine dinucleotide phosphate, NA: Sodium, K: Potassium, ATP: adenosine triphosphate, PLA2: Phospholipase A2, eNOS: Endothelial nitric oxide synthase 3, VEGF-A: Vascular endothelial growth factor A , PDGF: Platelet-derived growth factor, ET-1: Endothelin 1, CTGF: Connective tissue growth factor, TGF: transforming growth factor, VCAM: Vascular cell adhesion molecule, ICAM: Intercellular cell adhesion molecule, Egr-1: Early growth response protein 1, NF-kB: Nuclear factor kappa B, SP1: Specificity protein 1.

Open access, Reprinted from the Journal of circulation Research [24].

1.6 MicroRNAs

MicroRNAs (miRNAs) are endogenous regulatory non-coding RNA sequences that help in regulation of post-transcriptional gene expression [23], [24]. They are short non-coding RNA sequences usually 21-24 nucleotides long [23]. These miRNAs are present within an intron or untranslated region (UTR) of a protein coding messenger RNA (mRNA). They recognise the 3'-UTR of their target mRNA by partial base-pairing and control gene expression [24], [25].

Apart from post-transcriptional gene regulation, miRNAs are also known to be involved in intercellular signalling [26].

lin-4 is believed to be the first miRNA discovered 30 years ago in Caenorhabditis elegans. This miRNA is believed to be involved in development. Since then, over 2000 miRNAs have been discovered in humans and it is believed that these miRNAs regulate about 1/3 of the human genome (Hammond, 2015). In humans, the miRNA family let-7 is the most conserved and plays an important role in development, stem-cell renewal, cell differentiation and tumour suppression [23], [27].

1.6.1 MicroRNA biogenesis: transcription, modification, and regulation

Biogenesis of miRNAs occurs in a canonical pathway. It begins with nuclear transcription of a long primary miRNA transcript (pri-miRNA) by RNA polymerase II [23]. The pri-miRNA undergoes maturation to form the miRNA using endonuclease enzymes. This process occurs in 2 steps (Figure 1.9). In the first step, pri-miRNA is transcribed in the nucleus and cleaved to form a precursor miRNA (pre-miRNA) by the enzyme Drosha and its binding protein DiGeorge critical region 8 (DGCR8). The pre-miRNA has a stem loop structure. The pre-miRNA is then released into the cytoplasm by exportin5 in a Ran-GTPase fashion [23], [24]. In the cytoplasm, Dicer (RNase III enzyme) along with its binding protein TAR RNA binding protein (TRBP) cleaves the step loop structure of the pre-miRNA to form a 21-24 nucleotide double stranded miRNA [23]. One strand of this mature miRNA is loaded into the RNA-induced silencing complex (RISC) for regulation of post transcriptional gene expression, and the other strand is degraded [24].

Regulation in miRNA biogenesis is maintained by Argounate-2 (Ago2), one of the critical proteins in RISC. Ago2 binds to the mature miRNA and guides the activity of RISC to its target mRNAs. This guidance takes place by partial base-pairing and plays an essential role during mRNA destabilisation, translation repression and downregulation of mRNA expression [23], [24]

Most of the miRNAs discovered till date are produced by the steps mentioned above, called the canonical pathway of miRNA biogenesis. Some miRNAs such as mitrons (present within the exon region of mRNA) undergo biogenesis using the non-canonical pathway. This non-canonical pathway is independent of the activity of Drosha and Dicer [28].

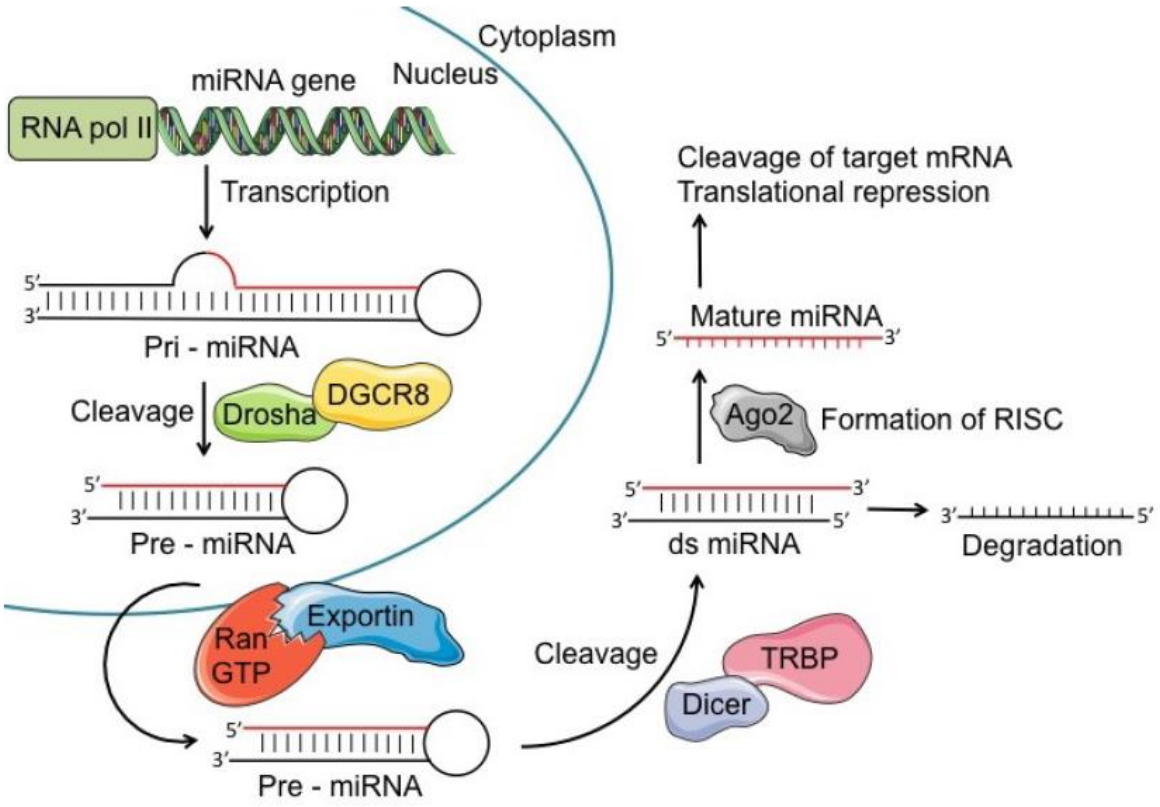

Figure 1-9. Schematic representation of miRNA biogenesis.

miRNA: microRNA, pri-miRNA: primary microRNA, pre-miRNA: precursor miRNA, RNA pol II: RNA polymerase II, Ago2: argonaute 2, dsmiRNA: double-stranded microRNA, RISC: RNA-induced silencing complex, DGCR8: DiGeorge critical region 8, TRBP: TAR RNA binding protein.

Adapted from [23] *and illustration made using Servier Medical ART: SMART.*

1.6.2 Biological role of microRNAs

MiRNAs mediate communication between different cells and signalling pathways involved in the body [25]. A single miRNA can regulate the expression of multiple target mRNAs and influence the expression of multiple gene regulatory networks [29], [30]. Previous literature demonstrates multiple miRNAs to regulate a single mRNA / gene and this interaction of multiple miRNAs is required to disrupt the expression of a single gene [31]. In this manner, the interaction between miRNAs and mRNAs are considered to be tightly regulated and dynamic [30]. The miRNA-mRNA interaction is also dependent on the cellular location of the miRNA, target mRNA, binding affinity of the mRNA and miRNA and miRNA abundancy. Some miRNAs are found freely circulating in body fluids, and some are found encapsulated within extracellular vesicles and exosomes and some are bound to membranes. This influences their mechanism of interaction with the target mRNA and miRNA silencing [24], [32].

Loss of a single step in miRNA biogenesis can result in developmental defects, loss of cellular action and even embryonic lethality [24]. Dysregulation in miRNA expression, as well as miRNA-mRNA interaction, leads to a disturbance in tissue homeostasis, tissue dysfunction and disease progression [23], [25]. This makes miRNAs an important regulator and modulator of cellular processes and instils the idea of their use as potential biomarkers for disease diagnosis and therapeutic agents [30] Since several miRNAs are found freely circulating in biofluids such as whole blood, saliva and urine, it makes them easily accessible and measurable in a non-invasive manner, further supporting their role as potential biomarkers [33].

Several miRNAs have been identified that regulate β-cell development and action, insulin resistance, glucose metabolism, bone remodelling and fracture risk. In this thesis, I am interested in identifying circulating miRNAs that are known to be important in diabetes and bone health and this will be discussed in detail in the sections to come.

1.7 Bone

Bones in the adult human skeleton comprise organic fibrous collagen matrix and inorganic alkaline mineral matrix. The organic collagen matrix comprises large structural proteins called type 1 collagen and smaller non-structural proteins called proteoglycans, glycosylated proteins and γ-carboxylated proteins [34]. Type 1 collagen makes up 90% of the total protein in bone and acts as the structural component of bone. In contrast, non-structural proteins control the metabolic and mineralisation activity of bone cells. The inorganic mineral matrix is composed of calcium and phosphorus which undergoes hydroxylation to hydroxyapatite as the bone mineralises. This hydroxyapatite mineral surrounds collagen fibres and provides strength and resistance to the bone. Additionally, matrix enzymes and water are present in the bone which helps to maintain the structure of type 1 collagen and control the uptake of ions and growth factors [34], [35].

The human skeleton is made up of 2 types of bone structures, cortical bone and trabecular bone. Cortical bone makes up 80% of the bone structures in the skeleton. It originates from the central lamellae of bone tissue called Haversian systems and surrounds the central blood vessels, creating a branch network within the bone. The bone thus formed is dense with a low surface area and mainly surrounds the marrow cavity. Trabecular bone makes up the remaining 20% of the bone structures in the skeleton and is less dense, spongy and has a higher surface area. Trabecular bone is a result of a trabeculae network separated by bone marrow spaces. It is mainly found in the centre of long bones, flat bones and the vertebra [34].

Bone provides (i) structural support to the skeleton, (ii) protection to organs, (iii) allows movement, acts as levers to the muscles and maintains mineral homeostasis, (iv) produces and stores blood cells, cytokines, hormones and growth factors and (V) creates a healthy hematopoietic niche within the bone marrow [35].

1.8 Bone cells

1.8.1 Osteoblasts and bone formation

Osteoblasts (bone-forming cells) originate from mesenchymal stromal cells in the bone marrow. They are cuboidal mononuclear cells responsible for bone formation and deposition of mineralised new bone collagen matrix [36], [37]. The transcription factors and cytokines that mediate cell proliferation, differentiation and maturation of osteoblasts are runt-related transcription factor 2 (runx2), transforming growth factor β (TGF-β), Wnt signalling, bone morphogenetic proteins (BMP), parathyroid hormone (PTH) and hedgehog signalling [38]–[40].

The progenitor cell committed to the osteoblast lineage expresses type 1 collagen and runx2. During the proliferation phase, it expresses bone-specific alkaline phosphatase (BAP). During the maturation phase, runx2 binds to the promoter of osteoblast-specific genes, turning on the genes of osteoblast maturation and formation of mature osteoblasts. Mature osteoblasts express osteocalcin (OC), type 1 collagen, BAP, osteopontin, osteonectin and proteoglycans [41]

1.8.2 Osteocytes

Terminally differentiated osteoblasts are referred to as osteocytes, and they express sclerostin, osteoprotegerin (OPG), receptor activator of NFκB Ligand (RANKL), macrophage colony-stimulating factor (M-CSF) and dentine matrix protein 1 (DMP1). Osteocytes are long-branched dendritic cells that remain deeply embedded in the mineralised bone matrix, forming a network with the nearby bone surface and lining cells [42], [43]. This dendritic network forms gap junctions and allows for cell-cell communications in the interstitial fluid [42].

The main role of osteocytes is to sense mechanical loading and stress created in the interstitial fluid via the dendritic network. The sensing of the mechanical loading acts as a stimulus which makes the osteocytes regulate bone formation by inhibiting the secretion of sclerostin and increasing Wnt signalling, promoting bone formation and osteoblast activity. During mechanical unloading, higher levels of sclerostin are secreted, inhibiting Wnt signalling and osteoblast formation [40], [44].

Osteocytes also help in the regulation of bone mineralisation and phosphate metabolism by secreting fibroblast growth factor 23 (FGF23) [42].

1.8.3 Regulation of bone formation

The most studied signalling pathway in regulation of bone formation and osteoblast differentiation is the Wnt signalling pathway. It regulates normal bone formation and homeostasis. The Wnt proteins (Wnt3a, Wnt5a, Wnt10b) are a family of 19 glycoproteins and they bind to their 7-transmembrane frizzled protein receptors and low-density lipoprotein receptor related proteins (LRP5 / LRP 6). This activates a signal to inhibit the activity of glycogen synthase kinase 3 beta (GSK3β) and thereby preventing the phosphorylation of β-catenin [45]–[47]. This allows β-catenin to remain in its stable and active form and increases the amount of β-catenin in the cytoplasm. The β-catenin then translocates into the nucleus where it binds to DNA-binding proteins and promotes the transcription of Wnt genes, activity of alkaline phosphatase and bone formation [47].

There are extracellular and intracellular proteins that can regulate and inhibit Wnt signalling. The most studied Wnt signalling inhibitors are sclerostin and dikkopf-related protein 1 (DKK1). These proteins can bind to the frizzled receptor and LRP5/6, thus inhibiting the binding of Wnt proteins and bone formation [44], [46], [48]

1.8.4 Osteoclasts and bone resorption

Osteoclasts (bone resorbing cells) are tissue specific multinuclear cells that originate from the CD14+ monocyte lineage. The osteoclasts are present as osteoclast precursor cells in circulation. Secretory proteins and cytokines such as macrophage colony-stimulating factor (M-CSF), tumour necrosis factor-α (TNF-α) and receptor activator of nuclear factor-ƙB ligand (RANKL) bind to their specific receptors on the osteoclast precursor cells and promote the differentiation and maturation of osteoclasts. The osteoclasts are usually present in the quiescent stage. They then migrate to the site of bone resorption via chemotactic factors, where they differentiate into mature osteoclasts and promote bone resorption [49], [50].

1.8.5 Regulation of bone resorption

The activation and maturation of osteoclasts is mediated by RANKL, osteoprotegerin (OPG), PTH, interleukins (IL-1/6), vitamin D and calcitonin [40], [50], [51].

The most studied signalling pathway in the regulation of bone resorption is RANKL/OPG signalling. RANKL and OPG are cytokines of the TNF superfamily. OPG is a secreted glycoprotein produced by the osteoblasts and stromal cells. OPG, a RANK like receptor molecule can act as a decoy and bind to RANKL thus inhibiting RANKL from binding to its receptor RANK on the surface of osteoclasts. This binding of OPG to RANKL inhibits osteoclast proliferation and differentiation and prevents bone resorption. In this manner, the

RANKL/OPG signalling pathway is important in regulating the amount of bone formed and resorbed; and determining bone mass and bone strength [51], [52].

1.9 Bone remodelling

The skeleton is metabolically active and undergoes continuous growth, modelling and remodelling to ensure good strength and structure of the bone and maintain mineral homeostasis. The process of bone remodelling in the adult skeleton is tightly regulated and occurs at a rate of 5-10% per year [53].

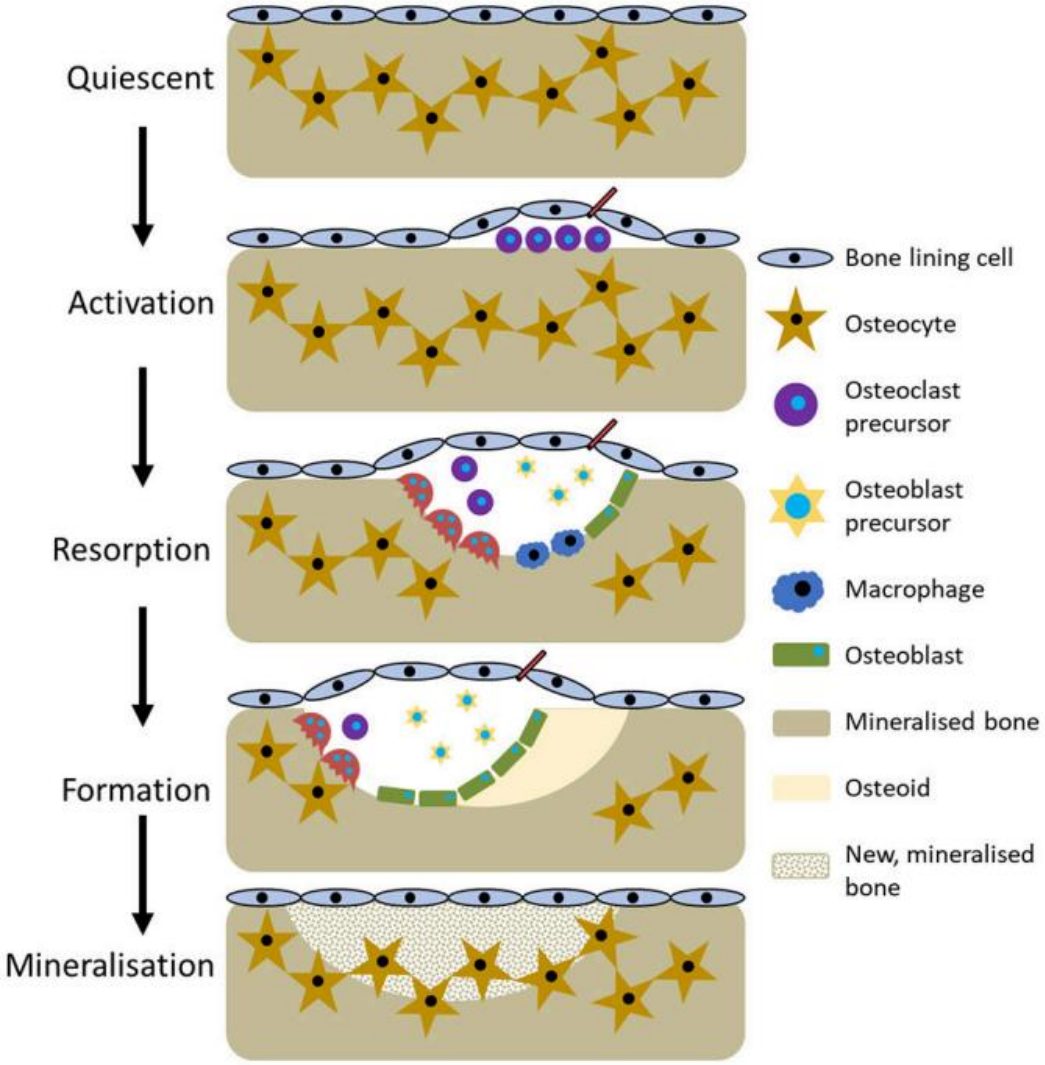

Figure 1-10. *Stages of bone remodelling in normal bone physiology*.

Open access, Frontiers in Bioengineering and Biotechnology [56].

Bone remodelling is triggered by microdamage and osteocyte apoptosis. During bone remodelling, the old and damaged mineralised bone is resorbed by osteoclasts and new bone is formed and mineralised by the osteoblasts. This process typically occurs in 5 highly regulated stages: (i) quiescence, (ii) activation, (iii) resorption, reversal/transition, (iv) formation and (v) mineralisation (Figure 1.10) [52], [54], [55]. The osteocytes first activate bone remodelling when they sense microdamage in bone and signal the quiescent bone lining cells to move from the bone surface and form the basic bone multinuclear units (BMUs). Chemotactic factors released from the site of bone damage activate the recruitment of osteoclast precursor cells from the monocyte lineage to the site of damage for resorption. In response to RANKL and M-CSF in circulation, the osteoclast precursor cells differentiate into mature osteoclasts. The mature osteoclasts secrete acids and specialised proteinases that degrade type 1 collagen and remove the quiescent bone lining cells before attaching to the bone surface. At this site of damage, bone resorption occurs, and once completed, the osteoclasts undergo apoptosis. The reversal phase is the transition from bone resorption to bone formation. The cavity in the matrix formed by the osteoclasts contains osteoclast precursor cells, which help form new bone. The osteoblasts proliferate and form new bone. They also secrete enzymes such as proteoglycans and pyrophosphates that help in the mineralisation of the new bone formed. This marks the end of the bone remodelling cycle [40], [52], [54], [56]. The activity of osteoclasts and osteoclasts is a coupled process activated in a tightly regulated sequence mediated via a strain gradient [40].

The rate of bone turnover in trabecular bone is high, as it is required for mineral metabolism, while the rate of bone turnover in cortical bone is low, which is needed to maintain bone strength [57].

Imbalances in the processes of bone formation and bone resorption leads to low bone mass and impaired bone strength. The low bone mass is characteristic of osteoporosis [58].

1.10 Osteoporosis

Osteoporosis is a disorder of low bone mass and low bone mineral density caused by deterioration of bone microstructure [59]. It predisposes a person to a fragile skeleton and increases the risk of low-impact fragility fractures [60]. Using the framework of the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) in 2019 and a systematic analysis approach, the authors reported that 37 million fragility fractures occur annually in individuals above 55 years, and 1 in 3 women and 1 in 5 men over 50 suffer from osteoporotic fractures [61]. Osteoporotic fractures are a public health concern due to their high healthcare costs, physical disability, increased mortality and reduced quality of life. Some of the risk factors of osteoporosis include ageing, gender, white ethnicity, family history of osteoporosis, lack of

physical activity and inadequate absorption of nutrition. There are also secondary causes of osteoporosis, such as primary hyperparathyroidism, vitamin D deficiency, renal disease, prolonged use of corticosteroids and hypogonadism [62]. There is growing evidence for hyperglycaemia and diabetes mellitus to harm bone [5].

Diagnosis of osteoporosis

According to the World Health Organisation (WHO), osteoporosis is diagnosed based on bone mineral density (BMD) T-scores. T-score describes a person's BMD in terms of the number of standard deviations (SD) by which it differs from the mean peak value of a young, healthy person of the same gender. The WHO uses a threshold of 2.5 SD below the mean of a young, healthy person as the criteria for the diagnosis of osteoporosis [63]. The BMD is measured using dual-energy X-ray absorptiometry (DXA) at the hip and spine, and this method is considered the gold standard for the diagnosis of osteoporosis [58].

1.11 Bone turnover markers

In addition to the measurement of BMD by DXA for the diagnosis of osteoporosis, abnormalities in bone turnover can also be measured using bone turnover markers (BTMs). BTMs are products secreted into circulation during the processes of bone formation and bone resorption. They reflect bone cell activity and help in understanding the state of skeletal turnover quantitatively [64]. Assessment of BTMs is helpful in the management of metabolic bone diseases such as postmenopausal osteoporosis and diabetes-induced bone fragility. Postmenopausal osteoporosis is characterised by increased osteoblast and osteoclast activity and increased bone loss and fracture risk [65] while in diabetes, the activity of bone formation and bone resorption are reduced, causing low/normal bone mass but an increase in fracture risk [5].

The commonly measured bone formation markers are the N-terminal propeptide of type I procollagen (PINP), osteocalcin (OC), and bone-specific alkaline phosphatase (BAP); and bone resorption markers are the C-terminal telopeptide of type I collagen (CTX) and tartrate-resistant acid phosphatase 5b (TRAcP5b). The BTMs can be measured in serum and plasma [64], [66].

1.12 Fracture risk in diabetes

Apart from microvascular complications such as retinopathy, neuropathy and nephropathy, the musculoskeletal system is also affected by long standing diabetes mellitus. Research to date provides evidence for increased bone fragility and osteoporotic fractures to be a severe complication of diabetes [5], [67]. In a recent systematic review and meta-analysis completed by Vilaca and colleagues, it has been reported that the relative risk of having an osteoporotic fracture at the hip is higher in a younger population (< 65 years old) of both type T1D and T2D [68].

T1D presents before peak bone mass is achieved, and research to date suggests that the absence of insulin in T1D is the main contributor to the observed low peak bone mass. As a result, these people with T1D are more susceptible to fractures [5], [69]. In T2D, the opposite is observed, where an increase in fractures and bone fragility is observed despite normal/high BMD.

The risk of fracture varies depending on the type of diabetes and the skeletal site affected. To date, many meta-analyses have reported an increase in fracture risk in people with diabetes. The findings of these different meta-analyses are reported in Table 1.1.

Table 1-1. Risk of hip and spine fractures in type 1 and type 2 diabetes according to meta-analyses.

			Hip fractures		Spine fractures	
Author, year	Population size	Age (years)	T1D	T2D	T1D	T2D
Vestergaard et al., 2007		43 - 73.5	6.94 (3.2– 14.78)*	1.38 (1.25–1.53)*		0.93 (0.63– 1.37)
Janghorbani et al., 2007	836,941	all ages	6.3 (2.6 - 15.1)*	1.7 (1.3 - 2.2)*		1.2 (0.7 - 2.2)
Fan et al., 2015	6,995,272	20-97	5.76 (3.66 – 9.07)*	1.34 (1.19-1.51)*		
Shah et al., 2015	4,391,425	> 20	3.78 (2.05-6.98)*		2.88 (1.71-4.82)*	
Vilaca et al., 2020	17,571,738	20 - 99	4.93 (3.06–7.95)*	1.33 (1.19–1.49)*		

**Statistically significant*

The risk of having a hip fracture in T1D ranged from 4 to 7 fold [68], [70]–[73] and from 1.33 to 1.7 fold in T2D [68], [71]–[73]. Shah et al. reported a 2.88 relative risk (RR) of having a vertebral fracture in T1D [73], however, no significant increase in the risk of vertebral fractures was reported by others [71], [72].

Janghorbani and co-authors evaluated fracture risks at skeletal sites apart from the hip and spine and reported a significant 1.3 RR of foot fractures in T2D [71]. The entire list is summarised in Table 1.2.

Table 1-2. Fracture risk in T2D reported by Janghorbani et al. 2007.

Site	Relative risk	95% CI
Hip	1.7 *	1.3 - 2.2
Any fractures	1.2 *	1.01 – 1.5
Foot	1.3 *	1.1 – 1.7
Distal forearm	0.98	0.8 - 1.2
Ankle	1.3	0.9 – 2.0
Proximal Humerus	1.3	0.8 – 2.2
Vertebra	1.2	0.7 – 1.2

**Statistically significant*

Vilaca and colleagues have also reported the RR of a non-vertebral fracture in T1D to be 1.92 and 1.19 in T2D. These results were significant [68].

1.13 Bone mineral density in diabetes

As described in Section 1.10, BMD measured by DXA is the gold standard for osteoporosis diagnosis and plays an important role in fracture risk evaluation. Usually, an inverse association is observed between BMD and fracture risk, and such is observed in T1D [72]. However, in T2D, irrespective of the observed normal / increase in BMD, the type 2 diabetic population is still at an increased risk for fractures. In contrast to results by Vestergaard et al., Vilaca et al. have reported a 1.33 RR of a hip fracture in T2D [68]. Results from the meta-analysis by Vestergaard et al. reflect the above (Table 1.3).

Table 1-3. BMD Z-score and fracture risk reported by Vestergaard et al. 2007.

Diabetes type	**Spine Z-score**	**Hip Z-score**
T1D	-0.22 ± 0.01*	-0.37 ± 0.16*
T2D	0.41 ± 0.01*	0.27 ± 0.01*
Expected relative fracture risk	**Any fracture**	**Hip fracture**
T1D	1.09	1.42
T2D	0.85	0.77

**Statistically significant*

Shwartz et al. have reported the association between BMD and fracture risk in T2D. For a given BMD T-score and age, patients with T2D had a higher risk of fracture when compared to those without diabetes. However, for a given fracture risk, patients with T2D had a higher BMD T-score when compared to patients without diabetes. This suggests that, in T2D, BMD underestimates the true fracture risk [74].

1.14 Bone microarchitecture in diabetes

1.14.1 Bone microarchitecture in type 1 diabetes

High-resolution peripheral quantitative tomography (HR-pQCT) is a volumetric bone imaging system that helps in imaging cortical and trabecular bone at the structural level, such as its microarchitecture. The summary of HR-pQCT findings in T1D are summarised in Table 1.4.

Research till date presents structural changes in bone microarchitecture at the radius and tibia in T1D. However, the findings are inconsistent. Research by Vilaca et al. and Shanbhogue et al. suggests no changes in bone microarchitecture in T1D without microvascular complications when compared to controls [75], [76]. However, research by Walle et al. and Rassmussen et al. suggests that there are changes in bone microarchitecture in T1D but if the T1D population studied includes those with microvascular complications is not specified [77], [78] A meta-analysis by Walle et al. identified adverse structural characteristics in trabecular bone in T1D at the radius, which is a non-weight-bearing site [78].

Table 1-4. Summary of findings on bone microarchitecture in T1D.

		Trabecular bone		Cortical bone	
Author, year	Population	Radius	Tibia	Radius	Tibia
Shanbhogue et al., 2015	26 T1D MVD+ 26 HC	low vBMD	low vBMD	low vBMD low Ct. Th.	
Shanbhogue et al., 2015	26 T1D MVD+ 29 T1D MVD-	low vBMD (18%) low Tb. Th. (12%)	low vBMD (20%) low Tb. Th.(14%) High Tb. Sp. (11%) high Tb.1/N.SD (16%)		
Vilaca et al., 2021	20 T1DN+ 20 T1DN-				high Ct. Po. (56%)
Walle et al., 2022	227 T1D 405 HC	low vBMD low Tb. N. high Tb.1/N.SD			
Rassmussen et al., 2023	109 T1D 160 HC			high vBMD high Ct. Th. low Ct. Po.	high vBMD

T1D = type 1 diabetes, MVD = microvascular disease, N = neuropathy, HC = healthy controls, vBMD = volumetric BMD, Tb. N. = trabecular number, Tb.1/N.SD = inhomogeneity of the trabecular network, Ct. Th. = cortical thickness, Ct. Po. = cortical porosity.

1.14.2 Bone microarchitecture in type 2 diabetes

The findings on bone microarchitecture in T2D are summarised in Table 1.5.

In trabecular bone in T2D, the most common finding was high vBMD at the radius and tibia [77]–[79] and high trabecular number (Tb. N.) at the radius [77], [78].

In cortical bone in T2D, the most common finding was high cortical porosity (Ct. Po.). Three studies reported high cortical porosity at the radius [78]–[80] and one study reported high

cortical porosity at the tibia [81]. A meta-analysis by Walle et al. reported the cortical bone in T2D to have more adverse structural characteristics compared to trabecular bone [78].

Table 1-5. Summary of findings on bone microarchitecture in T2D.

		Trabecular bone		Cortical bone	
Author, year	Population	Radius	Tibia	Radius	Tibia
Burghardt et al., 2010	19 T2D 19 HC		high vBMD adjacent to the cortex high Tb. Th	high Ct. Po.	
Shanbhogue et al., 2016	25 T2D MVD 26 HC			low vBMD low Ct. Th. High Ct. Po.	
Farr. et al., 2016	30 T2D 30 PM HC	no difference after adjsuting for covariates		no difference after adjsuting for covariates	
Samelson et al., 2018	129 T"D 940 HC				low vBMD high Ct. Po.
Walle et al., 2022	516 T2D 3067 HC	low Tb.1/N.SD high vBMD high Tb. N.	high vBMD	high Ct. Th. high Ct. Po.	high Ct. Th.
Rassmussen et al., 2023		high vBMD high Tb. N. low Tb. Sp.	high vBMD low Tb. Sp.	high vBMD high Ct. Th.	high vBMD high Ct. Th.

T2D = type 1 diabetes, MVD = microvascular disease, HC = healthy controls, PM = postmenopausal, vBMD = volumetric BMD, Tb. N. = trabecular number, Tb.1/N.SD = inhomogeneity of the trabecular network, Tb. Sp. = trabecular separation, Tb. Th. = trabecular thickness, Ct. Th. = cortical thickness, Ct. Po. = cortical porosity.

In a population of T2D with and without fractures, Samelson et al. have reported a decrease in Ct. Po. at the radius in those with a previous fracture compared to those without fractures. Interestingly, other parameters of cortical bone were also worse in T2D with a previous fracture (lower cortical vBMD, lower cortical thickness at the radius) [81].

1.15 Bone strength in diabetes

Bone strength is assessed by measuring estimated failure load and stiffness using finite element analysis. In T1D, Shanbhogue et al. have reported lower bone strength at the radius and tibia in patients with and without MVD [75]. However, Vilaca et al. assessed bone strength in a population of T1D and neuropathy and found no differences [76]. No studies have reported a difference in bone strength at the standard measurement site in T2D.

In a population of individuals with and without previous fractures, Samelson et al. have reported failure load to be lower at the radius and tibia in those with T2D (T2D with fractures vs T2D without fractures) and heathy controls, and also lower failure load at the tibia in the control population with a previous fracture compared to those without a previous fracture [81].

1.16 Bone turnover in diabetes

Diabetes, both T1D and T2D are characteristic of low bone turnover. The processes of bone formation and bone resorption in diabetes are dissociated, creating an imbalance in bone remodelling. This is reflected in the measurement of BTMs.

Several studies have reported a decline in bone turnover markers in diabetes [76], [80], [82]–[85]. A systematic review by Starup-Linde et al. reported OC to be decreased in T1D and T2D and alkaline phosphatase to be increased in T2D, suggesting a state of hyper mineralised bone [85]. A meta-analysis by the above team reported PINP, OC and IGF-1 to be lower in diabetic people with fractures compared to those without a previous fracture and sclerostin and BAP to be higher in diabetic people with fractures than those without a previous fracture. The authors found no difference in CTX between the groups [86]. Other authors have reported CTX to be lower in T1D and T2D compared to healthy people [76], [83]. Moreover, Vilaca et al. have reported CTX to be lower in T1D and neuropathy compared to a healthy control group.

Although there are some conflicting results in CTX, in general literature, there is strong evidence that BTMs are low in diabetes, suggesting a state of low bone turnover.

1.17 Effect of prolonged duration of diabetes on bone health

Limited evidence is available in literature on the effect of prolonged duration of diabetes on bone health.

Longitudinal studies on bone turnover and BMD in T1D have found no changes in bone turnover, and BMD after a mean follow up period of 2 years [87] and 10 years [88]. A study on 7 participants with T1D has reported a 0.5% decrease in bone mineral content in the trabecular compartment in insulin dependent diabetic patients after a follow up time of ~11 years [89].

In a five-year longitudinal study in T2D in those with and without a history of previous fractures, at baseline, higher cortical porosity (66.3%) and cortical porosity volume (71.9%) was observed at the tibia in the population of T2D with fractures when compared to those without fractures. Longitudinally, cortical porosity increased significantly over time at similar annual rates across the two groups. The population of T2D with fractures also had a higher annual decline in bone strength (4.7x and 6.7x greater decrease in failure load and stiffness respectively, when compared to T2D without fractures; and 14.1x and 22.2x greater decrease in failure load and stiffness when compared to a control group without diabetes and previous fractures) [90].

1.18 Cellular, molecular and structural mechanisms of diabetic bone disease

Evidence collected thus far suggests that apart from BMD and bone microarchitecture, there may be other changes in the bone matrix and microenvironment at the cellular and molecular level that may contribute to the increase in fracture risk in diabetes.

This section (Figure 1.11) describes the cellular and molecular mechanisms involved in the pathogenesis of diabetic bone disease.

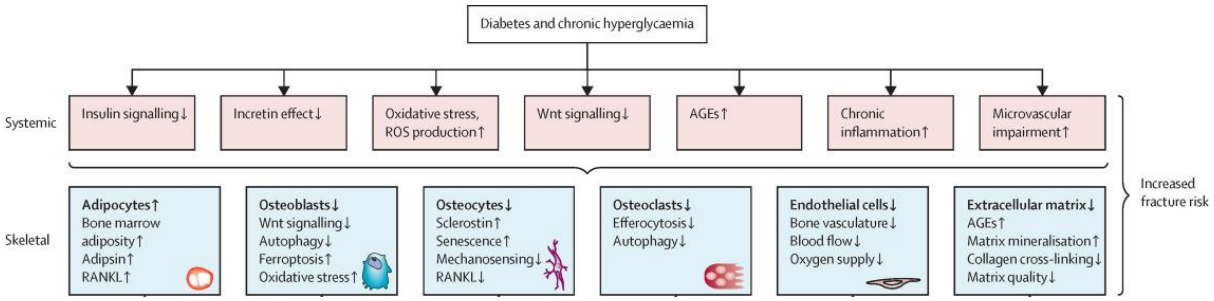

Figure 1-11. Cellular and molecular mechanisms contributing to bone fragility in diabetes.

The systemic signals and processes altered due to diabetes and chronic hyperglycaemia are mentioned in the top panel. Altered gene expression and cellular activity in the bone microenvironment are mentioned in the below panels [5].

ACE: Angiotensin-converting enzyme. AGE: Advanced glycation end product, PHD2: Prolyl hydroxylase domain protein 2, RAGE: Receptor for advanced glycation end products, RANKL: Receptor activator of NF-κB ligand, ROS=Reactive oxygen species.

Reprinted from The Lancet Diabetes & Endocrinology [5], with permission from Elsevier.

Accumulation of AGEs in the bone matrix: Diabetes and chronic hyperglycaemia increase the non-enzymatic binding of glucose to bone proteins (type 1 collagen), forming non-specific collagen crosslinks called AGEs. With the addition of renal impairment, which is a common diabetic complication, the degradation and clearance of AGEs and lipids is limited. As a result, the increase in AGEs and lipids deposits on the surface of the bone matrix impairs bone cell biology and makes the bone stiffer. The AGEs also contribute to oxidative stress to the bone microenvironment and make the bone more fragile [5].

Valcourt et al. have shown that an increase in pentosidine (a biomarker of AGEs) is negatively associated with osteoclast differentiation and activity. There is also evidence that AGEs suppress osteoblast activity, supporting low bone turnover in diabetes [91], [92].

In T2D, increased carboxymethyl-lysine (CML) (a biomarker of AGEs) has been reported compared to controls [93].

Osteocyte senescence and accumulation of microdamage: Due to the prolonged duration of hyperglycaemia and diabetes, osteocytes develop a phenotype associated with senescence and secrete pro-inflammatory cytokines, which impair their ability to control bone remodelling. It also accelerates osteocyte ageing. As a result, microdamage sensing by the osteocytes reduces, leading to more damage in the osteocyte canalicular network and a higher rate of osteocyte apoptosis. These structural alterations to the bone reduce its biomechanical properties, making it weak and more susceptible to fractures [5], [94]. In T1D, Kolibova et al. have shown signs of accelerated osteocyte apoptosis in the endosteal and periosteal side of human cortical bone; and local mineralisation of the osteocyte lacunae in the periosteal side of human cortical bone. Their results suggest increased micropetrosis and microdamage accumulation to impair bone remodelling (Figure 1.12) [94], [95].

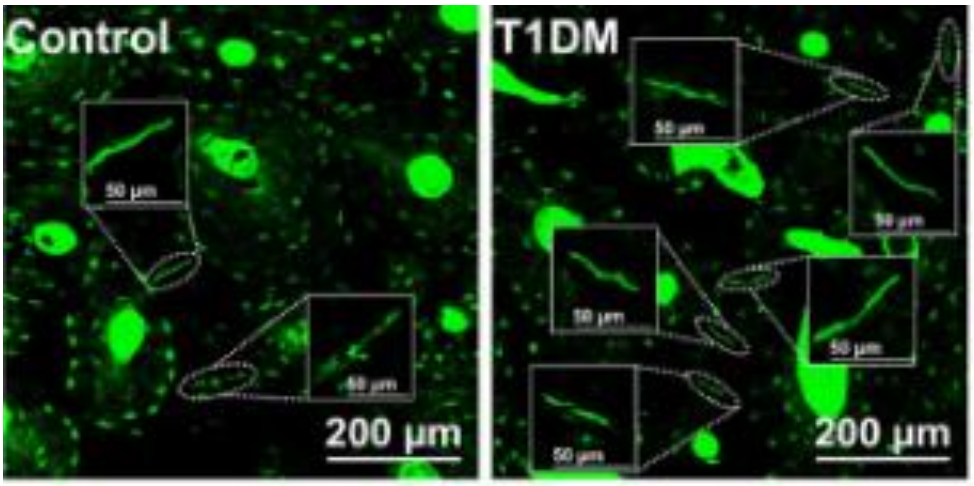

Figure 1-12. Increased accumulation of microcracks in the periosteal region of human cortical bone in type 1 diabetes when compared to a control group.

Microcrack accumulation has been visualised by the amount of infiltration of rhodamine-6G into the bone specimens and visualised by confocal laser scanning microscopy. Left = control, right = type 1 diabetes.

Open access, Journal of Acta Biomaterialia by Elsevier [95].

Increase in sclerostin production: Sclerostin is a Wnt antagonist produced by osteocytes. The increase in sclerostin by the osteocytes inhibits the Wnt signalling pathway important for bone formation and osteoblast activity. As a result, bone formation is reduced, creating a state of low bone turnover [96].

The early onset of diabetes (especially T1D, which occurs before peak bone mass is attained) results in abnormal production of insulin and insulin-like growth factor 1 (IGF-1). This affects the Wnt signalling pathway and osteoblast differentiation. The bone microenvironment is pushed into a state of oxidative stress, further inhibiting osteoblast differentiation. As a result, sclerostin increases and osteoblasts undergo ferroptosis (iron-dependent cell death) [44], [96].

Adipocytes in bone: Hyperglycaemia compromises osteoblast differentiation and promotes the differentiation of osteoblast progenitor cells to adipocytes, resulting in an increase in adipose tissue in the bone marrow and visceral regions. These adipocytes secrete pro-inflammatory cytokines (IL-1β and IL-18) and RANKL, which promotes osteoclast activity, high bone turnover and subsequently resulting in low bone formation in diabetes [5], [67].

Cortical porosity: Poor glucose control in diabetes impairs vasculature and vascular permeability and reduces blood flow to the bone. This results in microvascular hypertension and an increase in vascular permeability, narrowing of blood vessels, and lumen occlusion [5]. Further, a state of hypoxia is induced in the bone, which uncouples the process of bone remodelling in cortical bone. As a result, bone formation and microdamage repair decrease, and the cortical bone undergoes structural and morphological changes, which increase its porosity, making the bone more fragile [5], [76], [97].

Changes in bone microarchitecture have been reported in diabetes with microvascular complications but not in diabetic patients without microvascular complications. Vilaca et al. have reported cortical porosity at the tibia to be 56% higher in T1D with neuropathy when compared to patients with type 1 diabetes only. This increase in cortical porosity correlated with the severity of neuropathy [76].

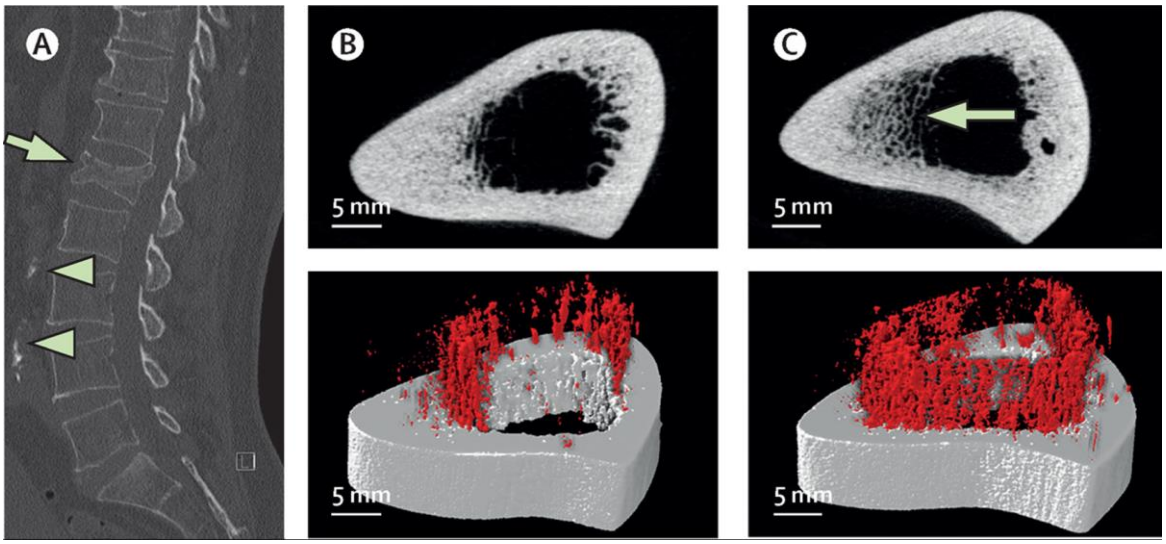

Figure 1-13. Vertebral fracture and poor bone microarchitecture in a person with type 2 diabetes.

(a) Image of a spine CT scan showing a fracture in the L1 vertebral region (arrow) in a 54-year-old man with poorly controlled type 2 diabetes, (BMD T score = -1.3). Arrow heads mark vascular calcification. (b) HR-pQCT scan of mid-diaphyseal tibial cross-section in person with long standing type 2 diabetes, (c) HR-pQCT scan of mid-diaphyseal tibial cross-section in person without diabetes arrow showing trabecularisation of cortical bone compartment. Red shows the amount of void spaces and increased porosity in cortical bone compartment.

CT: computed tomography, BMD: bone mineral density, HR-pQCT: High resolution peripheral quantitative computed tomography.

Reprinted from The Lancet Diabetes & Endocrinology [5] with permission from Elsevier.

1.19 MicroRNAs in diabetic bone disease

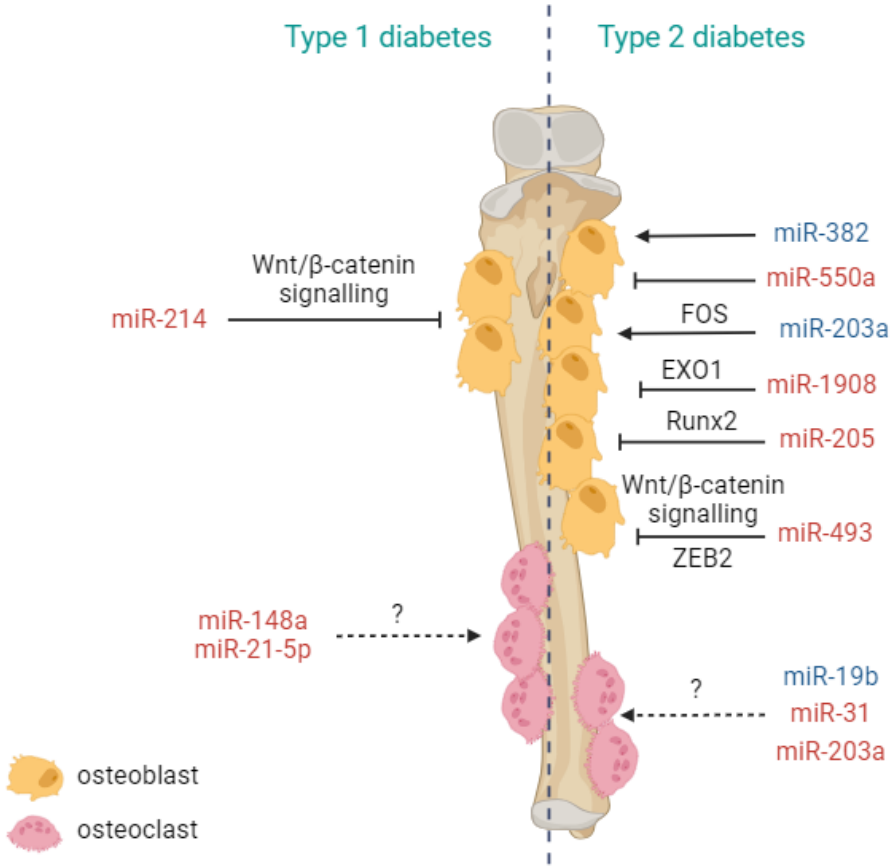

Figure 1-14. MicroRNAs of importance in type 1 and type 2 diabetic bone disease.

FOS: Fos proto-oncogene, EXO1: Exonuclease 1, Runx2: Runt-related transcription factor 2, ZEB2: zinc finger E-box-binding homeobox 2.

Adapted from [33]. *Illustration made using BioRender.*

1.19.1 MicroRNAs in type 1 diabetes and bone health

At present, only 2 research groups have reported on miRNAs in T1D and bone health.

Grieco and co-authors studied the serum expression of 6 miRNAs, miR-21, miR-24, miR-27a, miR-148a, miR-214 and miR-375, known to have a role in bone health (chosen from literature search) in 15 patients with T1D and 14 non-diabetic subjects [25]. They reported an increase in expression of miR-21 and miR-148a in patients with T1D when compared to the non-diabetic subjects. Although the authors found no correlation between these two miRNAs and metabolic parameters such as BMI and HbA1c, significant correlations were observed between these 2 miRNAs and parameters of bone metabolism. miR-148a was reported to be negatively associated with total body BMD (r = 0.37) and positively associated with PTH (r = 0.39); while miR-21 was reported to be negatively associated with bone mineral content at the femur (r = 0.37). Target gene prediction analysis for these 2 miRNAs revealed their possible

action in bone metabolism by regulating the FoxO signalling pathway and TGF-β signalling pathway. The identified validated gene targets for miR-21 and miR-148a and their role in bone metabolism are listed in Table 6.

Table 1-6. Validated target genes of miR-148a and miR-21 and their role in bone metabolism. [25].

Target miRNA	Gene	Function
miR-148a	Transforming growth factor, β 2 (TGFβ2)	Stimulates osteoblast differentiation and proliferation and bone formation
	Insulin-like growth factor 1 (IGF-1, somatomedin C)	Stimulates bone mineralization and positively regulates osteoblast differentiation
	Growth arrest and DNA-damage-inducible, α (GADD45α)	Positively regulates apoptosis
miR-21	Signal transducer and activator of transcription 3 (STAT 3)	Specifically inactivates osteoblast/osteocytes
	Transforming Growth Factor β Receptor 1 (TGFβR1)	Regulates bone resorption
	Insulin-like growth factor 1 receptor (IGF1R)	Involved in bone remodeling
	B-Cell lymphoma 6 (BCL 6)	Regulates osteoclast differentiation
	Transforming growth factor-β 2 (TGFβ 2)	Involved in osteoblast differentiation and bone remodeling
	Programmed Cell Death 4 (PDCD 4)	Involved in osteoclastogenesis through cFos regulation
	Superoxide dismutase 2 (SOD 2)	Controls osteoclastogenesis and maintains osteoblast differentiation
	Forkhead box O-3 (FOXO3)	Its overexpression increases bone resorption
	Ubiquitin-specific protease 7 (USP 7)	Involved in osteogenic differentiation
	Phosphatase and tensin homolog (PTEN)	Involved in adiponectin-induced osteogenesis
	Interleukin 10 (IL-10)	Inhibits bone resorption

Wang and colleagues have reported an upregulation in expression of miR-214 in bone specimen and bone marrow mesenchymal stromal cells (BMSCs) from patients with T1D when compared to healthy controls [98]. Their molecular studies reported an upregulation in miR-214-3p to inhibit osteogenic differentiation of BMSCs via β-catenin, an important protein downstream of the Wnt signalling pathway [98]. Of note, Grieco et al. did not find a significant upregulation in expression of miR-214 in serum in their study [25].

1.19.2 MicroRNAs in type 2 diabetes and bone health

Similarly, for T2D, limited evidence is available on miRNAs in T2D and bone disease.

A qPCR array analysis by Heilmeier et al. identified 48 miRNAs to be differentially expressed in T2D population with and without previous fractures. Upon validation by qPCR, they reported

miR-550a and miR-382 to be promising circulating miRNA biomarkers of T2D bone disease. Molecular analysis identified miR-382 to enhance osteogenic differentiation and impair differentiation of adipocytes, while miR-550a inhibited osteogenic differentiation and impaired adipogenic differentiation [30].

Another study by the same authors in an Icelandic Age, Gene/Environment Susceptibility cohort, studied the expression of 10 miRNAs (miR-550a, miR-203a, miR-19b, miR-942, miR-500a, miR-181c, miR-7, miR-96, miR-31, miR-382, chosen from a Pubmed Literature search) in a T2D population. Their results revealed miR-19b, miR-203a and miR-31 to be associated with incident fractures in T2D. The association with incident fractures was BMD dependent in the case of miR-19b and BMD independent in the case of miR-31 and miR-203a. On modelling the expression of all 3 miRNAs together, they reported a good diagnostic accuracy (AUC = 0.72) for incident fracture risk, and the diagnostic accuracy was higher when compared to fracture prediction using BMD alone [99]. Of note, in this population, the authors were unable to replicate their findings on miR-550a as reported above.

Stearoyl-coenzyme A desaturase (SCD1) is important in regulating β-cell response to physiological stress and production of saturated fatty acids. In relation to bone, SCD1 is important in regulating osteogenesis. Chen et al. have studied the role of SCD1 in diabetic bone disease in postmenopausal women with fractures and T2D and also if miRNAs mediate the role of SCD1. They reported the expression of SCD1 to be downregulated in BMSCs of T2D postmenopausal women with low trauma fractures compared to those with high trauma fractures. Further, SCD1 regulated the expression of miR-203a and miR-1908. The functional analysis reported SCD1 to inhibit the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A) and Fos proto-oncogene (FOS), and promote the expression of exonuclease 1 (EXO1) and (phospholipid scramblase family member (PLS1). miR-1908 was identified as a regulator of EXO1 expression, and miR-203a was identified as a regulator of FOS expression, suggesting SCD1 to control osteogenesis via a miR-203a/FOS and miR-1908/EXO1 regulatory pathway in T2D [100]. Further, SCD1 could be a beneficial treatment to reduce high fracture risk in T2D.

Zhang et al. have reported the expression of miR-205 to be increased in women with T2D and osteoporosis compared to healthy women. Although in mice, through functional analysis they report miR-205 to inhibit osteogenesis and promote adipogenesis via runx2 [101].

The Wnt/β-catenin signalling pathway is important for osteogenesis and bone remodelling. Zhai and co-authors have reported R-SMAD zinc finger binding protein 2 (ZEB2) as a downstream target of miR-493, which activates Wnt/β-catenin and promotes osteoblast formation. In the context of T2D and osteoporosis, they found increased levels of miR-493-5p

in BMSCs in high glucose conditions compared to those in low glucose conditions. This suggests that under hyperglycaemic conditions, miR-493 inhibits Wnt/β-catenin signalling, suppressing osteogenesis [102].

1.20 Perspective of microRNAs as biomarkers for diabetic bone disease

Evidence in the above section suggests that miRNAs mediate key roles in Wnt signalling, bone remodelling, and osteoblast and osteoclast differentiation in diabetic conditions. This suggests that miRNAs have the potential to act as promising biomarkers for diabetic bone disease. Importantly, miRNAs are highly stable, can be accessed in a non-invasive manner in body fluids such as blood and urine, and are easily detectable, making them suitable biomarkers [33], [103].

TAmiRNA have developed a panel of miRNAs for fracture risk prediction called the 'OsteomiR panel'. The 'OsteomiR panel' contains 19 microRNAs likely to play a supportive role in bone, and it uses an OsteomiR score to predict fracture risk. A higher OsteomiR score was associated with a lower BMD T-score. Moreover, the OsteomiR score predicted more incident fracture events compared to the Fracture Risk Assessment Tool (FRAX) 10-year probability score [104]. This supports developing a panel of miRNAs related to diabetes and bone health for better fracture risk prediction in diabetes and diagnosis of fractures solely based on the BMD T-score currently being used.

1.21 Justification for this research

Both T1D and T2D are associated with increased fracture risk. However, BMD underestimates true fracture risk in T1D and T2D. This calls for the need for additional markers to predict fractures in diabetes better. In this context, miRNAs are of interest. Currently, the literature provides limited evidence on the role of miRNAs in diabetes, more so in T1D. This calls for the investigation of whether miRNAs can be suitable biomarkers of diabetic bone disease and help in better fracture risk prediction in diabetes.

Additionally, the reasons for the increased fracture risk, irrespective of the normal/high BMD in T2D, are not known. We suspect an imbalance in bone formation and bone resorption in T2D is responsible for the increase in fractures, but the extent of the imbalance in these processes needs to be elucidated.

There is evidence of negative alterations in bone characteristics at the molecular (BMD, bone turnover) and structural level (bone microarchitecture) in diabetes, but the results are inconsistent. Some studies suggest that poor metabolic control and microvascular complications negatively impact bone, but data is limited and unclear. This calls for better characterising the negative impact on bone at the level of bone turnover, BMD and bone

microarchitecture in both T1D and T2D. Moreover, there are very few longitudinal studies on the impact of prolonged duration of diabetes and microvascular complications on bone. This information will be helpful to understand the extent of negative impact on bone due to diabetes and instil the need for better management and fracture risk prediction.

The role of miRNAs in diabetes and bone health has been investigated in studies of limited sample size and hence, the published results need to be taken with caution. Validating the potential miRNA biomarkers in large and well characterised clinical studies would provide us with a better understanding of their potential to be used as biomarkers.

1.21.1 Aims and objectives

The aims of this thesis were to identify miRNAs as suitable biomarkers for diabetic bone disease, understand the extent of imbalance in bone formation and bone resorption in T2D, and investigate the effect of prolonged duration of diabetes and microvascular complications on bone turnover, BMD, and bone microarchitecture. An additional aim of this thesis was to validate the 'OsteomiR kit' in an independent large and well characterised population of postmenopausal women.

Chapter 3

The aim of this chapter was to identify miRNAs as suitable biomarkers for diabetic bone disease using a systematic review of literature approach.

Chapter 4

The aims of this chapter were to

(i) Validate the findings from Chapter 3 to identify miRNA as suitable biomarkers for T1D bone disease.
(ii) Determine the effect of prolonged duration of diabetes (T1D) and microvascular complications on bone turnover, bone loss, BMD and bone microarchitecture.

Chapter 5

The aims of this chapter were to

(i) Validate the findings from Chapter 3 to identify miRNA as suitable biomarkers for T2D bone disease.
(ii) Understand the reasons for the increased fracture risk in T2D irrespective of normal / high BMD and the extent of imbalance in bone formation and bone resorption in T2D.

Chapter 6

The aim of this chapter was to validate the 'OsteomiR kit' in an independent well-characterised population of postmenopausal women with measurements of bone health made over six years.

In summary, this thesis provided evidence for miRNAs as suitable biomarkers for diabetic bone disease, investigated the imbalances in bone formation and bone resorption in T2D and the effect of prolonged duration of T1D and microvascular complications on bone health.

Chapter 2 – Methods

2.1 Systematic review of literature: principles and guidelines

The principles and guidelines for performing a systematic review of literature are listed below. These guidelines were referred to when performing the systematic review part of this thesis: dysregulated miRNAs in diabetes mellitus and altered bone phenotype.

2.1.1 What is a systematic review?

According to the Cochrane Glossary, a systematic review (or systematic overview) is

"A review of a clearly formulated descriptive research question that uses systematic and explicit methods to identify, select and critically appraise the relevant research, and to collect, analyse and summarise the collected data from the chosen studies part of the review" [105].

The Cochrane Handbook for Systematic Reviews of Interventions is the official guide that outlines in detail the process of preparing and maintaining Cochrane systematic reviews in the context of healthcare. The Handbook provides guidance on the methods applicable to performing a review (planning a review, searching and selecting studies, data collection, risk of bias assessment, statistical analysis and data interpretation) [106].

The aim of systematic reviews is to collect and summarise high quality evidence on a research topic in a systematic way while minimizing the risk of bias and random errors. The review question, review objectives as well as the inclusion and exclusion criteria for choosing the relevant study should be predefined. The methodology used to perform the systematic review should be clearly described before commencing the review and to allow for reproducibility of search results. The search should be broad enough to try and include all studies that may meet the defined eligibility criteria. Once selected, the studies will then need to be assessed for the validation of their findings and eliminate the risk of bias. The information collected form the eligible studies will then need to be organised, analysed and summarised in a clear and descriptive manner [105], [106].

2.1.2 Rationale of a systematic review

Systematic reviews are considered the highest and strongest level of evidence in research due to the following reasons –

(i) The chosen studies are capable of reporting data from large sample sizes, usually by pooling together many eligible studies to reach large sample sizes that could not be reached in individual studies.
(ii) It allows for gathering together all available data from the chosen studies, allowing for in-depth subject exploration and analysis of results. It acknowledges available evidence to address the research question and also reports if more information is required. This is particularly useful and helpful in guiding the next steps in research.
(iii) It reduces the problems of errors and flaws that are usually experienced while conducting single studies. Pooling in several studies reduces the effects of errors and flaws.
(iv) They allow for new insights into the research topic of interest by gathering evidence from different studies and sources and analysing them together.

2.1.3 Overview of performing a systematic review

A systematic review is performed in a structured manner to allow for transparency and reproducibility in the process. Before the review, the rationale must be assessed and the research question should be well established. A protocol to perform the review is then developed, allowing the review to be performed per high standards and in accordance with pre-defined strategies mentioned in the protocol. This is followed by reporting the review finding in a systematic and narrative manner.

2.1.3.1 Forming the review question

The review question or research question should be clear and broad enough to justify conducting the systematic review. The key principles that guide the formation of the research question in the context of observational studies are summarised by the acronym PECOS, P – participants, E – exposure, C – comparator, O – outcome and S – study design [105].

Participants: It is important to define the population being investigated. Several populations may have different characteristics that may influence the results of the exposure.

Exposure: It is important to define the risk group that the participants fall under, like a common disease condition or biological measure. This is because different risk exposures can influence the result of the review.

Comparator: It is important to define the reference group because different groups may have different baseline characteristics. An exposure has the capability to modify a feature that is directly related to the group that may be used as a reference. It is possible to assess multiple comparators, which can be addressed as the main reference group in the primary outcome and as additional reference groups in the secondary outcomes.

Outcome: The review question should clearly state and address a specific outcome of interest to allow for a clear definition of the research and review strategy and avoid misunderstanding.

Study design: The design of the studies to be included should be defined. This can range from literature reviews, randomised controlled trials to observational studies.

2.1.4 The study protocol

The study protocol describes the plans in carrying out the review to keep the process transparent and reproducible. It includes the subject background information and the research question to be addressed in the review. It also includes the methodology that will be used to carry out the review, such as the literature searches, study selection process, inclusion and exclusion criteria, quality assessment, data extraction, data synthesis and reporting. While developing the protocol, the team should try to anticipate potential challenges that could arise during the different steps of performing a review by a team and outline a plan on how to deal with them. The protocol should be finalised before conducting the review to avoid the risk of changing the review strategy motivated by the collected data. A study protocol allows for standardisation of the process, especially when a team is conducting a review. All study protocols should be registered on the PROSPERO website. This allowed for transparency and reproducibility in the review process, as well as avoid the possibility of multiple reviews addressing the same research questions simultaneously [105], [106]. However, the registered protocols can be amended, allowing space for justified modifications without compromising on the review transparency.

2.1.5 The review report

The review report describes the review process and the findings of the review.

The background information and review rationale

The first section of the report includes the background information, introduction to the research topic and explains the rationale for the review. The background information is important to conceptualise the review. It explains the condition or disease being explored and provides the rationale behind performing the review.

2.1.5.1 Review methodology: Search strategy and study selection

The second section of the report describes the methodology used to carry out the review for transparency and reproducibility. It includes the search strategy that will be used to perform the review – i.e. definition of the study sources, terminology that will be used to perform the study searches and the limits that will be applied. Usually, databases such as MEDLINE, Embase and The Cochrane databases are used to perform electronic searches. Conference abstracts and reference lists of key papers can also be used as a source of studies. It is important to note that the key search terms are selected using free-text and thesaurus terms (where possible) and combined with Boolean operations. Limitations such as study language, date of research article publication, human or animal studies can be applied while performing the search for the review. This section also includes the study selection process which should follow a systematic pattern [106].

2.1.5.2 Results of the review: Quality assessment, data extraction, data synthesis and reporting, and publication bias

The third section of the review describes the results of the review. In this section, the data collected undergoes a quality assessment and is then tabulated, described and analysed.

<u>Quality assessment</u>

Quality assessment is important to evaluate the internal and external validity of the chosen studies. Internal validity addresses the risk of bias while external validity addresses generalisation of results. The quality of results reported in any review is tied to the quality of data included. Many elements of the study like selection of study participants, study performance, detection of outcome of interest and participant's attrition might cause bias. Bias in the performance of studies can be reduced by defining an adequate measurement of the exposure, while bias in the detection of outcome can be reduced by defining an appropriate detection method and blindness of the assessment of the outcome of interest. In the base of

observational studies, bias can be reduced by the control for confounders. Additionally, attrition bias can be reduced by maintaining completeness in the follow-up.

A commonly used quality assessment tool in the context of systematic reviews is the Newcastle-Ottawa scale, which allows to assess the risk of bias in observational studies [107].

The scale includes three domains: selection of participants, selection of comparable population and assessment of the exposure of interest. To strengthen quality assessment, the Newcastle-Ottawa scale can be adapted to include an additional domain. For example, they can be additional questions to address the quality of exposure being assessed or any other feature that may be considered to be important in steering the quality of data collected.

The studies are awarded stars if they comply with each feature assessed in the follow, scoring a maximum of nine stars. In the case of the introduction of an additional domain, additional stars are provided for each question. Studies that score seven or more (on the standard Newcastle-Ottawa scale) are considered to be of high quality.

Data extraction

Data extraction should be done using standardised forms and these forms should be piloted. Data extracted should be checked preferably by a second reviewer.

Data synthesis and reporting

Data synthesis is usually done in a qualitative fashion and might also include a quantitative section like a meta-analysis. In systematic reviews, the data collected is usually reported using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The PRISMA is an evidence-based guideline that lists the minimum items that should be reported in systematic reviews and meta-analyses [108]. The PRISMA tool was originally designed to be used in reviews that summarise the findings of randomised trials but is now increasingly being used to summarise the findings of observational studies. The PRISMA reporting guideline suggests the use of flowcharts to summarise the study selection process (i.e. the number of studies identified and screened, the number of studies excluded in each phase of the selection process, the number of studies assessed for eligibility and the number of studies finally included in the review). The PRISMA guideline also includes a checklist of the main elements that should be included in the title, abstract, introduction, methods, results, and discussion. The review should always be checked against the PRISMA checklist [108], [109].

While performing a qualitative data synthesis, the data and quality assessment should be reported as a tabulation and a narrative synthesis to summarise the findings across all the eligible studies.

Discussion

In the last section, the forth section, the findings of the review are summarised and discussed in relation to the current literature. It also includes how the review adds value to the current literature, what the implications of the findings are with regards to current practice, the strengths and weaknesses of the review and what the next steps should be to investigate the chosen research topic in further detail.

2.1.6 Publication bias

The main source of data in the preparation of systematic reviews is published research articles. Large studies and those with large effect sizes are more likely to be published. In contrast, smaller studies and those with no or very small effect sizes are less likely to be published. This is important to keep in mind while assessing data from eligible studies as it can lead to asymmetry in data analysis, thereby causing publication bias.

2.2 Protocol for the systematic review part of this thesis

The protocol for the systematic literature review on identifying circulating miRNAs that are associated with diabetes mellitus and abnormal bone phenotype will be designed by myself and discussed with my supervisory team (Richard Eastell and Tatiane Vilaca). This discussion will allow for appraisal and contribution from members of the supervisory team. The protocol for this systematic review will be registered with PROSPERO. The research will focus on studies that report up-regulated or down-regulated circulating miRNAs in diabetes mellitus (type 1 and type 2 diabetes) and abnormal bone phenotype (previous fractures (>6 months since fracture), high / low bone mineral density and high bone turnover).

2.2.1 Review title

Circulating miRNAs associated with diabetes mellitus and abnormal bone phenotype: a systematic review

2.2.2 Review Question

Which are the circulating miRNAs that are either up-regulated or down-regulated in patients with diabetes mellitus and abnormal bone phenotype?

In this systematic review, PECOS guidelines will be used to define the review question and the search strategy will be performed as a two-step process.

Participants: Adults (aged 18 years and above). The population of interest is defined as adults (aged 18 years and above) because the epidemiology of diabetes mellitus, fractures, bone mineral density and bone turnover and their respective risk factors are different in children and adults. Moreover, type 1 diabetes is more common in children while type 2 diabetes is a common disorder that occurs in the older population. Hence, the population is defined as adults (aged 18 years and above) and the research question is focused on this specific population. During the search process, if studies are found that include data on children, animal models and cell culture, they will be excluded as these data would change the scope of this review.

Exposure: For search part 1, the exposure is defined as those adults who had a diagnosis of either type 1 or type 2 diabetes, and for search part 2, the exposure is defined as those adults who have been characterised by abnormal bone phenotype (increased fracture risk, high / low bone mineral density, high bone turnover). In the case where the study population consists of people with previous fractures, only those who had the outcome measured more than 6 months after the occurrence of the fracture will be included. This is because fracture healing, which usually occurs up to 6 months from the time of fracture can change the measurement of the outcome of interest.

In the review described in this thesis, data will be collected on circulating miRNAs that are either up-regulated or down-regulated in patients with type 1 or type 2 diabetes and abnormal bone phenotype.

Comparator: Different population groups have different baseline characteristics and they play a direct relationship in influencing or modifying the exposure of interest. Hence, a single comparator group is defined for each search part of this review. For search part 1, the comparator group is healthy adults without type 1, type 2 or any other form of diabetes and for search part 2, the comparator group is healthy adults without the bone phenotype in exposure.

Outcome: In this review, the outcome is pre-defined as the expression level of a circulating miRNA measured by real-time – quantitative polymerase chain reaction (RT-qPCR), next-generation sequencing (NGS) and micro-arrays.

Study design: Since the exposure of interest is diabetes and abnormal bone phenotype, the review will focus on data from observational studies. The chosen effect of exposures is usually well described in observational studies. Interventional studies, reviews, letters and editorials, studies using bioinformatics tools for prediction of miRNA expression levels, studies not published in full text in English and studies performed in animal models or cell culture will be excluded.

2.2.3 Review objective

This review aims to identify circulating miRNAs that are up-regulated or down-regulated in people with diabetes mellitus (type 1 and type 2 diabetes) and abnormal bone phenotype (increased facture risk, high / low bone mineral density, high bone turnover).

2.2.4 Background and rationale for performing this review

In Chapter 1, I have shown evidence for bone fragility and increased fracture risk to be a major complication of diabetes. I have also shown evidence for aBMD measured by DXA to underestimate true fracture risk, especially in T2D. The other methods used to assess bone health, fracture risk and bone turnover such as the Fracture Risk Assessment Tool (FRAX), measurement of BTMs and monitoring levels of calcium, vitamin D and PTH also have certain limitations. This underscores the need for novel biomarkers such as miRNAs to compliment routine clinical practice and bone health management.

In Chapter 1, I have also shown evidence for miRNAs to be critical molecular regulators of diabetes pathophysiology and bone remodelling, making them suitable biomarkers for diabetic bone disease. However, there is limited information on the expression of miRNAs in diabetic bone disease.

Hence, this systematic review aims to identify circulating miRNAs that are either up-regulated or down-regulated both in diabetes (type 1 and type 2 diabetes) and in individuals with abnormal bone phenotype (increased fracture risk, high / low bone mineral density, increased bone turnover).

2.2.5 Methodology of this review

In this review, the study search will be conducted in two parts, one for each exposure of interest. The study search will include free-text and thesaurus terms (where applicable). Search part 1 will focus on identifying circulating miRNAs that are either up-regulated or down-regulated in diabetes mellitus (type 1 and type 2 diabetes). Search part 2 will focus on identifying circulating miRNAs that are either up-regulated or down-regulated due to abnormal bone phenotype (increased fracture risk, high / low bone mineral density, and high bone turnover). The next step will be to identify those miRNAs that overlap between the two exposures of interest using a manual approach. This will be help us identify those circulating miRNAs that are either up-regulated or down-regulated in both diabetes and abnormal bone phenotype, prospective for the study of diabetic bone disease.

2.2.5.1 Search strategy

Search part 1 will be conducted by combining terminology for miRNAs and diabetes mellitus and related synonyms. The following medical subject headings (MeSH) will be used: (‘microRNA’ OR ‘miRNA’ OR ‘non-coding RNA’) AND (‘diabetes mellitus’ OR ‘type 1 diabetes’ OR ‘type 2 diabetes’ OR ‘high glucose’ OR ‘hyperglycaemia’).

Search part 2 will be conducted by combining terminology for miRNAs and abnormal bone phenotype and related synonyms. The following MesH terms will be used: (‘microRNA’ OR ‘miRNA’ OR ‘non-coding RNA’) AND (‘fracture*’ OR ‘fracture risk’ OR ‘bone turnover’ OR ‘bone turnover markers’ OR ‘BTMs’ OR ‘bone mineral density’ OR ‘BMD’ OR ‘bone’ OR ‘bone phenotype’). As mentioned above, the search terms will be combined using Boolean operators and database-specific syntax.

Both searches will be conducted using bibliographic databases such as PubMed, MEDLINE and Web of Science. The study search will be limited to published articles in the English language available in full-text and to observational studies carried out in humans. However, no date restriction will be applied to the searches. Abstracts from relevant meetings and conferences such as ASBMR and ECTS will be checked for further relevant information. Experts in the field will be consulted in order to identify any additional relevant information for inclusion.

2.2.5.2 Study selection

Study selection will follow a systematic flow by importing them into a reference management software. The duplicate records will first be removed and next the records will be assessed against the inclusion and exclusion criteria described in this protocol.

Eligible studies will be chosen in two steps. Firstly, the records will be assessed on the basis of their title and abstract. This will be done by a single reviewer and the second reviewer will verified the retrieved records. It is during this step that majority of the records are excluded because they are usually found to be irrelevant. Subsequently, the remaining records will undergo assessment at the full-text level. This is done by one reviewer and verified by the second reviewer. In the case of discrepancies or doubts between the two reviewers, decision of members of the supervisory team (Richard Eastell and Tatiane Vilaca) are final. The selected articles that will be included in the review will undergo quality assessment and data extraction.

Eligibility criteria for study selection in search part 1 (miRNAs in diabetes)

Inclusion criteria

Population

- Humans (18 years of age and above)

Exposure

- Patients who have a confirmed diagnosis of either type 1 or type 2 diabetes

Comparison

- Humans (18 years of age and above) who do not have diabetes mellitus (healthy controls)

Outcome

- Significant difference in miRNA expression level between control and test population, measured using next-generation sequencing (NGS), RT-qPCR or microarrays

Study design

- Observational studies

Exclusion criteria

Population

- Studies lacking a clear definition/diagnosis of diabetes mellitus
- Studies without a clear understanding of when diabetes mellitus was diagnosed and when the miRNAs were measured

Comparison

- Studies lacking a clear description of the comparison group

Outcome

- Studies reporting miRNAs based on methods other than RT-qPCR, microarrays and next-generation sequencing (NGS)
- Study results lacking statistical significance in miRNA expression levels
- Studies with unclear/missing data

Study design

- Interventional studies, reviews, letters and editorials
- Studies not published in full text in English
- Studies carried out using animal models and cell lines

Eligibility criteria for study selection in search part 2 (miRNAs in abnormal bone phenotype)

Inclusion criteria

Population

- Humans (18 years of age and above)

Exposure

- Patients characterised by altered bone phenotype (increased fracture risk, high/low bone mineral density, high bone turnover)
- Patients who have experienced fractures (> 6 months since fracture)

Comparison

- Humans (18 years of age and above) without the bone phenotype in exposure / healthy controls

Outcome

- Significant difference in miRNA expression level between control and test population, measured using RT-qPCR, NGS or microarrays

Study design

- Observational studies

Exclusion criteria

Population

- Studies lacking a clear description/characterisation of bone phenotype
- Studies without a clear understanding of when the diagnosis of abnormal bone phenotype was done and when the miRNAs were measured

Comparison

- Studies lacking a clear description of the comparison group

Outcome

- Studies reporting miRNAs based on methods other than RT-qPCR, microarrays and NGS
- Study results lacking statistical significance in miRNA expression levels
- Studies with unclear/missing data

Study design

- Interventional studies, reviews, letters and editorials
- Studies not published in full text in English
- Studies carried out using animal models and cell lines

2.2.5.3 Data Extraction

Data will be extracted by one reviewer (with no blinding to authors or journals) and cross-verified by the second reviewer using a piloted data extraction form. Any discrepancies will be resolved by discussion between the reviewers, or a third reviewer may be consulted.

The following key data items will be extracted.

- Name of first author, title, year of publication
- Population size and characteristics (age, gender)
- Method of diagnosis of diabetes / characteristic of altered bone phenotype
- The biological sample type in which the miRNA was measured
- The method used for quantification of miRNA expression
- Significantly expressed miRNAs were reported along with the direction of expression in comparison to the control group

2.2.5.4 Data synthesis: Identification of miRNAs overlapping between the two searches

Data from the eligible studies will be reported in a tabulated form. The overlapping miRNAs between the two study searches will be identified using BioVenn [110]. The overlapping miRNAs will then be subject to bioinformatics analysis using miRNet 2.0 [111] to understand which tissue type they are expressed in, which diseases they play a role in, and which signalling pathway they regulate.

2.2.5.5 Quality assessment

Quality of the study and risk of bias assessment will be done using a modified version of the Newcastle-Ottawa Scale (NOS) / Newcastle-Ottawa quality assessment tool. The modified tool includes 2 additional questions to assess the quality of miRNA measurement in the eligible studies.

A reviewer will perform quality assessment and cross-verify it with a second reviewer. Any discrepancies will be resolved by discussion between the reviewers, or a third reviewer may be consulted.

Below are the questions the studies will be assessed on as per the modified NOS.

The studies will be awarded a maximum of one star for each criteria within study selection and exposure categories. A maximum of 2 stars will be given for comparability and additional questions to assess quality of miRNA measurement.

If the study follows the answer mentioned in *, they get a point. A study can earn a maximum of 11 points.

Study selection

1. Is the case definition adequate? (i.e. ascertainment of diabetes (OR) fracture (OR) BMD < -1)

a. *Requires independent validation (eg: primary medical records such as blood testing/x-ray scans/hospital records)*
b. Record linkage or self-reporting with no reference to primary record
c. no description

2. Representativeness of cases

a. *All eligible cases with outcome of interest over a defined time period, all cases from a defined area, all cases in a defined hospital or clinic or group of hospitals or an appropriate sample of those cases*
b. Not satisfying conditions in part (a), or not stated

3. Selection of controls: this question assesses whether the control group used in the study is derived from the same population as the cases and essentially would have been cases had the outcome been present

a. *Community controls (i.e. same community as cases and would be cases if they had outcome)*
b. Hospital controls, within same community as cases but derived from the hospital population (i.e. from the same city/region)
c. No description

4. Definition of controls

a. *no history of disease outcome*
b. No description of history of outcome

Comparability

1. Comparability of population (one star if study controls for age, two stars if study controls for sex)

a. *age*
b. *sex*

Exposure

1. Ascertainment of exposure (diabetes (OR) fracture (OR) BMD < -1)
a. *Secure record (e.g. surgical record, test reports)*
b. *Structured interview where blind to case/control status*
c. Interview not blinded to case/control status
d. Written self-report or medical record only
e. No description

2. The same method of ascertainment for cases and controls
a. *yes*
b. No

3. Non-response rate (list with explanation)
a. *Same rate for both groups*
b. Non-respondents described
c. Rate different and no designation

Additional questions to assess the quality of miRNA measurement

1. Biological sample used for miRNA isolation (one star for either serum or plasma)
a. *Serum*
b. *Plasma*
c. Whole blood/other tissue types
d. No information provided

2. Comparability in miRNA measurement (one star for relevant spike in and haemolysis control)
a. *Studies reporting use of relevant spike in controls / reference RNA and haemolysis control*
b. No controls used
c. No description available

2.2.5.6 Sensitivity analysis

The overlapping miRNAs identified between the two searches will be subject to a sensitivity analysis. This will help in ranking the miRNAs and identifying the top scoring miRNAs. A miRNA can earn a maximum score of 13. The top scoring miRNAs will be considered as the most promising miRNAs and reported in the review.

The sensitivity analysis will be performed based on the questions below.

1. Based on study quality after NOS scoring (max score = 3)
   High quality = score of 7 or more out of 9
   Intermediate quality = score of 4-6 out of 9
   Poor quality = Score of less than 3 out of 9

   Score for study quality
   High quality = 3
   Intermediate quality = 2
   Poor quality = 1
   Based on this, NOS score for each study will be recorded and an average will be taken. Next, an average of NOS scoring for each miRNA for diabetes and abnormal bone phenotype combined will be calculated.

2. Based on scoring for 2 additional questions added to assess the quality of miRNa measurements (sample source, use of appropriate controls for miRNA measurement) (max possible = 2)
   Studies scoring 2 = 2
   Studies scoring 1 = 1
   Studies scoring 0 = 0
   Categorized by miRNA, similarly, the additional NOS score for each study will be recorded and an average will be taken. Next, an average of the additional NOS score for each miRNA for diabetes and abnormal bone phenotype combined will be calculated.

3. Consistency in miRNA expression across the eligible studies (max score = 3)
   In cases where a mixed direction of expression is observed, the majority will be considered.
   The same direction of expression in diabetes and bone = 3
   The same direction of expression between diabetes and bone based on majority = 2
   The mixed direction of expression (cannot choose a majority) between diabetes and bone = 1
   Different direction of expression in whole set/majority in diabetes and bone = 0

4. miRNA of interest described in multiple studies (max score = 5)
   miRNA of interest described only in 1 study in diabetes and abnormal bone phenotype = 1
   miRNA of interest described in only 1 study in diabetes / abnormal bone phenotype and 2 or more studies in diabetes / abnormal bone phenotype = 2
   miRNA of interest described in only 2 studies in diabetes and abnormal bone phenotype = 3
   miRNA of interest described in only 2 studies in diabetes / abnormal bone phenotype and 2 or more studies in diabetes / abnormal bone phenotype = 3
   miRNA of interest described in only 3 studies in diabetes and abnormal bone phenotype = 4
   miRNA of interest described in only 3 studies in diabetes / abnormal bone phenotype and 3 or more studies in diabetes / abnormal bone phenotype = 4
   miRNA of interest described in 4+ studies in diabetes and abnormal bone phenotype = 5

2.3 Study design and study participants

2.3.1 Longitudinal study on type 1 diabetes and bone health

Study design

This study has been registered under STH20992 (IRAS: 303770): A longitudinal study of microRNA expression levels, bone mineral density, bone turnover markers, bone microarchitecture and bone strength in people with type 1 diabetes with and without neuropathy and the comparison with healthy controls. This study is an extension to STH19719 (IRAS: 222726): An evaluation of bone microarchitecture in people with type 1 diabetes with and without neuropathy.

This study was a longitudinal, single-centred, observational, cross-sectional study to evaluate changes in microRNA expression levels, bone turnover, aBMD, bone microarchitecture and bone strength for 4 and a half years in people with T1D with and without neuropathy and the comparison with healthy controls.

Study participants

As this study is a continuation of STH19719 (IRAS: 222726), participants who took part in this study (baseline visit) were invited to participate in the follow-up study STH20992 (IRAS: 303770) [76]. Participants with T1D were recruited from the outpatient clinic at Sheffield Teaching Hospitals and the Sheffield Teaching Hospital's Diabetes Database between October 2017 and October 2018 (baseline visit). The follow-up visit took place during August 2022 and December 2022.

Healthy controls were recruited from the Mellanby Centre for Musculoskeletal Research Database and emails sent to the Trust employees. Participants with type 1 diabetes were men and women older than 18 years, diagnosed with type 1 diabetes for more than five years, with and without neuropathy, and without chronic kidney disease (CKD) (eGFR > 60 ml/min.m^2) (estimated glomerular filtration rate, eGFR). This resulted in three groups: type 1 diabetes with neuropathy (T1DN+), type 1 diabetes without neuropathy (T1DN-) and healthy controls. During the baseline visit, the study participants were matched for age and gender as closely as possible, as these two features are known to affect bone structure. As skeletal size is known to affect bone mineral content in DXA and the site of the bone scanned by HR-pQCT, efforts were made to match participants in a 5 cm interval in height to minimise the effect of potential confounders. The baseline study was approved by Liverpool Research Ethics Committee (IRAS: 222726, 17/NW/0291), and this follow-up study was approved by Harrow Research Ethics Committee (IRAS: 303770, 21/PR/1712). The participants who provided written informed consent for the baseline study were approached to participate in this follow-up study.

During this follow-up study, all participants provided written informed consent in accordance with good clinical practice guidelines.

Sample size

Power calculations using G*Power was used to calculate the sample size. From previous studies, the rate of bone loss at the distal tibia (Tt.BMD) in a healthy population between ages 20 and 90 is 37%, estimating to a rate of bone loss of ~0.5% each year [112]. No data is available on rate of trabecular bone loss. The estimated rate of bone loss is 30% higher in patients with type 1 diabetes compared to a healthy control population. The coefficient of variance (CV) for trabecular volumetric BMD (Tb. vBMD) at the distal radius is 0.9% [113].
The clinically significant difference in rate of bone loss in patients with T1DN- is 2.6% and 3.2% in patients with T1DN+. This results in a total sample size of 32. This sample size has 80% power to detect a significant difference at $p<0.05$.

G*Power (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower)

Inclusion and exclusion criteria

Below are the inclusion criteria used during participant recruitment for the baseline study (IRAS: 222726) [76]. The same criteria were used during the re-recruitment of participants for this follow-up study.

Inclusion criteria

- Participants must have been part of the baseline study
- Male and female participants aged 18 and older
- Participants with diabetes: Patients with T1D who have been diagnosed with T1D for more than 5 years (HbA1c > 5.7% (39 mmol/mol)) and without CKD (eGFR > 60 ml/min.m^2). The participants with T1D were appropriately evaluated and classified according to the presence of neuropathy using the Toronto Clinical Neuropathy Score (TCNS) and DPNCheck (DPN = diabetic peripheral neuropathy).
- Healthy controls: Haemoglobin A1c levels (HbA1c) less than 5.7% (39 mmol/mol), according to American Diabetes Association standards
- Sufficiently mobile to undergo scanning
- Able to remain motionless for the duration of the scans
- Able and willing to participate in the study and provide written informed consent

Exclusion criteria

Below are the exclusion criteria that were used during participant recruitment for the baseline study. The same criteria were referred to during re-recruitment of participants for this follow up study.

- Previous orthopaedic surgery or fractures which preclude imaging at all sites
- History of any long term immobilisation (duration greater than three months)
- High or low trauma fracture less than one year prior to recruitment
- History of bilateral fractures at tibia and/or radius
- Current pregnancy or trying to conceive
- Delivery of last child less than one year prior to recruitment
- Breastfeeding less than one year prior to recruitment
- Women in the perimenopause period, including 5 years after menopause
- History of or current conditions known to affect musculoskeletal health, diabetes and/or neuropathy evaluation or bone metabolism, including:
    - Diagnosed skeletal disease
    - Osteoarthritis at study measurement sites
    - Chronic renal disease
    - Malabsorption syndromes
    - Hypocalcaemia or hypercalcemia
    - Diagnosed with a restrictive eating disorder
- Women in the perimenopause period, including 5 years after menopause
- Conditions which prevent the analysis of the DXA scans or the interpretation of their results
- Conditions which prevent the analysis of the HR-pQCT scans or the interpretation of their results
- Use of medications or treatment known to affect musculoskeletal health, diabetes and neuropathy evaluation or bone metabolism including depot medroxyprogesterone or the combined oral contraceptive pill
- Alcohol intake of greater than 21 units per week
- Competitive athlete, defined as participating in competitive sport at amateur or professional level
- Markedly abnormal clinical laboratory parameters that are assessed as clinically significant by the PI
- For healthy controls, abnormal levels of fasting glucose (fasting glucose levels > 126 mg/dL) or HbA1c > 5.7% (39 mmol/mol)

Study procedures (at the follow up visit)

All participants attended two visits and both visits were at the clinical research facility (CRF) at the Northern General Hospital, Sheffield (NGH). Table 2.1 lists the procedures undertaken during each visit.

Table 2-1. Procedures the participants will undertake during the study visits.

Visit	Procedure
Visit 1	Informed consent, blood sample, urine sample, neurophysiology evaluation*
Visit 2	Height, weight, BMI, pregnancy test (premenopausal women only), DXA, HR-pQCT

**only for participants with T1D*

Processing of blood and urine sample

After an overnight fast, a blood sample of approximately 20 ml was collected from each participant. Some of the blood sample collected was used to measure HbA1c at the Department of Clinical Chemistry, Northern General Hospital, Sheffield Teaching Hospitals NHS Foundation Trust. HbA1c was measured using capillary electrophoresis. The remainder of the blood sample was processed accordingly to separate them into serum and plasma. The serum and plasma samples were aliquoted and stored at -80°C until their use for further analysis. The plasma sample was used to measure miRNAs and bone turnover markers (PINP and CTX). A urine sample was also collected from all the participants to measure their urine albumin/creatinine ratio and additionally, in premenopausal women, a confirmatory urine stick pregnancy test was performed prior to visit 2. This was also done at the Department of Clinical Chemistry, Northern General Hospital, Sheffield Teaching Hospitals NHS Foundation Trust.

2.3.2 Study related to bone health in type 2 diabetes (DiabOsteo, ExtremeCT and Fat and Bone Study)

Study design

This was an observational, case-controlled study to evaluate aBMD in patients with T2D and establish if there is a relationship between aBMD, bone turnover and bone balance in T2D. In this study I was also interested in identifying miRNAs as biomarkers to assess bone fragility in T2D.

Study participants

The DiabOsteo Study was a three-year prospective cohort study conducted in Denmark which recruited men and women with T2D. In this study, participants with T2D who took part in the

DiabOsteo Study were included (baseline visit only). These participants were recruited during routine medical evaluation from the diabetic outpatient clinic at the endocrinology department at the Odense University Hospital (OUH) and from the "Fyn Diabetes Database (FDDB)" between May 2017 and October 2018. Healthy controls were chosen from previous studies run by our research group in Sheffield, namely, 'Evaluation of the XtremeCT device for the assessment of bone (XtremeCT Study)' and 'The effects of obesity on bone structure and strength (Fat and Bone Study)'. These participants were recruited from General Practices in Sheffield or from the list of individuals previously consented to be approached for future studies at the Osteoporosis Clinical Trials Unit in Sheffield. Participants in the XtremeCT Study were recruited between May 2009 and May 2010; and participants in the Fat and Bone Study were recruited between August 2011 and June 2013. A material transfer agreement was set up between University of Sheffield and Southern Denmark University to facilitate the transfer of research samples and clinical data.

Participants with T2D were men and women older than 18 years, diagnosed with T2D for more than 5 years and without end stage CKD (eGFR <30ml/min.m^2). Diagnosis of T2D was ascertained based on HbA1c ≥ 7.0% / 39 mmol/mol (or) fasting plasma glucose ≥ 126 mg/dL (in accordance with WHO guidelines). Participants clinical data and biochemical parameters (absence of glutamic acid decarboxylase antibodies and high C-peptide levels > 700 pmol/L), and the use of and response to anti-diabetic medications where applicable was also reviewed. The measurement of glutamic acid decarboxylase antibodies is essential to rule out the possibility of a person having insulin-dependent diabetes mellitus, and high C-peptide levels (> 700 pmol/L) suggest high insulin production and may indicate T2D [114]. HbA1c (using capillary electrophoresis) and eGFR were measured at the Central Clinical Laboratory at Odense University Hospital. Healthy controls were participants without T2D and without any illness known to alter bone metabolism. This resulted in two groups: type 2 diabetes and healthy controls. Best efforts were taken to match the study participants by gender, age (±3 years) and BMI (±3 kg/m^2) as close as possible as these three features are known to affect bone structure. As skeletal size is known to affect bone mineral content measured by DXA, best efforts were made to match participants in a 10 cm interval in height to minimise the effect of potential confounders. The serum samples used in this study were collected from the participants after an overnight fast.

The research ethics board in Denmark approved the DiabOsteo Study. The study titled 'Evaluation of the XtremeCT device for the assessment of bone' was approved by the Sheffield Research Ethics Committee (09/H1308/55) and registered under STH15265; and the study titled 'The effects of obesity on bone structure and strength' was approved by the Sheffield Research Ethics Committee (REC 10/H1308/61) and registered under STH15688. For the use

of samples (healthy controls) from the above-mentioned studies in this research, ethics approval was obtained from the South Yorkshire North Derbyshire Musculoskeletal Biobank (SYNDMB), Mellanby Centre for Musculoskeletal Research, University of Sheffield. All study participants had given written consent for the use of their research samples and collected clinical data for future research studies in accordance with good clinical practice guidelines.

Inclusion and exclusion criteria

Inclusion criteria

- Male and female participants aged 18 and older
- Patients with diabetes: Patients with type 2 diabetes who have been diagnosed with T2D for more than 5 years (in accordance with WHO guidelines – HbA1c ≥ 7.0%, 39 mmol/mol, fasting plasma glucose ≥ 126 mg/dL, and without end stage CKD (eGFR <30 ml/min.m^2)
- Absence of glutamic acid decarboxylase antibodies
- C-peptide levels greater than > 700 pmol/L
- Provided written consent for their blood sample and clinical data to be used for future research studies

Exclusion criteria

- Known metabolic bone disease other than osteoporosis
- Patients with liver dysfunction (ALT > 3 times the upper limit of normal) (ALT = alanine transaminase)
- Women who are pregnant
- Individuals unable to consent for the use of their blood sample and clinical data for future research studies

2.3.3 Osteoporosis and Ultrasound Study

Study design

The Osteoporosis and Ultrasound (OPUS) study was a population-based, longitudinal study consisting of five European centres: Aberdeen (UK), Sheffield (UK), Kiel (Germany), Berlin (Germany) and Paris (France). A detailed description of the OPUS study design and participant recruitment has been described [115] and is briefly summarised here. The study was coordinated by “Medizinische Physik” in Kiel, Germany. All investigations were performed in accordance with the Declaration of Helsinki, after ethical approval was obtained from the local ethics committees of each participate study site and after obtaining written informed consent from each participant. The study participants had consented to using their research samples and clinical data in future research studies. For the use of research samples from the

OPUS Study in this research project, ethics approval was obtained from the South Yorkshire North Derbyshire Musculoskeletal Biobank (SYNDMB), Mellanby Centre for Musculoskeletal Research, University of Sheffield.

Study participants

The overall aim of the OPUS Study was to assess quantitative ultrasound methods and their association with prevalent vertebral fractures and compare it with bone densitometry. Participants from the different study sites part of the OPUS study were recruited at random from government-provided registries, population-based health registries and general practitioners during the period of April 1999 and April 2001, and they had a follow-up visit approximately 6 years later. At baseline, 2837 participants were recruited in total across the five different study sites. They were all women, out of which 2374 women were in the older age category of 55 – 79 years and 463 women were in the younger age category of 20 - 39 years. Of these, 1604 participants returned for the follow up visit 6 years later. A detailed description of the inclusion and exclusion criteria based on which the study participants were chosen can be found in [115].

For the purpose of this study, 1329 older women aged 55 – 79 years from the OPUS study population were included. The serum samples used in this study were collected from the participants in the non–fasted state.

Fracture assessment

At the baseline visit, prevalent vertebral fractures (i.e. osteoporotic fractures and deformities at the spine) were assessed by quantitative morphometry. A threshold of vertebral height ratio of <0.80 was used, followed by visual radiological differential diagnosis. At the follow up visit, incident vertebral fractures were assessed using a threshold ratio of <0.80 of the baseline vertebral height and quantitative morphometry. For consistency, all spine radiographs were read by an experienced radiographer at the Berlin Centre.

At the baseline and 6-year follow-up visit, participants were required to fill out the OPUS Risk Factor Questionnaire which captured information on previous fractures. Prevalent non-vertebral fractures (i.e. hip, shoulder and wrist) were captured through this questionnaire filled at baseline. Incident non-vertebral fractures were captured through self-report and questions detailed in the OPUS Risk Factor Questionnaire filled at the follow-up visit. Radiographs or medical records then verified this information.

2.4 Estimation of glomerular filtration rate

eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (National Institute of Diabetes and Digestive and Kidney Diseases Handbook).

$$eGFR\left(\frac{mL}{min \times 1.73\ m^2}\right) = 141 \times \min\left(\frac{Scr}{k}, 1\right)^{\alpha} \times \max\left(\frac{Scr}{k}, 1\right)^{-1.209} \times 0.993^{age} \times 1.018\ [if\ female] \times 1.159\ [if\ black]$$

Scr = serum creatinine (mg/dL)

K = 0.7 for females and 0.9 for males

α = -0.329 for females and -0.411 for males

min = minimum of Scr/K or 1

max = maximum of Scr/K or 1

2.5 Neurophysiology evaluation

In accordance with the Toronto Consensus meeting, DSPN is defined as length-dependent sensorimotor polyneuropathy. DSPN is chronic, symmetric and is associated with longstanding hyperglycaemia and cardiovascular risk factors. Symptoms of neuropathy are linked to abnormalities in nerve conduction testing. In order to categorise participants with T1D type 1 diabetes into groups of with and without neuropathy, the TCNS and DPNCheck was used.

2.5.1 Toronto Clinical Neuropathy Score

The TCNS is a simple and comprehensive assessment used to evaluate DSPN. The assessment includes questions and tests that assesses symptoms, reflexes and sensations in small and large nerve fibres in both limbs (Table 2.2).

The presence of abnormalities in symptoms, reflexes and sensations is graded up to a maximum score of 19. The symptoms scores are assessed in each limb as present (1) and absent (0) by the examiner. The reflexes scores are assessed as absent (2), reduced (1) or normal (0). The sensory scores are assessed as abnormal (1) or normal (0). The participants are categorised into their respective group based on the scoring: no neuropathy (0 - 5), mild neuropathy (6 - 8), moderate neuropathy (9 - 12) and severe neuropathy (> 12).

Table 2-2. List of symptoms, reflexes and sensory tests assessed by the Toronto Clinical Neuropathy Score.

Symptoms	Reflexes	Sensory tests
Foot Pain	Knee	Temperature
Numbness	Ankle	Light touch
Tingling		Pinprick
Weakness		Vibration
Ataxia		Position
Upper limb symptoms		

2.5.2 DPN Check

The DPN Check (Neurometrix Inc., Waltham, MA) is a point-of-care device used to perform sural nerve conduction assessments. The DPN Check device assesses nerve amplitude potential (sural nerve action potential - µV) and conduction velocity (m/s). The nerve conduction assessment requires the probes to be placed along the lateral region of the ankle and a biosensor is located 9.22 cm from the probes that covers the area above the ankle to record the response from the stimulation (Figure 2.1). The device stimulates the sural nerve, the longest sensory nerve orthodromically and the responses are read from a display screen. This device has been validated for clinical use and has demonstrated reliability and acceptable accuracy for the diagnosis of diabetic neuropathy [116].

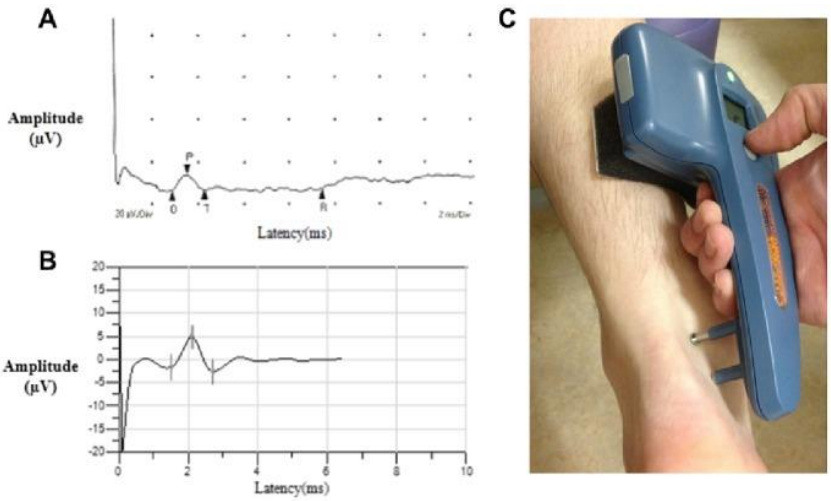

Figure 2-1. DPNCheck device in use.

Sample nerve conduction recordings from standard NCS (A) and the point-of-care device (B) from a 60-year-old female with type 2 diabetes and an image of the point-of-care procedure (C). Panel A: Sample standard NCS recording. Sural nerve amplitude potential was 6.8 mV and conduction velocity was 48.3 m/s. Panel B: Sample recording from the point-of-care device. Sural nerve amplitude potential was 8 mV and conduction velocity was 56 m/s. Panel C: The device was placed on the lateral aspect of the leg and the sural nerve was stimulated and recorded by the electrical probes and biosensor, respectively. NCS: Nerve conduction study, mV: milli volts.

Open Access, Plos One [116].

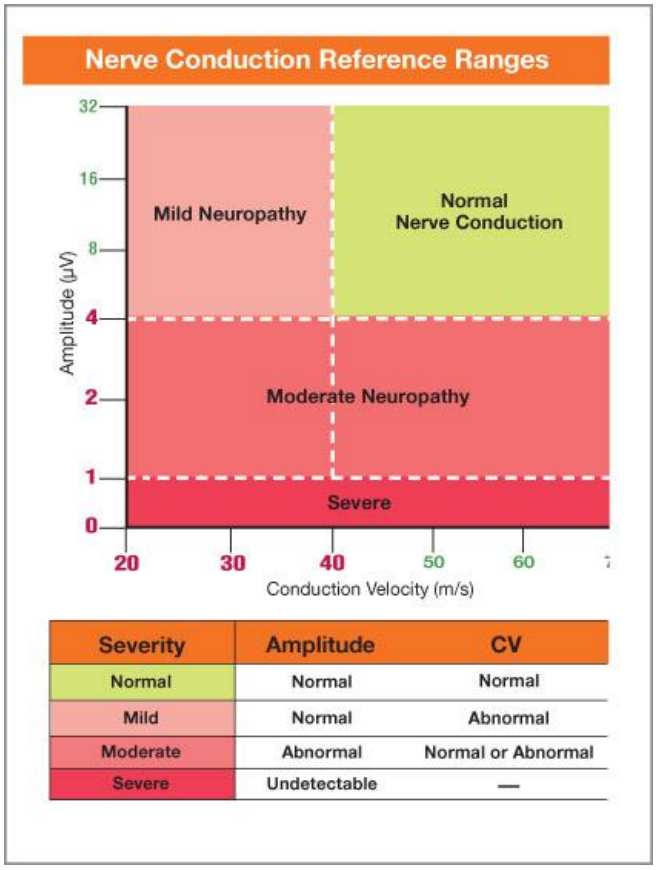

Figure 2-2. DPNCheck interpretation guide.

[DPNCheck device handbook - Copyright © 2024 NeuroMetrix, Inc.]

2.5.3 Group allocation for participants with type 1 diabetes

This study used DSPN as the main characteristic to classify participants with type 1 diabetes into the groups of with neuropathy (T1DN+) and without neuropathy (T1DN-). T1DN- participants were categorised as those with a TCNS score of ≤ 5 (indicative of no neuropathy) and a normal nerve conduction assessment using the DPN Check. T1DN+ participants were categorised as those with a TCNS score of > 6 (indicative of neuropathy) and an abnormal nerve conduction assessment using the DPN check.

2.6 Dual energy X-ray absorptiometry

Scan acquisition and evaluation

In this thesis, dual-energy X-ray absorptiometry (DXA) was used to assess aBMD at the lumbar spine (L1-L4) and hip (total hip and femoral neck). DXA provides a two-dimensional projection of the anatomical region of interest for which the total area and aBMD are measured. The aBMD is a measure that is used to calculate a score that compares the person's individual measurement with predefined populations which is used in assessment and interpretation of results. Several studies have shown evidence for the use of aBMD by DXA to predict fracture risk and is used as a surrogate for bone strength. The aBMD scores obtained from DXA are helpful in the diagnosis of osteoporosis (BMD 2.5 SD or more below the mean of young reference group in clinical practice [117]–[119].

Principle of DXA

Different tissues in the body attenuate X-ray differently depending on their density. This is the principle behind the working of DXA.

By alternating the voltage of the X-ray tube built in the DXA machine, an X-ray source emits two distinct energy levels: one high (140kVp) and one low (60kVp). DXA generally uses a low radiation dose (lumbar spine = 13.3 µSv, proximal femur = 9.3 µSv) [120]. The beams from the X-ray source pass through a collimator that produces a beam in the form of a fan. Different tissues in the body attenuate the X-ray beams differently depending on their density; the less dense a tissue, the lesser it will attenuate the X-ray beam. During this process, some photons are absorbed by the tissue and some get scattered. A detector opposite the X-ray source detects the difference in energy between the two original energy levels.

The human body is composed of tissues and bones. Due to the ability of different tissues to attenuate X-ray differently, the DXA machine is capable of detecting bone and differentiating it from soft tissue. This allows the machine to detect bone area and BMD. The output is a two-dimensional measurement and BMD is expressed as g/cm^2. BMD can also be expressed as scores, called the Z-score and T-score.

BMD follows a normal distribution and hence, means and standard deviations are calculated using population databases. The measured BMD score of an individual is placed between a population data distribution and this value is expressed as a standard deviation from the mean population in a given database. To obtain BMD as a Z-score, a database from a population at the same age is used for comparison. BMD as a Z-score expresses how far the result is (in

standard deviation) from the mean for a given age-matched population. On the other hand, T-score refers to a population at the peak of their bone mass [119].

2.6.1 DXA procedure

Standard operating procedures provided by the manufacturer were used to obtain DXA images at each site of interest. My research group has previously described this (Evans et al., 2015). In all the studies described in this thesis, DXA scans of the lumbar spine (L1 – L4) and hip (total hip and femoral neck) were obtained in a posterior – anterior projection.

In the study on T1D with and without neuropathy, DXA scans were captured using the Hologic Discovery A densitometer (Hologic Inc., Bedford, MA, USA) and the appropriate Hologic software. In the DiabOsteo Study, DXA scans were captured using a Discovery A densitometer (Hologic Inc., Bedford, MA, USA) and the appropriate Hologic software. In the XtremeCT Study and Fat and Bone Study, DA scans were captured using a Discovery A densitometer and Horizon A densitometer (Hologic Inc., Bedford, MA, USA) and the appropriate Hologic software. In the OPUS study, DXA scans were captured using the postero-anterior projection (Hologic QDR-4500; Hologic, Bedford, MA, USA in the Kiel, Paris, and Sheffield centres) or in antero-posterior projection (Lunar Expert devices; GE Lunar, Madison, WI, USA in the Aberdeen and Berlin centres).

2.6.1.1 DXA scan procedure - hip

The participant was made the lay flat on their back on the scan table within the marked scan limits. A head positioner was used to ensure that the participant was comfortable and well positioned. The foot on the side to be scanned was placed on a hip positioner to keep the leg stationary and rotate the hip internally by 25° (Figure 2.3). Care was taken to make sure that the participants hands were not in the scanning area and were rather placed on their chest as shown in figure 2.3. During all scans, an express scan was performed to assess body positioning: at least 3 cm of straight femoral shaft below the lesser trochanter was included in the scan field. Positioning was corrected where necessary.

Figure 2-3. Participant positioned for a dual-energy X-ray absorptiometry scan of the proximal hip. Courtesy of Dr. Margaret Paggiosi.

For a DXA proximal hip scan analysis, the region of interest of the hip was positioned using the following guidelines: (i) The lateral border was positioned 5 scan lines away from the edge of the greater trochanter, (ii) The medial border was positioned 5 scan lines away from the edge of the femoral head of the hip, (iii) The bottom border was positioned 10 scan lines below the lesser trochanter, and (iv) the upper border was positioned 5 scan lines away from the edge of the femoral head of the hip. Using these guidelines, the hip was correctly positioned and the bone map was identified. The central axis of the femur was used to place the midline. The neck box was placed closer to the greater trochanter. The neck box also included an equal amount of soft tissue on either side of the femoral neck of the hip (Figure 2.4).

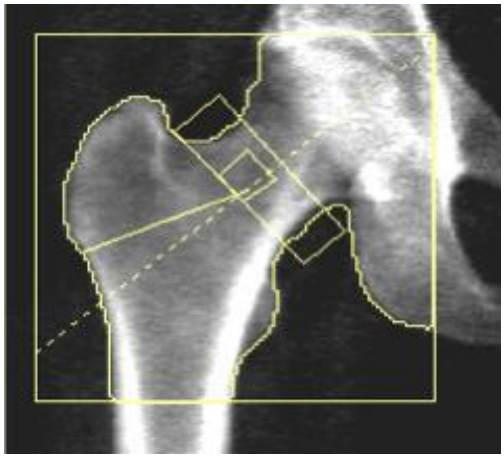

Figure 2-4. Region of interest for a dual-energy X-ray absorptiometry scan analysis of the proximal femur. Courtesy of Dr. Margaret Paggiosi.

2.6.1.2 DXA scan procedure - lumbar spine

For a DXA scan of the lumbar spine, a spine positioning block was used to elevate the participant's legs and the hip positioner was removed (Figure 2.5). The scan region of interest included sections L1 - L4. Just like at the hip, an express scan was performed to assess correct body positioning.

Figure 2-5. Participant positioned for a dual-energy X-ray absorptiometry scan of the lumbar spine. Courtesy of Dr. Margaret Paggiosi.

For a DXA lumbar spine scan analysis, the top border was positioned within the T 12-L 1 intervertebral space and the bottom border was positioned within the L4 - L5 intervertebral space (Figure 2.6). The lines placed in each intervertebral space allowed identification of each vertebra.

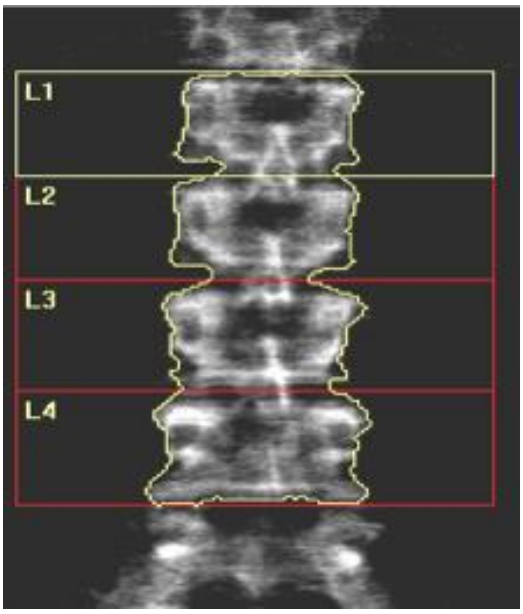

Figure 2-6. Region of interest for a dual-energy X-ray absorptiometry scan analysis of the lumbar spine. Courtesy of Dr. Margaret Paggiosi.

2.6.2 Quality control of DXA scanners

2.6.2.1 Quality control of the DXA scanner used in the longitudinal study on T1D study and bone health

A trained scanning technician performed all DXA scans. Following the manufacturer's standard operating procedures and recommendations, quality control assessments were performed regularly to ensure stability and precision during the scans. An anthropometric bone phantom containing four single density semi-hydroxyapatite 'vertebrae' was used for quality control assessments. The measurements of the bone phantom 'vertebrae' were plotted graphically against pre-specified acceptable limits set by the manufacturer. aBMD was within limits (Figure 2.7, Figure 2.8) and the coefficient of variation was below 0.3%.

The DXA scans at baseline and follow up were performed by the same scanning technician using the same Discovery A densitometer (Hologic Inc., Bedford, MA, USA) and Hologic software (version 12.6). The same bone phantom 'vertebrae' was also measured at both time points. This allowed to reduce the possibilities of any major precision error.

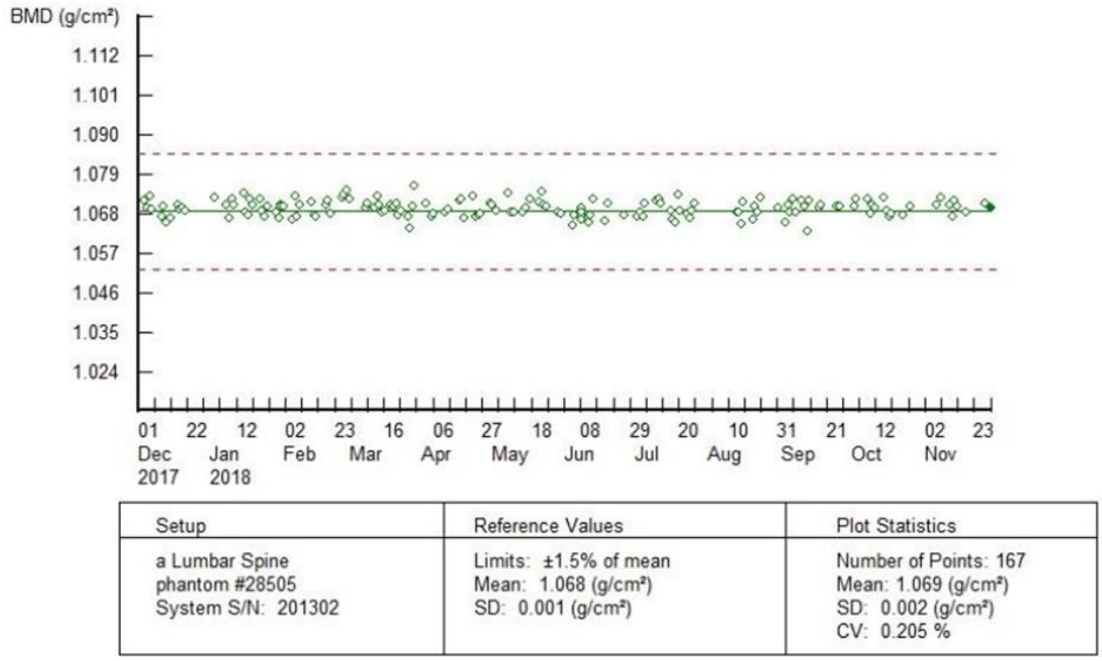

Figure 2-7. Quality control plot for arial bone mineral density measured using dual-energy X-ray absorptiometry during the type 1 diabetes and bone study at baseline (January – November 2018).

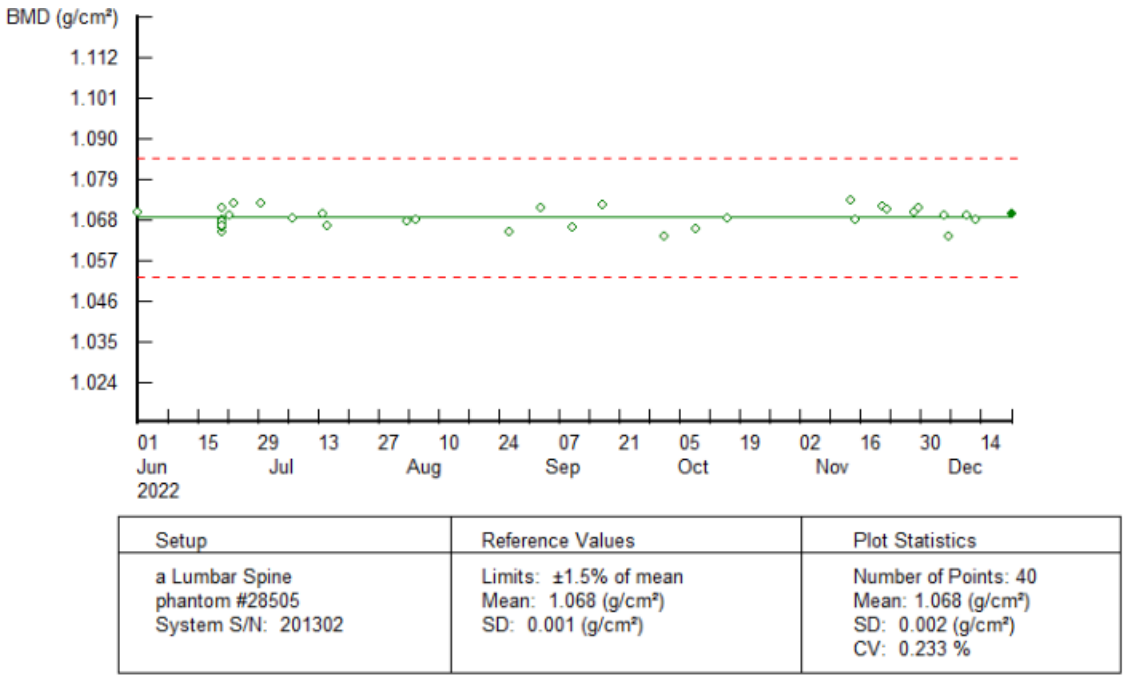

Figure 2-8. Quality control plot for arial bone mineral density measured using dual-energy X-ray absorptiometry during the type 1 diabetes and bone study at follow-up (August – November 2022).

2.6.2.2 Quality control of the DXA scanner used in the study on T2D and bone health

All DXA scans in the DiabOsteo Study (Odense, Denmark) and XtremeCT and Fat and Bone Study (Sheffield, UK) were performed by a trained scanning technician at each site. In accordance with the manufacturer's standard operating procedures and recommendations, quality control assessments were performed regularly to ensure stability and precision during the scans. Cross calibration using an anthropometric bone phantom could not be done at both sites because the Odense site had upgraded to a newer densitometer and the older densitometer was no longer available on site. However, at both sites, respective anthropometric bone phantoms were measured during the study duration to assess for quality control of the densitometers. At both sites, aBMD was within limits and comparable with the phantom used to check for quality control. Figure 2.9 shows the QC plot for aBMD measured on the scanner used in the Diabosteo Study, and Figure 2.10 and Figure 2.11 shows the QC plots for aBMD measured on the scanners used in the FAB Study and XtremeCT Study respectively. The coefficient of variation of aBMD measured was below 0.41% at both sites.

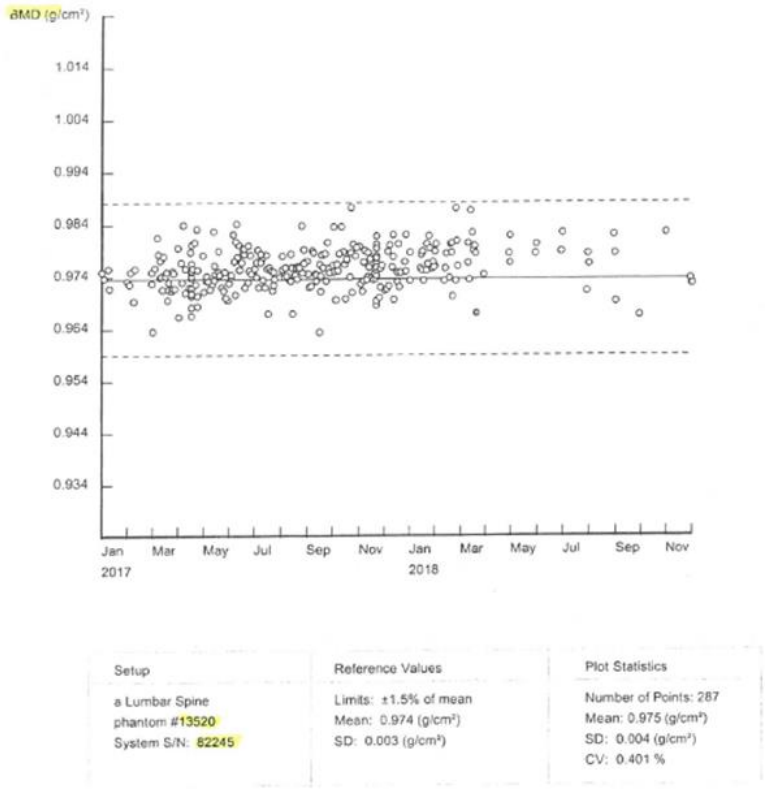

Figure 2-9. Quality control plot for arial bone mineral density measured in Odense using dual-energy X-ray absorptiometry in the DiabOsteo Study (May 2017 – October 2018).

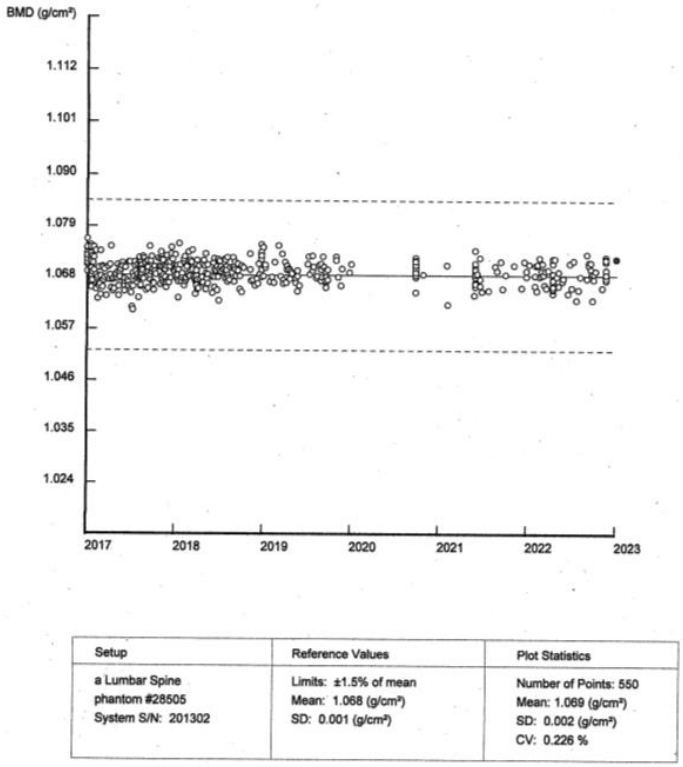

Figure 2-10. Quality control plot for arial bone mineral density measured in Sheffield using dual-energy X-ray absorptiometry in the Fat and Bone Study.

The QC data from the time of recruitment was unavailable as the scanner saves the QC plot for only 10 years. Participant recruitment for the Fat and Bone Study was during August 2011 and May 2013.

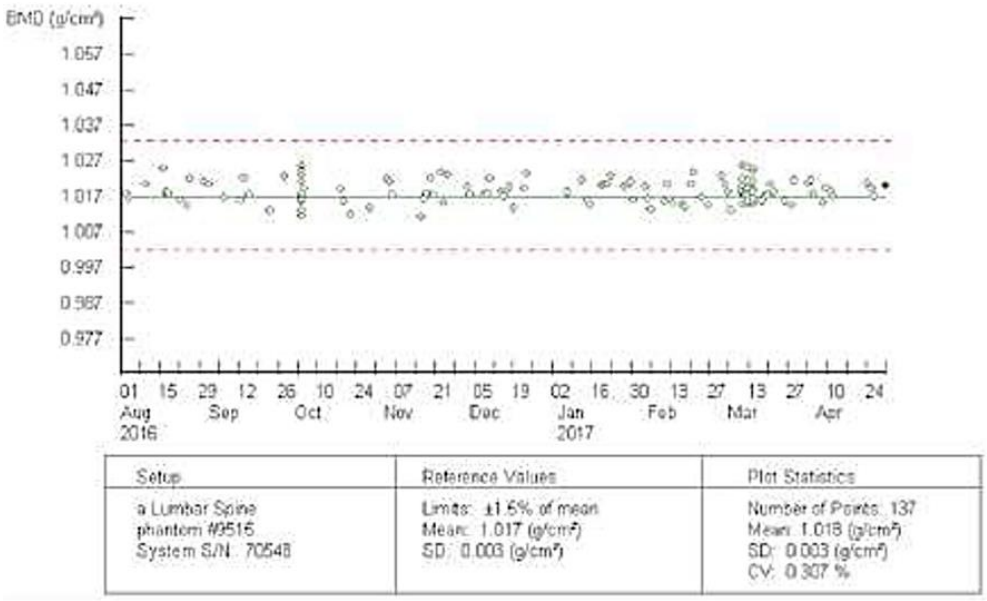

Figure 2-11. Quality control plot for arial bone mineral density measured in Sheffield using dual-energy X-ray absorptiometry in the the XtremeCT Study.

The QC data from the time of recruitment was unavailable as the scanner saves the QC plot for only 10 years. Participant recruitment for the XtremeCT Study was during May 2009 and May 2010.

2.7 High resolution peripheral quantitative computed tomography

Principle

High resolution peripheral quantitative computed tomography (HR-pQCT) uses a low radiation dose to provide high resolution images of the distal appendicular skeleton. In this study, a first generation XtremeCT device and SCANCO Image Processing Language Software (IPL, version 5.08-B) (SCANCO Medical AG, Bruttisellen, Switzerland) were used to measure volumetric BMD and the bone microstructure at the radius and tibia.

The device contains a rotating X-ray tube and a stationary 2D array for detection. The X-ray beams generated by the rotating X-ray tube are made to pass through a section of the limb being scanned and the spatial distribution of the attenuation of the X-ray beams is computed onto a blank matrix. This is used to generate an image [121]. A series of parallel consecutive image slices are generated. These slices are computed together to form a high resolution 3D image (Figure 2.12).

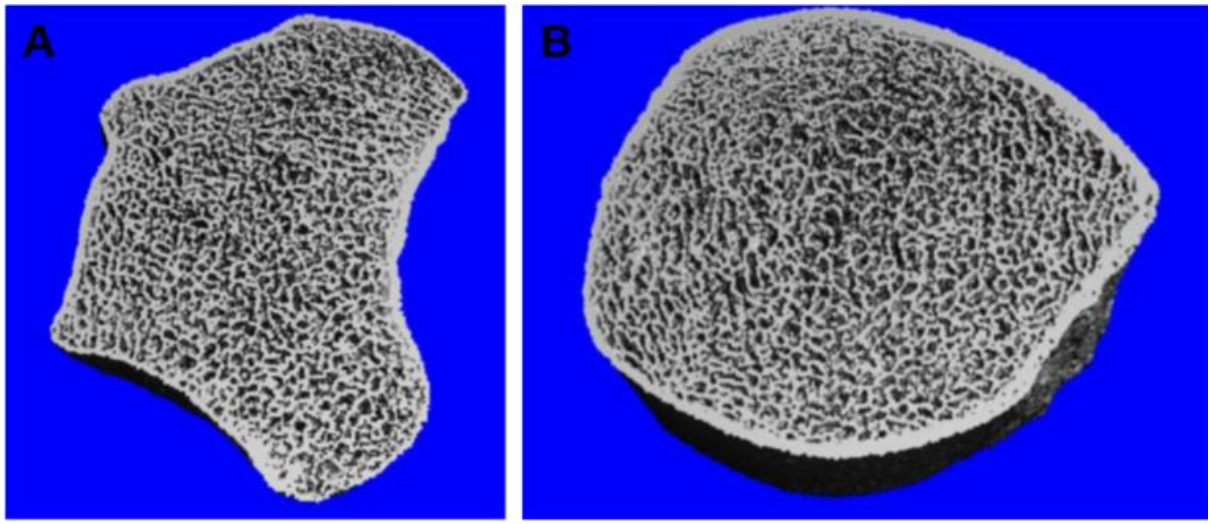

Figure 2-12. 3 Dimensional images of a section of the (A) radius and (B) tibia obtained from high-resolution peripheral quantitative computed tomography.

Courtesy of Dr. Margaret Paggiosi.

In order to calculate volumetric BMD (vBMD), pre-calibration using a phantom is required. For a HR-pQCT scan, calibration is done using a phantom containing five hydroxyapatite resin compartments. The five compartments of the phantom consists of progressively increasing densities ranging from 0mgHA/cm^3 (equivalent to soft tissue containing no mineral content) to 800mgHA/cm^3 (equivalent to bone material) (Figure 2.13). After the phantom is scanned, attenuation of the image slices is calculated, which is then converted to vBMD values in mgHA/cm^3.

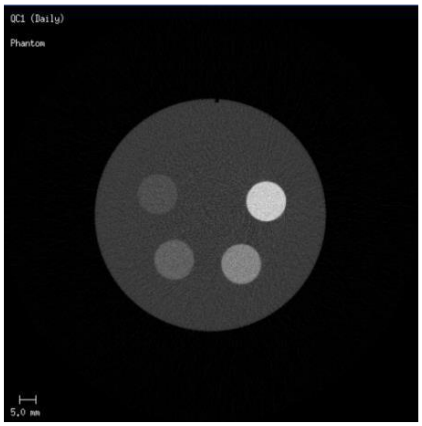

Figure 2-13. A scanned image of the calibration phantom used during high-resolution peripheral quantitative computed tomography.

Courtesy of Dr. Margaret Paggiosi.

2.7.1 HR-pQCT scan procedure

A trained scanning technician performed all HR-pQCT scans. In HR-pQCT scans, the same non-dominant limb was scanned at both time points (baseline and follow up), unless the participant reported a previous fracture on the limb scanned at baseline. At a given site (radius or tibia) the radiation dose for each HR-pQCT scan is 3 µSv [122]. A source potential of 60kVp and high resolution mode of image matrix 1536 x 1536 was used to obtain the scans. The current passing through the rotating X-ray tube was set at 900mA and the integration time was 100ms. The area scanned at baseline was used as a reference during the follow-up scan. This was done to ensure the same area was measured at both time points. A reference line was placed at the region of interest to place the first scanning line. From this reference line, 110 slices were acquired within a stack height of 9.8mm.

The scanning technician requested the participant to remain motionless during the scans and image acquisition. The quality of each acquired scan image was evaluated using a visual grading system [123].

Grade 1: Perfect, no noticeable artefacts
Grade 2: Slight artefact, small streaking
Grade 3: Pronounced artefact, large streaking, particularly near the cortex
Grade 4: Unacceptable artefacts: discontinuous from the cortex

Scan images with grade 1 and 2 were accepted for analysis. Scan images with grade 3 and 4 were repeated up to a maximum of 2 times. Repeat scan images with grade 4 were excluded from the analysis.

2.7.1.1 HR-pQCT scan procedure at the distal radius (standard site)

The participant was asked to sit in a particular chair recommended by the manufacturer. This chair allowed for required positioning and adjustment. The participant’s hand and lower arm was positioned in a forearm cast and an arm pad was used to stabilise the arm. The arm was then placed into the device and secured (Figure 2.14).

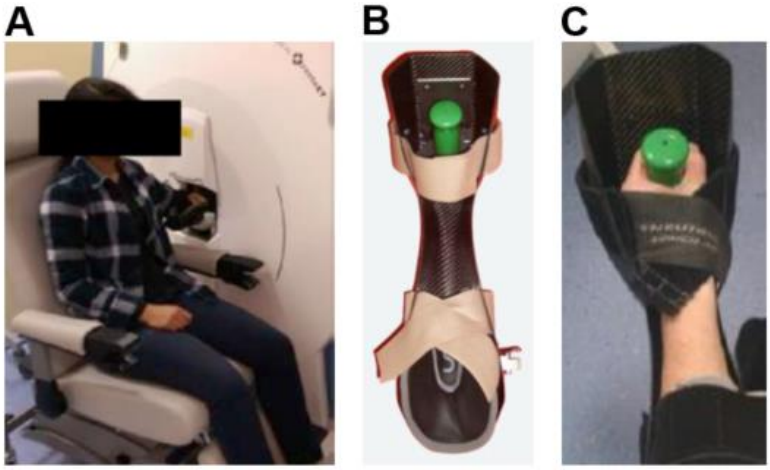

Figure 2-14. Position of the arm for a high-resolution peripheral quantitative computed tomography scan of the distal radius.

(A). Participant's seating position, (B) arm cast, (C) participant's arm positioned inside the arm cast.

Courtesy of Dr. Margaret Paggiosi.

A scout scan was performed after the arm was positioned to determine the measurement using standard procedures. This scout scan also helped to align the follow up scan based on the positioning of the arm during the baseline scan. In the image obtained from the scout scan, the notch of the articular surface of the distal radius was identified. A reference line was placed at this site. The measurement occurred 9.5 mm from the reference line (Figure 2.15).

Figure 2-15. Position of the reference line on the radius scout scan for measurement at the standard site.

Courtesy of Dr. Margaret Paggiosi.

2.7.1.2 HR-pQCT scan procedure at the distal tibia (standard site)

The participant was asked to sit in a particular chair recommended by the manufacturer and this time for the tibia scan, the participant had to place their foot and lower leg in a tibia cast. The participant's leg was positioned by placing it on a leg support. The chair height was adjusted to ensure that the leg height was the same as the height of the gantry (Figure 2.16).

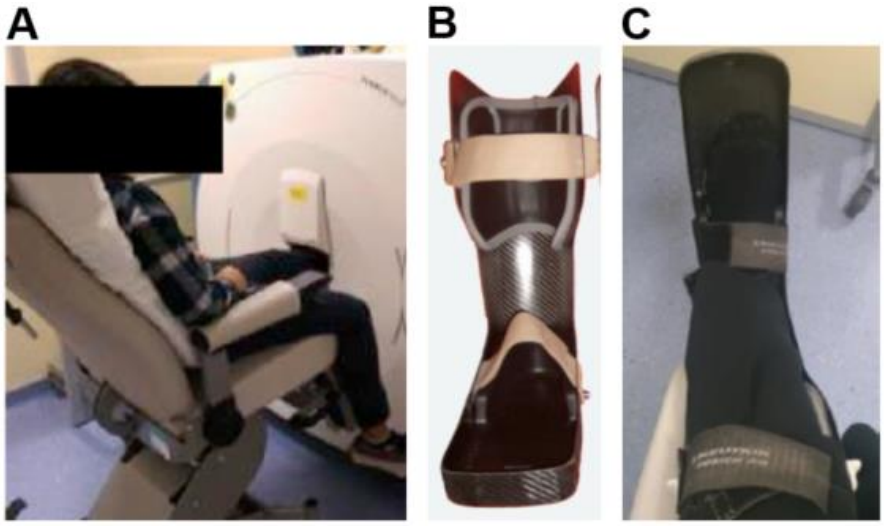

Figure 2-16. Position of the leg for a high-resolution peripheral quantitative computed tomography scan of the distal tibia. Radius.

(A). Participant's seating position, (B) leg and foot cast, (C) participant's leg positioned inside the leg and foot cast.

Courtesy of Dr. Margaret Paggiosi.

After the leg was positioned, a scout scan was performed using standard procedures to determine the measurement. This scout scan also helped to align the follow-up scan based on the positioning of the leg and foot during the baseline scan. In the scout scan, a reference line was placed at the endplate of the distal tibia (Figure 2.17). The first slice was measured 22.5mm from the reference line.

Figure 2-17. Position of the reference line on the tibia scout scan for measurement at the standard site.

Courtesy of Dr. Margaret Paggiosi.

2.7.2 HRpQCT scan analysis and image registration

Small differences in a participant's limb positions in a follow up study can result in variations of the axial position and in the degree of tilt of the limb (Figure 2.18). This reduces the ability to accurately detect longitudinal changes along the length of the bone, especially cortical geometry [124]. To reduce positioning errors and allow for measurement of the same region of interest at baseline and follow up, a method of image registration was used in this study.

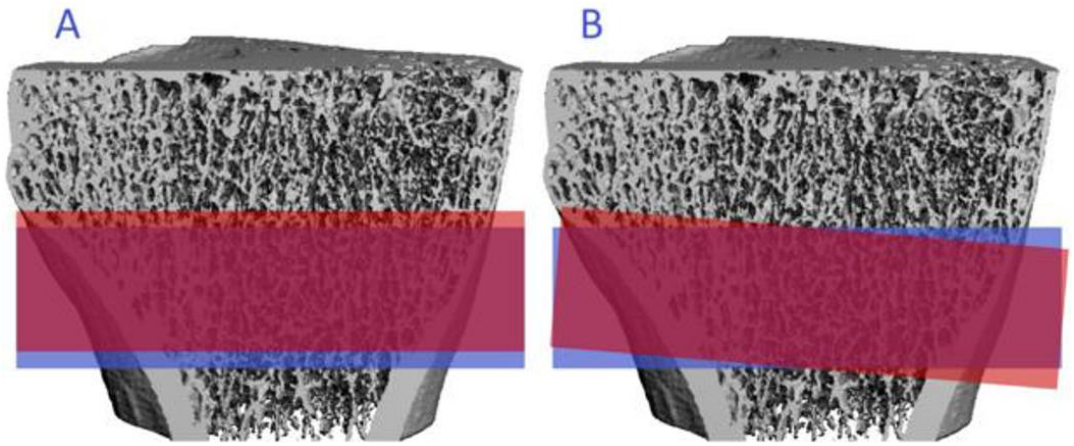

Figure 2-18. Examples of (A) axial and (B) angular offsets in scan regions captured at baseline (blue) and follow up (red) during a high-resolution peripheral quantitative computed tomography scan.

Reprinted from Osteoporosis International [119], with permission from Springer Nature.

There are two types of image registration: 2D image registration and 3D image registration.

2D image registration

2D image registration is a standard registration technique that uses the total cross-sectional area of each slice to determine an optimal offset between longitudinal scans. This results in a common volume of interest with a subset of the original slices acquired. In contrast, some scan data especially those from the periphery of the scan region of interest are lost. 2D registration allows for correcting changes in an axial direction, but cannot correct variation in tilt of the limb [124].

3D image registration

3D image registration takes into account both axial positioning and angular differences in images by determining a necessary 3D transform (translational and rotational) between the two images. 3D registration involves optimally overlapping all image data within a volume of interest and hence noise and structures outside of the region of interest doesn't interfere with the registration. The masks of the scan region between the two time points are transformed and the resulting 3D transform is used to identify a common region or volume between multiple

scans in a series. This allows to prevent image degradation associated in image interpolation. The common region or volume identified is then used to modify the contours of the cortex (Figure 2.19) [124], [125].

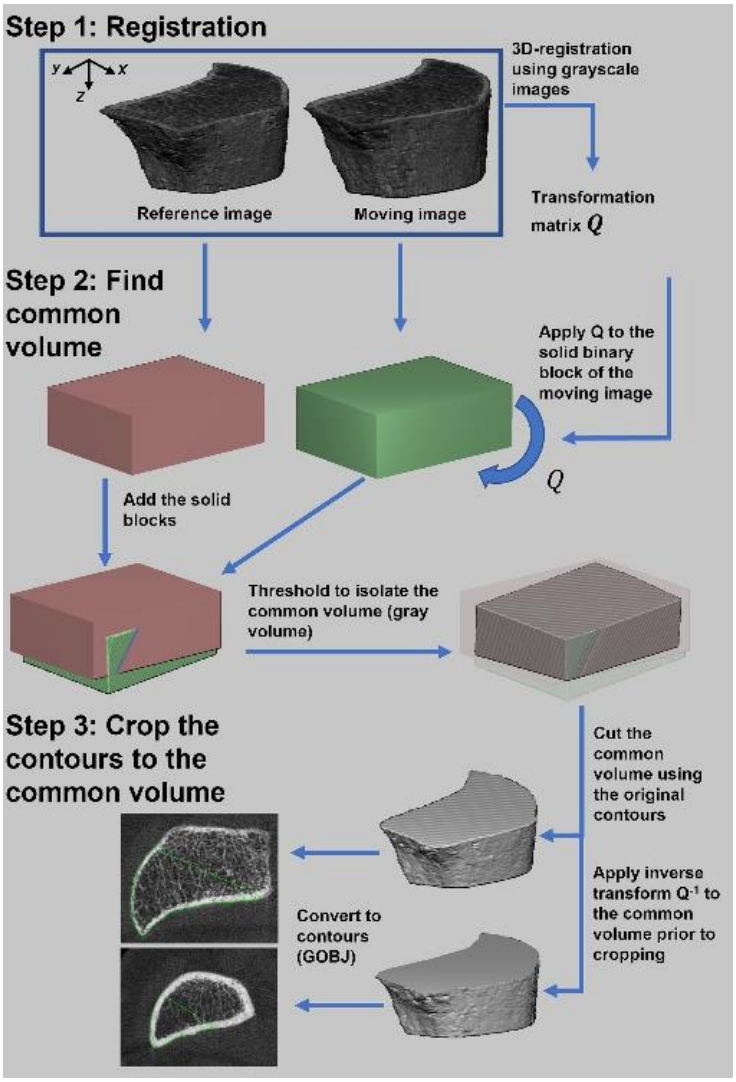

Figure 2-19. Workflow of the 3-dimensional image registration process.

Workflow of the 3D registration process to align two images and to find the common region or volume to measure density and microstructural outcomes. The first step was to find the transformation matrix that would align the moving image onto the reference image. In the second step, solid blocks were created and aligned to find the common volume. Finally, during the third step, the common volume was used to modify the original contour lines (ie, Scanco GOBJ files). Q = transformation matrix.

Reprinted from the Journal of Bone and Mineral Research [120], with permission from Oxford University Press.

3D image registration works great for longitudinal comparisons in morphological parameters. It allows to track local changes. Unfortunately, it doesn't work well for micro finite element analysis [124], [125]. The demarcation of boundary limits for non-parallel surfaces resulting from common regions of interest from 3D registration in micro finite element analysis is not trivial and the reproducibility of micro finite element analysis has not found to be greater with

3D registration compared to 2D registration. At the moment, literature recommends the use of 2D registration for micro finite element analysis and assessment of bone strength in longitudinal studies.

In the longitudinal study on T1D, 3D image registration was used to measure morphological changes in bone microstructure over time. To measure longitudinal changes in bone strength using micro finite element analysis, 2D image registration was used.

2.7.3 HR-pQCT outcomes

The following HR-pQCT outcomes were measured in this study.

Table 2-3. Outcomes of a HR-pQCT scan measured in this thesis.

Measurement	Abbreviation	Unit	Source of measurement
Volumetric total BMD	vBMD	mg HA/cm^3	Total mineral mass divided by the total bone volume
Trabecular vBMD	Tb.vBMD	mg HA/cm^3	Trabecular mineral mass divided by the volume inside the cortical bone
Trabecular thickness	Tb.Th	µm	Mean thickness of trabeculae within the trabecular compartment
Trabecular number	Tb.N	mm^{-1}	Mean number of trabeculae per mm within the trabecular compartment
Trabecular separation	Tb.Sp	µm	Mean distance between trabeculae within the trabecular compartment
Trabecular bone volume fraction	BV/TV	%	Derived by dividing Tb.vBMD by an assumed 100% mineralisation of 1200 mg HA/cm^3
Cortical vBMD	Ct.vBMD	mg HA/cm^3	Cortical mineral mass divided by the cortical volume
Cortical thickness	Ct.Th	µm	Mean thickness between the periosteal and endosteal surfaces
Cortical porosity	Ct.Po	%	In a given cortical region, cortical porosity = the volume of pores (Po.V, mm^3) / total volume of cortical bone compartment (Ct.V, mm^3)

2.7.4 Micro finite element analysis

The micro finite element analysis software was used to measure bone strength from the collected HR-pQCT data (version 1.13, FE-solver included in the image Processing Language Software, Scanco Medical AG, Zurich, Switzerland). The software uses mathematical modelling to simulate strength-determining biomechanical tests using micro-architectural features of trabecular and cortical bone. The software is fully automated and validated. It provides an in vivo assessment of bone strength [126].

2.7.4.1 Micro finite element analysis outcomes

The following Micro finite element analysis outcomes were measured in this study.

Table 2-4 Outcomes of micro finite element analysis measured in this thesis.

Outcome	Abbreviation	Unit	Source of measurement
Stiffness	Stiffness	kN/mm	Resistance to deformation when load is applied; total reaction force divided by displacement
Estimated failure load	Est.Fail.Load	kN	Maximum load the bone can bear before a fracture occurs; when 2% of the bone is strained beyond 3500 µstrain

2.8 Measurement of bone turnover markers and hormones important for bone remodelling

PINP, CTX, OC, BAP and TRAcP 5b were measured in serum / plasma using the IDS-iSYS Multi-Discipline Automated System (IDS, Boldon, UK). Intact PTH, 25-hydroxy vitamin D and 1,25-dihydroxy vitamin D were also measured in serum using the IDS-iSYS Multi-Discipline Automated System (IDS, Boldon, UK). This system uses a chemiluminescence immunoassay as its method of detection (Figure 2.20). This chemiluminescence immunoassay uses two antibodies for the measurement and detection; an anti-analyte antibody labelled with biotin and an antibody labelled with acridinium. The serum or plasma sample is loaded onto the autoanalyzer. Once the two antibodies are added, magnetic micro-particles coated with streptavidin bind to the biotin forming a complex. This mixture is incubated to allow the magnetic particles to bind to the magnet. This is followed by a wash step. After washing, the unbound substances are removed and the acridinium conjugate stimulates to emit light in the form of photons. The photons are detected by a luminometer within the wavelength of 300 – 500 nm. The reaction is measured and expressed as relative light units (RLUs). These RLUs detected are proportional to the concentration of the analyte present in the sample that is being measured.

The inter-assay CV for OC, CTX, BAP and TRAcP 5b was less than 5%, less than 7% for PTH, 25-hydroxy vitamin D and 1,25 –dihydroxy vitamin D, and less than 9% for PINP.

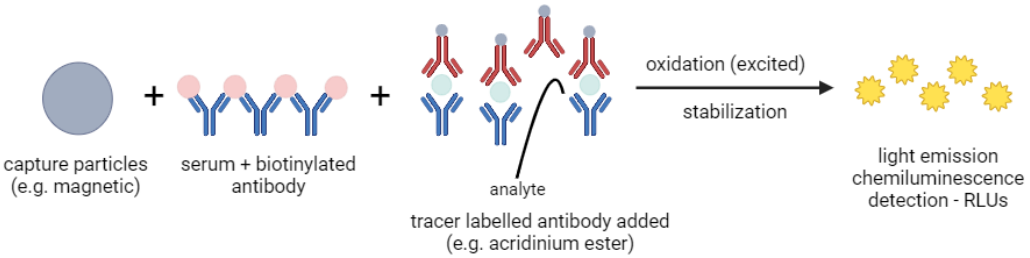

Figure 2-20. An overview of the principle behind chemiluminescence method of detection.

Illustration made using BioRender.com.

Figure 2-21. IDS IDS-iSYS Multi-Discipline Automated System.

[Source: IDS-iSYS Multi-Disciple Automated System Handbook]

2.9 Measurement of Sclerostin

Sclerostin was measured in fasted serum samples using a human sclerostin ELISA kit (Biomedica Medizinprodukte GmbH, Austria). This kit uses a method called sandwich enzyme immunoassay or ELISA for a quantitative measurement of human sclerostin. A sandwich ELISA is based on the principle of combination of specific antibodies (in this case, anti-human sclerostin antibodies) to detect the antigen (in this case, sclerostin) (Figure 2.22).

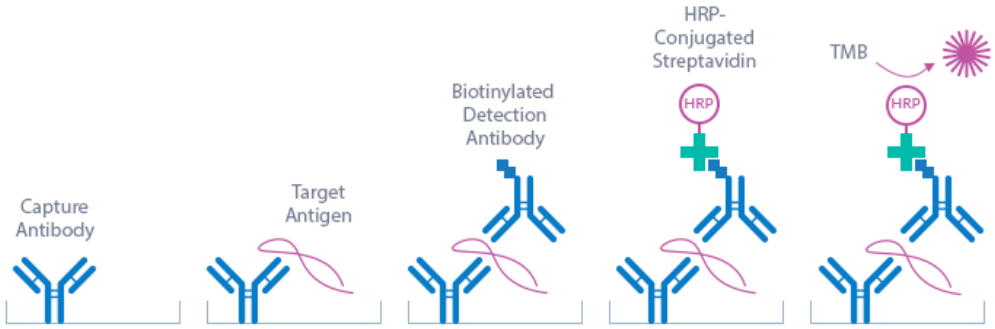

Figure 2-22. Principle of sclerostin sandwich ELISA.

Capture antibody: polyclonal goat anti-human Sclerostin antibody, Detection antibody: monoclonal mouse anti-human Sclerostin antibody, biotin labelled, Target antigen: human Sclerostin, HRP: horseradish peroxide, TMB: tetramethylbenzidine.

[Source: Sclerostin ELISA Handbook (BI-20492), Biomedica Medizinprodukte GmbH]

The assay is performed in several steps. In the first step, the assay buffer is pipetted into the wells of a 96-well plate. Next, a serum sample and detection antibody (biotin labelled monoclonal mouse anti-human sclerostin) is pipetted into the wells, which are pre-coated with polyclonal goat anti-human sclerostin antibody, called the capture antibody. Any sclerostin that is present in the serum sample binds to the pre-coated antibody in the well and forms a sandwich with the detection antibody. The next step involves washing, where all non-specific unbound material is removed. During step 4, the conjugate (streptavidin-HRP) is pipetted into the wells, and this streptavidin-HRP reacts with the detection antibody. Another washing step is done, following which the substrate (tetramethylbenzidine, TMB) is pipetted into the wells. The causes an enzyme-catalyzed color reaction of the substrate. The absorbance of the reaction is read at 450 nm using a standard microtiter plate reader. The colour emitted is directly proportional to the amount of sclerostin present in the serum sample. The concentration of human sclerostin in the serum is determined directly from a dose response curve drawn using standards provided in the kit (Figure 2.23). The inter assay CV for sclerostin was less than 10%.

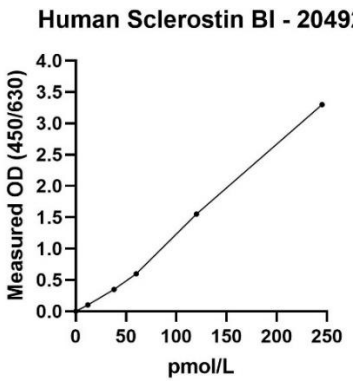

Figure 2-23. Standard curve for human sclerostin obtained from measurement using enzyme linked immunosorbent assay.

2.10 Measurement of microRNAs

Measurement of miRNAs involves a three step process: (i) isolation of RNA, (ii) cDNA synthesis and (iii) quantification of cDNA using real-time quantitative polymerase chain reaction (RT-qPCR).

The RNA template is first converted into complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for amplification using PCR. Real-time quantitative PCR allows for reliable, specific, sensitive, and reproducible quantification of RNA.

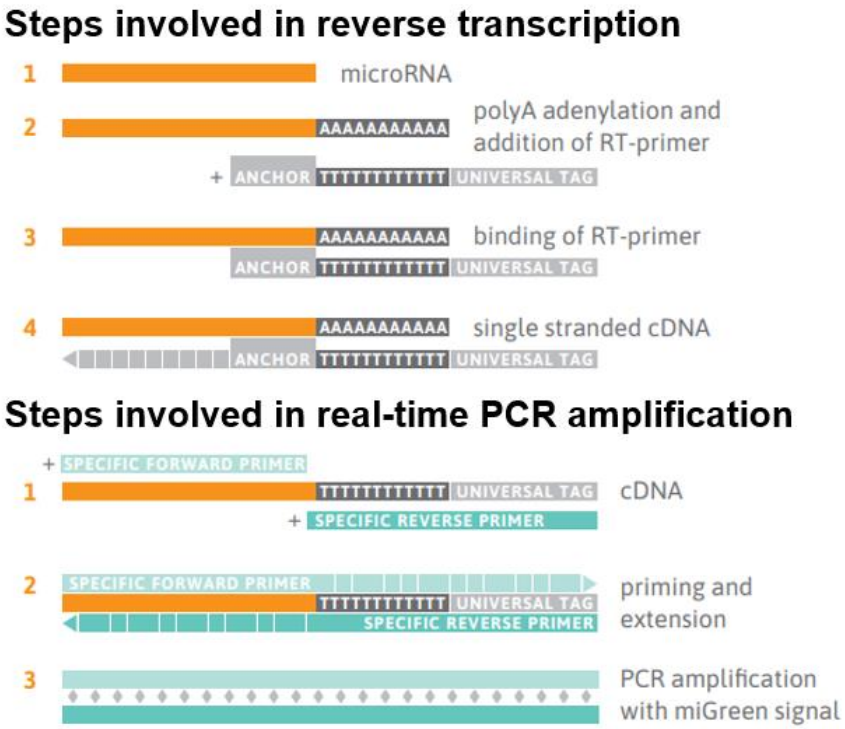

Figure 2-24. Principle of reverse transcription and product amplification using polymerase chain reaction.

[Adapted from the OsteomiR handbook, TAmiRNA GmbH, Austria]

2.10.1 Protocol used for measurement of miRNAs

<u>Isolation of RNA</u>

RNA was isolated from plasma using the miRNeasy Serum/Plasma Kit (TAmiRNA GmbH, Austria). The frozen plasma samples were thawed on ice and centrifuged for 5 min at 12,000 x g at 4°C. After centrifugation, 200 µL of plasma was added to 60 µL of lysis solution provided in the kit along with 1 µL of UniSp4 spike-in control and vortexed at high speed for 5s. UniSp4 spike-in control was used to monitor RNA isolation and quantification of cDNA using qPCR.

This was followed by incubation for 3 min at room temperature. After incubation, 20 µL of protein precipitation solution was added to the sample mixture and vortexed at high speed for 20 sec followed by incubation for 1 min at room temperature. The sample mixture was centrifuged at 12,000 x g for 3 min at room temperature, following which 200 µL of the upper aqueous phase of the sample mixture was separated. 2 µL of glycogen was added to the separated upper aqueous phase of the sample. The separated sample mixture was transferred to filter columns for precipitation of RNA. RNA was precipitated using 270 µL of isopropanol and wash solutions. The RNA was eluted in 30 µL of nuclease-free water and stored at -80 °C until further use.

cDNA synthesis

4 µL of isolated RNA was used for cDNA synthesis using the method of reverse transcription and the Universal cDNA Synthesis KIT II (TAmiRNA GmbH, Austria). Synthetic cel-miR-39-3p was added as a spike-in control to monitor enzyme inhibition during the process of conversion of RNA to cDNA. The reaction mixture was incubated at 42°C for 60 min, followed by heat inactivation at 95°C for 5 min. cDNA samples were stored at -20°C until further use.

qPCR

For qPCR analysis, the cDNA samples were diluted 50-fold and 5 µL of the cDNA sample was used in individual 10 µL PCR reactions using the ExiLENT SYBR Green Master Mix and LNA-enhanced miRNA primer assays along with UNISp3 as a spike in control (TAmiRNA GmbH, Austria). Custom made PCR plates (Qiagen) containing the 61 chosen miRNAs and 3 miRNA spike-in controls were used for the analysis. The PCR plates were measured using the LC 480 Real-Time PCR system (Roche) using the following conditions: 95 °C for 10 min, 40 cycles of denaturation (95 °C, 10s) and annealing (60 °C , 60s).

Quality check and quantification of miRNA expression

The presence of hemolysis in the serum / plasma sample has a substantial impact on the amount of cell-free miRNA present [127]. The presence of haemolysis in the serum / plasma samples was controlled by calculating the ratio of miR-23a-3p and miR-451a.

$$\text{Haemolysis ratio} = Cq_{\text{miR-23a-3p}} - Cq_{\text{miR-451a}}$$

miR-23a-3p is known to be less abundant in red blood cells while miR-451a is known to be highly abundant in red blood cells and therefore their ratio changes based on the degree of haemolysis present in the sample. Samples with haemolysis ratio greater than 7 were excluded from the analysis.

The quality of data collected from each sample was checked using synthetic miRNA spike-in controls during each step of the workflow: RNA isolation (UniSp4), cDNA synthesis (cel-miR-39-3p) and real time qPCR amplification (UniSp3). A melting curve analysis was done. The cycle of Cq values was calculated using the second derivative method.

Technical and manual handling variation between the samples was reduced by normalising the data collected. Data normalization can be done using 2 methods: normalization using an exogenous control (UniSp4) and global mean normalization.

Normalization using UniSp4 is done using the below equation.

$$\text{Normalised Cq} = \text{Cq}_{\text{Unisp4}} - \text{Cq}_{\text{measured miRNA}}$$

Normalization using the global mean approach is done using the below equation.

$$\text{Global mean normalised Cq} = \text{Raw Cq}_{\text{chosen miRNA}} - \text{mean Cq}_{\text{all measured miRNAs}}$$

miRNA data in the study on T1D was normalized using UniSp4 and data in the OPUS study was normalised using the global mean approach.

2.11 T-score bone marker plot

A mathematical model called the T-score bone marker plot previously described by our research group [128] was used to demonstrate a relationship between bone turnover and bone balance in type 2 diabetes.

This method uses the T-scores of the bone formation marker PINP and bone resorption marker CTX to calculate bone turnover and bone balance [128], allowing them to be assessed simultaneously in a single model.

Step 1: Logarithmic transformation

The bone formation marker PINP and bone resorption marker PINP are $\log_{10}$-transformed and normalised to a young, healthy population of 226 premenopausal women aged between 30 and 39.

Step 2: Calculation of T-scores for PINP and CTX

The T-scores of PINP and CTX for the study population were calculated from the mean and standard deviation of the results of the premenopausal women.

$$\text{T-score} = \text{BTM} - \text{mean BTM} / \text{standard deviation BTM}$$

Step 3a: Calculation of bone turnover from the T-scores of bone formation (PINP) and bone resorption (CTX)

Bone turnover = (T-score $_{\text{bone formation}}$ + T-score $_{\text{bone resorption}}$) / 2

Step 3b: Calculation of bone balance from the T-scores of bone formation (PINP) and bone resorption (CTX)

Bone balance = T-score $_{\text{bone formation}}$ – T-score $_{\text{bone resorption}}$

Representation of data using a 95% confidence ellipse

Bone turnover and bone balance obtained after log transformation of the bone turnover markers (PINP and CTX) were plotted against each other forming a four-quadrant graph (Figure 2.25). A bivariate 95% confidence ellipse was drawn to surround the data for patients with type 2 diabetes and healthy controls. The Mahalanobis distance between the centres of the ellipses of the 2 study groups was calculated as described in [129]. It involves a multi-dimensional measurement of a number of standard deviations from the centre of the premenopausal ellipse to the centre of the ellipse for the 2 study groups, along with taking into account the covariance between bone turnover and bone balance.

Example: When sizes are defined as n_1 and n_2, means as $\bar{x}_1$ and $\bar{x}_2$ and variances as S_1 and S_2, the Mahalanobis distance is calculated using the equation:

$D^2=(\bar{x}_1-\bar{x}_2)S^{-1}(\bar{x}_1-\bar{x}_2)^T$, where S is the pooled variance from the covariance matrix

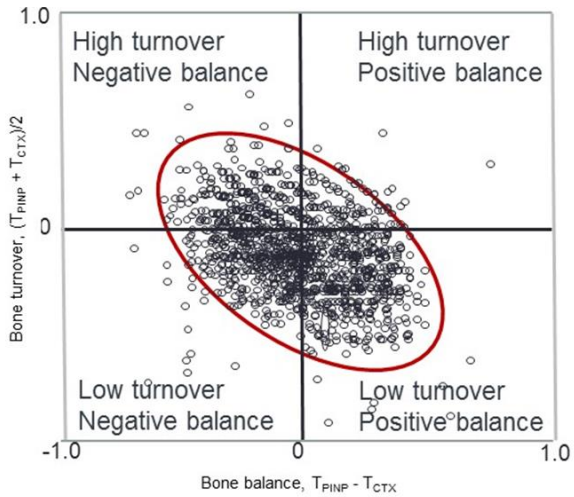

Figure 2-25. An illustration of the T-score bone marker plot.

Bone turnover and bone balance calculated from the T-scores for PINP and CTX were plotted against each other for each individual and a bivariate 95% confidence ellipse surrounded the data.

PINP: Procollagen type I N-terminal propeptide, CTX: cross-linked C-telopeptide of type I collagen.

Open access, White Rose eTheses Online [130].

Chapter 3 – MicroRNAs in diabetic bone disease: a systematic review of literature

3.1 Introduction

Diabetes mellitus and osteoporosis are regarded as important public health concerns associated with high healthcare costs, mortality and morbidity [131]. The alarming global prevalence of diabetes goes hand in hand with the increase in osteoporotic fractures. By 2025, the projections for hip fractures are expected to increase to 2.6 million [132]. Emerging evidence from large observational studies supports a direct concerning and detrimental impact of diabetes, both type 1 and type 2 diabetes, on skeletal health.

In Chapter 1, I have shown evidence for both T1D and T2D to be characteristic of an increased risk for hip fractures and other non-vertebral fractures. I have also shown evidence for low aBMD in T1D to be one of the reasons for the increase in fracture risk. However, in patients with T2D, their normal or high bone mineral density contradicts the increase in fracture risk. Thus, it becomes obvious that, especially in T2D, the measurement of bone density underestimates true fracture risk.

The International Osteoporosis Federation recommends using standard references of bone turnover markers (PINP and CTX) to monitor response to osteoporosis treatment in routine clinical practice [65]. However, in a population of T2D, part of the Health, Ageing and Body Composition (Health ABC) Study, PINP, OC and CTX were not associated with increased fracture risk [133]. This calls for additional measures for better fracture risk prediction in people with diabetes. In this context, miRNAs are of particular interest.

Recently, miRNAs have received much attention as non-invasive biomarkers for disease diagnosis, including diabetes and osteoporosis. In Chapter 1, I have shown evidence for the role of miRNAs in diabetes and bone metabolism and their capability of being suitable biomarkers.

At present in literature, there are very few studies that comment on the role of miRNAs in both diabetes (T1D and T2D) and bone health and their association with markers of bone strength and metabolism. Rather, literature provides substantial evidence on the role of miRNAs in T1D and T2D, and osteoporosis, fractures, aBMD and bone turnover, individually. Hence, the aim of this chapter was to perform a systematic review to identify miRNAs that are differentially expressed both in diabetes and in abnormal bone phenotype (fractures, aBMD, bone turnover).

3.2 Methods

The protocol for this systematic review was registered with PROSPERO (CRD42020212451).

3.2.1 Search strategy and selection criteria

The search strategy was to identify miRNAs previously reported to be differentially expressed in diabetes mellitus (T1D and T2D) and abnormal bone phenotypes (including fractures, bone turnover, and aBMD). The search was conducted using three databases: PubMed, Ovid MEDLINE, and Web of Science. The search was performed in two parts, independent of each other.

The searches were limited to observational studies, human subjects >18 years of age, and the English language and were completed in November 2020. In search part 1, miRNAs differentially expressed in diabetes mellitus were captured. In search part 2, miRNAs differentially expressed in abnormal bone phenotype were captured. The MeSH terms used for both searches have been previously mentioned in Chapter 2. Experts in the field were consulted for additional relevant articles.

The whole list of inclusion and exclusion criteria applied for this systematic review has been mentioned in Chapter 2. In short, for both searches, studies that did not include a control group lacked clear case description/diagnosis, did not report any significance (*p<0.05/95% CI) in miRNA expression levels, studies on miRNA expression level prediction using bioinformatics and algorithm-based tools, narrative reviews, editorial letters or commentary articles were excluded. Additionally, studies on animal models and cell line-based models were excluded.

In search part 1, original articles that analysed miRNA expression in patients with diabetes ((both T1D and T2D, including its three well-characterised complications, namely retinopathy, neuropathy and nephropathy) (cases)) and non-diabetic/healthy subjects (controls) were included. Studies related to other diabetic complications were excluded. In search part 2, original articles that analysed miRNA expression in people with low aBMD ((in patients with osteoporosis or osteopenia), previous fractures (> 6 months since fracture), abnormal / low bone turnover (cases)) and people with normal aBMD/healthy subjects (controls) were included. Studies on fracture healing were excluded.

The identified searches were reviewed in line with the inclusion and exclusion criteria at the title and abstract level by one reviewer and was checked by the second reviewer. Disagreements if any were resolved through discussion or a third reviewer was involved.

3.2.2 Data extraction, quality assessment, sensitivity analysis and data analysis

The selected list of articles were reviewed at the level of full-text and this was also verified by a second reviewer. Data was extracted from the eligible studies using a data extraction form and similarly here, the extracted data was verified by a second reviewer. These studies reviewed at the full-text level were included in this review and information on miRNAs from these studies were used to identify the potential miRNA overlaps between the two study searches. Disagreements at any step were resolved through discussion or involvement of a third reviewer. The BioVenn online tool [110] was used to identify the dysregulated miRNAs overlapping between the two study searches, diabetes and altered bone phenotype.

The eligible studies were subject to quality assessment using the Newcastle-Ottawa Scale (NOS). As described in Chapter 2, 2 more additional questions were added to assess the quality of miRNA measurements in the eligible studies. The eligible studies were further subject to a sensitivity analysis scored based on the 4 parameters mentioned in Chapter 2. The studies were scored out of a total of 13.

miRNet 2.0, a network-based visual analytics platform was used to perform a miRNA functional analysis and gather information on the organs these chosen miRNAs are abundant in and the diseases they target [111].

3.3 Results

3.3.1 Search part 1 – miRNAs in T1D and T2D

The search and study selection process of primary studies included in search part 1, miRNAs in T1D and T2D, is described in Figure 3.1 as a PRISMA diagram. 3286 studies were identified. After removing the duplicates, 3106 studies were excluded after assessment at their title and abstract level. The remaining 180 studies were subject to assessment at the full-text level and 102 studies met the inclusion criteria. miRNA expression levels analysed in these 102 studies and those found to be significant ($p < 0.05$) were included to identify a potential overlap with search part 2.

Of these 102 studies, 4 studies did not mention the type of diabetes the miRNAs were identified in [134]–[137]. 2 studies mentioned the use of miRNAs as potential biomarkers in both T1D and T2D [138], [139]; 4 studies mentioned the use of miRNAs as potential biomarkers in T1D alone [140]–[143] and the remaining studies mentioned about the use of miRNAs as potential biomarkers in T2D.

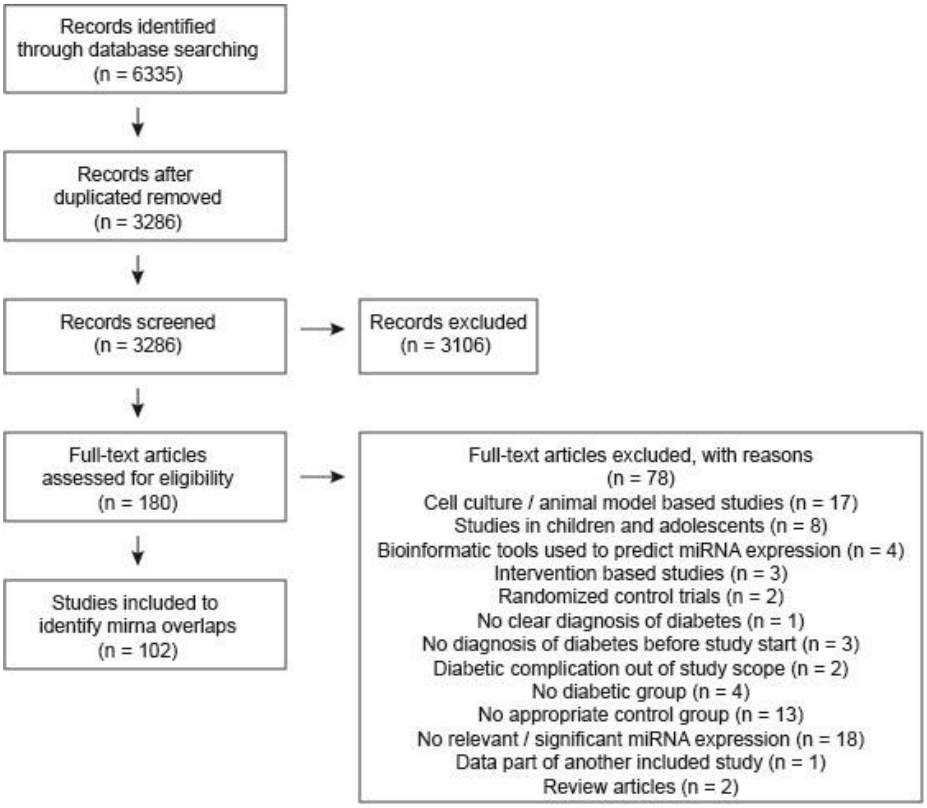

Figure 3-1. PRISMA flowchart of search part 1 - microRNAs in type 1 and type 2 diabetes.

3.3.2 Search part 2 – miRNAs in abnormal bone phenotype

The search and study selection process of primary studies included in search part 2, miRNAs in abnormal bone phenotype (fractures, BMD, bone turnover), is described in Figure 3.2 as a PRISMA diagram. 7129 studied were identified. After removing the duplicates, 4115 studies were excluded after assessment at their title and abstract level. The remaining 150 studies were subject to assessment at the full-text level and 45 studies met the inclusion criteria. miRNA expression levels analysed in these 45 studies and those that were found to be significant ($p < 0.05$) were included to identify a potential overlap with search part 2.

Of these 45 studies, 14 studies [144]–[157] did not mention how osteoporosis or osteopenia was diagnosed. In the remaining studies, osteoporosis was diagnosed using BMD T-scores.

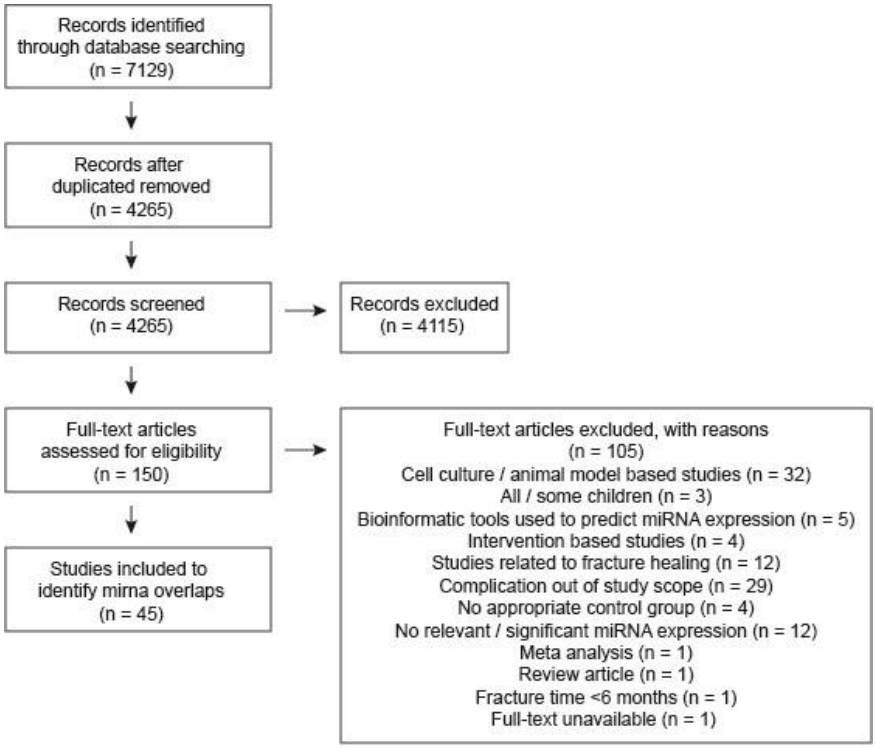

Figure 3-2. PRISMA flowchart of search part 2 - microRNAs in abnormal bone phenotype.

3.3.3 Extracted data from the eligible studies

Data from the eligible studies of both searches was extracted using a self-made form. Table 3.1 summarizes the dysregulated miRNAs and characteristics of eligible studies identified in search part 1 (Diabetes) and Table 3.2 summarises the dysregulated miRNAs and characteristics of eligible studies identified in search part 2 (altered bone phenotype).

Table 3-1. Dysregulated microRNAs and characteristics of eligible studies identifies in search part 1 (type 1 and type 2 diabetes).

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
MicroRNA-194 Modulates Glucose Metabolism and Its Skeletal Muscle Expression Is Reduced in Diabetes [158]	5 pre-diabetes 6 T2D 5 HC	pre-diabetes 52 ± 2 T2D 53 ± 2 HC 48 ± 3	M	T2D	NA	OGTT, fasting glucose	microarray and RT-PCR	skeletal muscle	miR-194 - downregulated
Plasma levels of miR-29b and miR-200b in type 2 diabetic retinopathy [159]	Total of 186 all T2D Breakdown: 91 T2D wo DR 46 NPDR 49 PDR 20 HC	T2D wo DR 60.3 ± 8.3 NPDR 60 ± 9.2 PDR 63.8 ± 6.6	M & F	T2D	retinopathy	ADA guidelines	RT-PCR	plasma	miR-200b - downregulated HC vs T2D without DR
Type 2 Diabetes Monocyte MicroRNA and mRNA Expression: Dyslipidemia Associates with Increased Differentiation-Related Genes but Not Inflammatory Activation [160]	34 T2D 25 HC validation cohort 64 T2D 44 HC	T2D 61 (37 – 85) HC 44 (32-87)	M & F	T2D	NA	ADA guidelines	microarray and RT-PCR	monocytes	miR-34c-5p, miR-576-3p - upregulated
MiR-92a contributes to the cardiovascular disease development in diabetes mellitus through NF-κB and downstream inflammatory pathways [161]	69 T2D 68 HC	T2D 64.29 ± 3.77 HC 62.98 ± 7.42	M & F	T2D	NA	self-report and confirmed with medical records	RT-PCR	serum	miR-92a - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Circulating ectosomes: Determination of angiogenic microRNAs in type 2 diabetes [162]	15 T2D 15 HC	T2DM 67 (65-71) HC 65 (63-71)	M & F	T2D	NA	OGTT Polish Diabetes Association Guidelines	microarray and RT-PCR	platelet depleted plasma ectosome enriched miRNA	miR-193b-3p, miR-95-3p, let-7i-5p, miR-199a-3p, miR-26b-5p, miR-30b-5p, miR-374a-5p - upregulated miR-409-3p - downregulated
MicroRNA-19a-3p enhances the proliferation and insulin secretion, while it inhibits the apoptosis of pancreatic β cells via the inhibition of SOCS3 [163]	45 T2D 20 HC	NA	NA	T2D	NA	NA	RT-PCR	plasma	19a-3p - downregulated
Plasma-derived exosome characterization reveals a distinct microRNA signature in long duration Type 1 diabetes [140]	36 T1D 36 HC	T1D 46.1 ± 14.4 HC 44.2 ± 10.8	M & F	T1D	NA	NA	microarray and RT-PCR	plasma	miR-16-5p, miR-302d-3p, miR-574-5p - downregulated
Diabetes induces the activation of pro-ageing miR-34a in the heart, but has differential effects on cardiomyocytes and cardiac progenitor cells [164]	11-14 T2D (in different durations of T2D) 32 HC	NA	M & F	T2D	NA	NA	RT-PCR	plasma, deceased human myocardial tissue	miR-34a - upregulated
Increasing the miR-126 expression in the peripheral blood of patients with diabetic foot ulcers treated with maggot debridement therapy [165]	93 T2D 79 HC	HC female 62.1 ± 8.2 Male 63.5 ± 7.4 T2D male 66.7 ± 6.8 Female 65.3 ± 7.5	M & F	T2D	NA	OGTT, random plasma glucose	RT-PCR	plasma	miR-126 - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Plasma Exosome MicroRNA Profiling Unravels a New Potential Modulator of Adiponectin Pathway in Diabetes: Effect of Glycemic Control [166]	18 T2D 12 HC	T2D 57.2 ± 9.6 HC 48.5 ± 12.4	M & F	T2D	NA	NA	microarray and RT-PCR	plasma/PBMC	miR-326 - upregulated let-7a, let-7f - downregulated
Circulating miRNA Profiles in Patients with Metabolic Syndrome [167]	46 HC 50 T2D	HC 45.7 ± 11.3 T2D 44.2 ± 8.4	NA	T2D	NA	fasting glucose		blood	miR-17, miR-197, miR-509-5p, miR-652, miR-130a, miR-92a - downregulated miR-150, miR-192, miR-27a, miR-320a - upregulated
Role of MicroRNA 126 in Screening, Diagnosis, and Prognosis of Diabetic Patients in Egypt [168]	100 HC 100 T2D	HC 45.7 ± 8.4 T2D 48.2 ± 8.2	M & F	T2D	retinopathy, nephropathy, neuropathy	ADA guidelines	RT-PCR	serum	miR-126 - downregulated
Salivary microRNA 155, 146a/b and 203: A pilot study for potentially non-invasive diagnostic biomarkers of periodontitis and diabetes mellitus [134]	10 diabetes 14 non-diabetes	non-diabetes 25.92 ± 6.72 diabetes 32.4 ± 14.38	M & F	NA	NA	HbA1c	RT-PCR	saliva	miR-155, miR-146,a miR-146b, miR-203 - downregulated
Clinical Value of Circulating miR-1 and miR-21 in Evaluating the Diagnosis of Acute Heart Failure in Asymptomatic Type 2 Diabetic Patients [169]	45 HC 45 T2D	HC 60.23 ± 6.27 T2D 61.50 ± 5.08	M & F 22/23 each group	T2D	NA	OGTT (controls) and ADA guidelines (DM)	RT-PCR	serum	miR-1 - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Differential expression of microRNAs in plasma of patients with prediabetes and newly diagnosed type 2 diabetes [170]	50 HC 50 T2D	HC 45.52 ± 6.215 T2D 46.22 ± 6.897	M & F	T2D (newly diagnosed)	NA	plasma glucose	microarray and RT-PCR	plasma	miR-1249, miR-320b - downregulated miR-572 - upregulated
Change in circulating microRNA profile of obese children indicates future risk of adult diabetes [171]	101 diabetes 82 NGT	diabetes 57.5 ± 12.2 NGT 49.3 ± 7.73	NA	T2D	NA	FPG	RT-PCR	serum	miR-15b, miR-146b, miR-486 - upregulated
Unique molecular signatures of microRNAs in ocular fluids and plasma in diabetic retinopathy [138]	11 Controls wo diabetes undergoing eye surgery 5 T1D PDR 7 T2D PDR 4 T2D NPDR	30-80	M & F	T1D T2D	retinopathy	NA	microarray and RT-PCR	plasma, aqueous humor and vitreous humor	let-7b, let-7c - downregulated T2D-PDR T2D-NPDR
Down-regulation of miR-23a inhibits high glucose-induced EMT and renal fibrogenesis by up-regulation of SnoN [172]	10 T2D nephropathy 10 HC from nephrectomy as part of their tumor treatment	T2D nephropathy 51 ± 12	7 M 3 F	T2D	nephropathy	NA	RT-PCR	kidney tissue	miR-23a - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
The variant at TGFBRAP1 is significantly associated with type 2 diabetes mellitus and affects diabetes-related miRNA expression [173]	468 T2DM 2855 NGT	T2D 61.61 ± 10.21 NGT 60.29 ± 10.74	M & F	T2D	NA	fasting glucose	microarray and RT-PCR	plasma	miR-150-5p, miR-328-3p, miR-335, miR-30b-5p, miR-93-5p, miR-320a, miR-139-5p, miR-191-5p, miR-574-3p, miR-720 - upregulated miR-511-5p, miR-126-3p - downregulated
A unique plasma microRNA profile defines type 2 diabetes progression [174]	9 NGT 9 T2D	NGT 57.9 ± 8.9 T2D 60.2 ± 8	M & F	T2D	NA	OGTT	microarray	plasma	miR-148a-3p - upregulated miR-222-3p, miR-342-3p - downregulated
Circulating levels and characterization of microparticles in patients with different degrees of glucose tolerance [175]	53 NGT 68 T2D	NGT 57 ± 1 T2D 60 ± 1	M & F	T2D	NA	OGTT/HOMA IR to assess NGT/PreDM FPG to assess PreDM. DM diagnosis - no clear description	RT-PCR	plasma microparticles	miR-126-3p - downregulated
Expression of miR-18a and miR-34c in circulating monocytes associated with vulnerability to type 2 diabetes mellitus and insulin resistance [176]	117 T2DM newly diagnosed 105 HC	30-65	M & F	T2D	NA	FPG	RT-PCR	PBMC	miR-34c - downregulated miR-18a - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Serum microRNA profiling and bioinformatics analysis of patients with type 2 diabetes mellitus in a Chinese population [177]	10 T2DM 5 HC	T2D 48-66 (58.2±7.7 years) HC 51-61 (56.4±3.7 years)	M & F	T2D	NA	ADA guidelines	RT-PCR array	serum	miR-409-3p, miR-665, miR-766-3p - downregulated miR-455-5p, miR-454-3p, miR-144-3p, miR-96-5p - upregulated
Circulating and visceral adipose miR-100 is down-regulated in patients with obesity and Type 2 diabetes [178]	16 T2D 16 HC	30-60	NA	T2D	lean and obese	FPG, HbA1c	RT-PCR array	blood	miR-100, miR-210, miR-378, miR-181a - downregulated in obese and lean diabetes miR-125b - downregulated in lean diabetes
Molecular and functional characterization of circulating extracellular vesicles from diabetic patients with and without retinopathy and healthy subjects [141]	7 HC 7 T1D 7 T1DR	HC 41.0 ± 10.6 T1D 46.1 ± 11.7 T1DR 39.3 ± 5.9	M & F	T1D	retinopathy	NA	microarray and RT-PCR	plasma	T1D and T1DR miR-21-3p - upregulated T1DR miR-150-5p, miR-30b-5p - downregulated
Expression Level of Circulating Cell Free miR-155 Gene in Serum of Patients with Diabetic Nephropathy [179]	61 T2DM 22 HC	general mean age 55.65 ± 10.48	M & F (Ref 38)	T2D	nephropathy	ADA guidelines	RT-PCR	serum	miR-155 - downregulated
T2DM inhibition of endothelial miR-342-3p facilitates angiogenic dysfunction via repression of FGF11 signaling [180]	18 T2D 15 HC	NA	NA	T2D	NA	NA	RT-PCR	CD34+ cells	miR-342-3p - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	11 T1D 9 HC	T1D 23.5 ± 3.9 HC 25.1 ± 3.2	M & F	T1D	NA	NA	micro array	PBMC	let-7f let-7g miR-101 miR-10a miR-126 miR-126*, miR-1275, miR-140-3p, miR-148a, miR-148b, miR-15b*, miR-16, miR-186, miR-18b, miR-195, miR-199a-3,p miR-19a, miR-20a*, miR-20b, miR-21, miR-26b, miR-27b, miR-201a, miR-30e*, miR-32, miR-324-5p, miR-335, miR-338-3p, miR-33,a miR-340*, miR-342-3p, miR-423-5p, miR-424, miR-450a, miR-454, miR-542-3p, miR-548c-3p, miR-7, miR-720, miR-766, miR-940, miR-98 - upregulated
Circulating endothelium-enriched microRNA-126 as a potential biomarker for coronary artery disease in type 2 diabetes mellitus patients [181]	45 T2D 45 HC	T2D 61 ± 12 HC 53 ± 8.6	M & F	T2D	NA	OGTT fasting glucose HbA1c	RT-PCR	blood	miR-126 - downregulated
Impaired miR-146a expression links subclinical inflammation and insulin resistance in Type 2 diabetes [182]	20 T2D 20 NGT	NGT 42.0 ± 4.7 T2D 43.7 ± 5.1	NA	T2D	NA	plasma glucose OGTT	RT-PCR	PBMC	miR-146a - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
miRNA signatures of insulin resistance in obesity [183]	15 T2D with obesity 12 controls	T2D with obesity 45 ± 8 controls 44 ± 9	F	T2D	obesity	fasting glucose HbA1c and were taking oral 8 on hypoglycemics 1 diet-controlled T2D	micro array	plasma	let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, miR-15b, miR-23a, miR-23b, miR-26a, miR-26b, miR-28-5p, miR-29a-5p, miR-29b-2-5p, miR-30e-3p, miR-99b, miR-103, miR-139-5p, miR-151-3p, miR-151-5p, miR-155, miR-181a, miR-182, miR-197, miR-200c, miR-374b, miR-485-3p, miR-584, miR-2110 - upregulated miR-19a, miR-19b, miR-29c, miR-32, miR-34a, miR-101, miR-122, miR-136, miR-144, miR-193b, miR-365, miR-451, miR-497 - downregulated
Determination of 14 Circulating microRNAs in Swedes and Iraqis with and without Diabetes Mellitus Type 2 [184]	33 T2D 119 control	45-65	M & F	T2D	NA	OGTT Plasma glucose for controls	RT-PCR	plasma	miR-15a, miR-24, miR-320a - upregulated
Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study [185]	19 NGT 18 T2D	NGT 41.00 ± 2.616 T2D 47.33 ± 2.617	M & F	Recent diagnosis of T2D	NA	OGTT and FPG	RT-PCR	serum	miR-29a, miR-34a, miR-146a, miR-375, miR-30d, miR-9, miR-124a - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Decreased expression of microRNAs targeting type-2 diabetes susceptibility genes in peripheral blood of patients and predisposed individuals [186]	37 control 40 T2D	Control 49 (19–69) T2D 59 (35–75)	M & F	T2D	NA	Medical records	RT-PCR	blood	let-7b-5p, miR-1-3p, miR-24-3p, miR-34a-5p, miR-98-5p, miR-133a-3p - downregulated
MicroRNA-342, microRNA-191 and microRNA-510 are differentially expressed in T regulatory cells of type 1 diabetic patients [143]	5 T1D 6 HC	T1D 19-56	M & F	T1D	NA	NA	array	Treg cells	miR-342, miR-191 - downregulated
Circulating miR-103 family as potential biomarkers for type 2 diabetes through targeting CAV-1 and SFRP4 [187]	50 HC 48 diabetes	HC 45.62 ± 8.58 Diabetes 52.6 ± 9.13	M & F	T2D	NA	OGTT	RT-PCR	plasma	miR-103a - upregulated miR-103b - downregulated
Platelet-derived miR-103b as a novel biomarker for the early diagnosis of type 2 diabetes [188]	46 HC 43 T2D	HC 51.4 ± 9.4 T2D 64.8 ± 9.8	NA	T2D	NA	OGTT, FPG	RT-PCR	leukocyte-depleted platelets	miR-103b - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Upregulation of Circulating Cardiomyocyte-Enriched miR-1 and miR-133 Associate with the Risk of Coronary Artery Disease in Type 2 Diabetes Patients and Serve as Potential Biomarkers [189]	30 T2D 30 HC	T2D 58 ± 11.5 HC 56 ± 5.1	M & F	T2D	NA	OGTT, HbA1c, FPG	RT-PCR	blood	miR-1, miR-133 - upregulated
Roles of microRNA-124a and microRNA-30d in breast cancer patients with type 2 diabetes mellitus [190]	36 T2D 36 HC	T2D 53.7 ± 9.8 HC 54.8 ± 9.2	F	T2D	NA	medical history/self report and FPG, random glucose or OGTT	RT-PCR	blood	miR-124a, miR-30d - upregulated
Changes of serum Mir-217 and the correlation with the severity in type 2 diabetes patients with different stages of diabetic kidney disease [191]	495 T2D 195 HC	T2D 55.64 ± 12.24	M & F	T2D	nephropathy	ADA 2010 guidelines	RT-PCR	serum	miR-217 - upregulated increasing with T2D micro and micro albuminuria
Increased MicroRNA-146a Levels in Plasma of Patients with Newly Diagnosed Type 2 Diabetes Mellitus [192]	90 T2D 90 HC	T2D 48.50 (42.00-56.00) HC 48.00 (41.75-55.00)	M & F	T2D newly diagnosed	NA	OGTT and/or FPG	RT-PCR	plasma	miR-146a - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Plasma level of miR-93 is associated with higher risk to develop type 2 diabetic retinopathy [193]	140 T2D 75 with DR 65 without DR 127 HC	T2D 25-72 HC 23-76; 47.25 ± 9.75	M & F	T2D	retinopathy	WHO	RT-PCR	plasma	miR-93 in DR and NDR - upregulated DR vs NDR sig different
Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-hyperglycemic patients with type 2 diabetes mellitus [194]	27 T2D 16 HC	T2D 52.8 ± 9.1 HC 52.9 ± 6.7	M & F	T2D	NA	HbA1c and glucose	micro array	PBMC	T2D-H miR-186, miR-222, miR-29b, miR-342-3p - upregulated miR-199a-3p, miR-199a-5p - downregulated T2D-N miR-101, miR-10a*, miR-1260, miR-142-5p, miR-146b-5p, miR-186, miR-222, miR-29b, miR-302b, miR-30e, miR-32, miR-338-3p, miR-424 - upregulated miR-107, miR-148b, miR-151-5p, miR-224, miR-23b - downregulated
Co-expression analysis among microRNAs, long non-coding RNAs, and messenger RNAs to understand the pathogenesis and progression of diabetic kidney disease at the genetic level [137]	85 in total	NA	NA	diabetes	nephropathy CKD	NA	micorarray and RT-PCR	plasma	miR-223-3p - downregulated further downregulated in CKD

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Expression and DNA methylation status of microRNA-375 in patients with type 2 diabetes mellitus [195]	100 T2D 100 NGT	T2D 51.33 ± 11.75 NGT 48.55±12.41	M & F	T2D	NA	WHO criteria	RT-PCR	plasma	miR-375 - upregulated
Circulating Exosomal miR-20b-5p Is Elevated in Type 2 Diabetes and Could Impair Insulin Action in Human Skeletal Muscle [196]	20 NGT 21 T2D	NGT 60 ± 1 T2D 61 ± 1	M	T2D	NA	NA	microarray RT-PCR	serum exosomes	miR-20b-5p, miR-150-5p - upregulated
MicroRNAs from urinary extracellular vesicles are non-invasive early biomarkers of diabetic nephropathy in type 2 diabetes patients with the 'Asian Indian phenotype' [197]	40 NGT 40 T2D 40 T2D micro albuminuria 40 T2D macro albuminuria	NGT 44 ± 6.3 T2D 46 ± 7.7 T2D micro albuminuria 51 ± 6.0 T2D macro albuminuria 54 ± 6.2	NA	T2D	nephropathy	WHO guidelines	microarray and RT-PCR	urine Evs	miR-135b-5p - upregulated in T2D-NA miR-15b-5p - upregulated in T2D neuropathy let-7i-5p, miR-24-3p, miR-27b-3p, miR-30a-5p, miR-135b-5p - downregulated in T2D nephropathy
Altered circulatory levels of miR-128, BDNF, cortisol and shortened telomeres in patients with type 2 diabetes and depression [198]	40 NGT 40 T2D	NGT 48±10 T2D 54±6.0	M & F	T2D	NA	WHO guidelines OGTT	RT-PCR	serum	miR-128 - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Circulatory miR-98-5p levels are deregulated during diabetes and it inhibits proliferation and promotes apoptosis by targeting PPP1R15B in keratinocytes [199]	5 HC 5 T2D	HC 34 ± 6.26 T2D 42 ± 7.16	NA	T2D	NA	fasting glucose	microarray RT-PCR	serum	miR-98-5p, miR-423-3p, miR-6131, miR-1246 - downregulated miR-99b-5p, miR-1304-5p, miR-302d-3p - upregulated
Identification of the differential expression of serum microRNA in type 2 diabetes [200]	Screening 56 HC 40 T2D Validation T2D 69 HC 74	screening 63 control 58.7 T2D Validation 60.8 control 61.1 T2D	M & F	T2D	NA	blood glucose ≥11.1 mmol/L fasting blood glucose (FBG) ≥7 mmol/L glucose tolerance testing 2-h blood glucose ≥11.1 mmol/L	microarray RT-PCR	serum	miR-320d - upregulated miR-3960, miR-451a, miR-572, miR-4534 - downregulated
Profiling of Circulating MicroRNAs Reveals Common MicroRNAs Linked to Type 2 Diabetes That Change With Insulin Sensitization [201]	45 NGT 48 T2D	NGT 38-65 (48.1 6+/-10.1) T2D 44-65 (54 +/-10)	M	T2D	obesity	NA	RT-PCR	plasma	miR-140-5,p miR-142-3p, miR-222 - upregulated miR-423-5p, miR-125b, miR-130b, miR-126a, miR-192, miR-195 - downregulated miR-532-5p downregulated only in control vs T2D wo obesity

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
MicroRNA signature in patients with hepatocellular carcinoma associated with type 2 diabetes [202]	200 T2D 225 HC	HC 49.92 ± 7.14 T2D 53.68 ± 9.48	NA	T2D	NA	NA	microarray RT-PCR	serum	miRNA-16, miRNA-23b-3p, miRNA-34a, miRNA-199a-3p, miRNA-221 - upregulated miRNA-122-5p, miRNA-198 - downregulated
Serum miR-23a, a potential biomarker for diagnosis of pre-diabetes and type 2 diabetes [203]	20 NGT 24 T2D	NGT 46.65 ± 16.181 T2D 51.13 ± 9.214	M & F	T2D	NA	fasting glucose, OGTT	deep seq RT-PCR	serum	miR-23a, miR-96, miR-486, miR-186, miR-191, miR-192, miR-146a - downregulated let-7i - upregulated
Regulatory T cells in the pathogenesis of type 2 diabetes mellitus retinopathy by miR-155 [204]	20 T2D 20 NGT 20 background T2D retinopathy 20 proliferative T2D retinopathy	T2D mean age 52 (range 38-70) NGT mean age 50 (range 36-65)	M & F	T2D	retinopathy	NA	RT-PCR	PBMCs	miR-155 - upregulated increasing with retinopathy
A potentially functional polymorphism in the regulatory region of let-7a-2 is associated with an increased risk for diabetic nephropathy [205]	104 T2DM 108 T2D DN 62 HC	35-65	M & F	T2D	nephropathy	NA	microarray RT-PCR	venous blood	let-7a - upregulated in T2D let-7a - downregulated in DN vs T2D
Potential value of circulating microRNA-126 and microRNA-210 as biomarkers for type 2 diabetes with coronary artery disease [206]	20 HC 54 T2D	HC 58.1 ± 1.1 T2D 56.5 ± 7.7	M & F	T2D	NA	fasting glucose, HbA1c	RT-PCR	blood	miR-126 - downregulated miR-210 - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
A novel transthyretin/STAT4 /miR-223-3p/FBXW7 signaling pathway affects neovascularization in diabetic retinopathy [207]	46 NPDR 54 PDR 50 non DM	30-80	M & F	T2D	retinopathy	NA	RT-PCR	serum, aqueous humor	miR-223 - upregulated increase with retinopathy
MicroRNA-152 regulates insulin secretion and pancreatic β cell proliferation by targeting PI3Kα [208]	50 T2D 20 Controls	T2D 45-60 Controls 44-60	M & F	T2D	NA	NA	RT-PCR	blood	miR-152 - downregulated
Circulating microRNA-155 is associated with insulin resistance in chronic hepatitis C patients [209]	19 HC 21 T2D	HC 39.4 ± 1.2 T2D 44.2 ± 1.6	M & F	T2D	NA	ADA guidelines	RT-PCR	serum	miR-155 - downregulated
Apelin-13 serum levels in type 2 diabetic obese women: possible relations with microRNAs-107 and 375 [210]	50 obese 50 T2D obese	T2D Obese 62.38 ± 8.9 obese 55.25 ±12.3	M & F	T2D	obesity	fasting blood glucose HbA1c WHO criteria	RT-PCR	serum	miR-107, miR-375 - upregulated
DNA methylation of microRNA-375 in impaired glucose tolerance [211]	54 T2D 53 NGT	T2D 52.9 ± 9.7 NGT 52.9 ± 9.4	M & F	T2D	NA	HbA1c WHO criteria	RT-PCR	plasma	miR-375 - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Sequence variation in microRNA-34a is associated with diabetes mellitus susceptibility in a southwest Chinese Han population [212]	135 T2DM 30 DN 117 control	30-75	M & F	T2D	nephropathy	NA	RT-PCR	plasma	miR-34a - upregulated increases with nephropathy
Sex Differences in Plasma MicroRNA Biomarkers of Early and Complicated Diabetes Mellitus in Israeli Arab and Jewish Patients [213]	30 HC 29 T2D 29 late stage T2DM with renal/vascular problems	20-80	M & F	T2D	NA	fasting glucose random glucose, OGTT symptoms, HbA1c DCCT guidelines	RT-PCR	plasma	miR-486-3p - upregulated miR-423 - downregulated
Hyperglycaemia suppresses microRNA expression in platelets to increase P2RY12 and SELP levels in type 2 diabetes mellitus [214]	28 obese DM2 19 non-DM obese 23 healthy individuals	T2D 53 (50-59) obese controls 53 (48-57) HC 53 (34-60)	M & F	T2D	NA	NA	RT-PCR	leukocyte depleted platelets platelet poor plasma	miR-223, miR-26b, miR-140, miR-126 - downregulated in plasma miR-223, miR-26b - downregulated in platelets
Expression and clinical significance of microRNA 146a in peripheral blood mononuclear cells from type 2 diabetic neuropathy patients [215]	34 HC 37 T2D 44 diabetic neuropathy	HC 49.08±11.09 T2D 49.44±8.69 diabetic neuropathy 54.10±9.22	M & F	T2D	neuropathy	HbA1c fasting glucose WHO guidelines	RT-PCR	PBMC	miR-146a - downregulated in T2D w/o DN

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Four-microRNA signature for detection of type 2 diabetes [216]	102 T2D 68 HC	NA	NA	T2D	NA	fasting glucose, post prandial glucose, OGTT	RT-PCR	serum	miR-148, miR-22, miR-130, miR-19a - upregulated
Elevated Circulating hsa-miR-106b, hsa-miR-26a, and hsa-miR-29b in Type 2 Diabetes Mellitus with Diarrhea-Predominant Irritable Bowel Syndrome [217]	NA	18-75	NA	T2D	NA	NA	microarray RT-PCR	plasma	miR-106b, miR-26a, miR-29b - upregulated
Study on inflammation-related genes and microRNAs, with special emphasis on the vascular repair factor HGF and miR-574-3p, in monocytes and serum of patients with T2D [218]	44 HC 64 T2D	HC 53 (32–87) T2D 61 (37–85)	M & F	T2D	NA	HbA1c fasting glucose ADA guidelines	RT-PCR	serum	miR-146a, miR-574-3p - downregulated
Identification of Neuroendocrine Stress Response-Related Circulating MicroRNAs as Biomarkers for Type 2 Diabetes Mellitus and Insulin Resistance [219]	112 T2D 94 HC	30-65	M & F	T2D	NA	fasting glucose 2h glucose	RT-PCR	plasma	let-7b, miR-29a, miR-144 - upregulated miR-142 - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Effect of diabetes on exosomal miRNA profile in patients with obesity [135]	18 HV 16 HV + obesity 13 DM + obesity	HV 38.61±7.96 HV + obesity 31.38±8.76 DM + obesity 42.77±13.03	M & F	NA	obesity	fasting glucose WHO guidelines	RNA-seq	serum exosomes	miR-150-3p, let7b-5p, miR-664a-5p, miR-320b, miR-320a, miR-424-3p, miR-3960, miR-423-5p, miR-483-5p - upregulated
MicroRNA-130a expression is decreased in Xinjiang Uygur patients with type 2 diabetes mellitus [220]	42 NGT 30 T2D	Peripheral blood samples NGT 49 ± 13 T2DM 56 ± 10 Omental samples HC 53 ± 14 T2D 57 ± 11	M & F	T2D	NA	fasting glucose or random glucose or 2h PP glucose	microarray RT-PCR	peripheral blood and omental fat	peripheral blood miR-130a, miR-10b, miR-143 - downregulated omental fat miR-130a - downregulated
Serum microRNA-221 as a biomarker for diabetic retinopathy in patients associated with type 2 diabetes [221]	33 HC 37 T2D 34 NPDR 30 PDR	HC 63.52±4.91 T2D 66.14±3.96 NPDR 63.50±4.99 PDR 64.67±5.17	M & F	T2D	retinopathy	NA	RT-PCR	serum	miR-221 - upregulated, increases with retinopathy
Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment [222]	39 control 10 T2D not metformin treated and 31 T2DM metformin treated	control 61.3 ± 6.1 T2D 63.0 ± 8.7 met-treated T2D 61.7 ± 5.8	M & F	T2D	NA	NA	microarray RT-PCR	plasma	plasma miR-122-5p, miR-192-5p, miR-483-5p, miR-885-5p, miR-99a-5p, miR-339-5p, miR-146a-5p, miR-323b-3p - upregulated EV depleted plasma miR-122-5p, miR-192-5p, miR-483-5p, miR-885-5p, miR-99a-5p, miR-339-5p, miR-146a-5p, miR-23a-3p, miR-203a-3p, miR-10b-5p, miR-142-3p - upregulated EVs miR-203a-3p, miR-126-3p, miR-23a-3p, miR-26a-5p, miR-122-5p, miR-192-5p, miR-323b-3p, miR-885-5p - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Gene polymorphism and plasma levels of miR-155 in diabetic retinopathy [223]	139 control 244 T2D 161 NPDR 141 PDR	NDR 60.1 ± 9.3, NPDR 60.8 ± 9.0, PDR 62.4 ± 8.3	M & F	T2D	retinopathy	HbA1c ADA guidelines	RT-PCR	plasma	miR-155 - downregulated decreasing with retinopathy
Circulating miR-135 May Serve as a Novel Co-biomarker of HbA1c in Type 2 Diabetes [224]	40 HC 40 T2D	HC 33.59 ± 7.58 T2D 53.69 ± 5.69	M & F	T2D	NA	WHO guidelines FPG, 2h PP glucose, HbA1c	RT-PCR	plasma	miR-135a - upregulated
Serum miR-17 levels are downregulated in obese, African American women with elevated HbA1c [136]	23 normal HbA1c 46 high HbA1c	diabetic 62.27 ± 1.23 non-diabetic 60.11 ± 1.89	F	Diabetes – not specified	obesity	HbA1c	RT-PCR	serum	miR-17 - downregulated
Plasma miR-135a; a potential biomarker for diagnosis of new type 2 diabetes (T2DM) [225]	30 HC 30 T2D	HC 24.7± 2.8 T2D 53.8 ± 5.6	M & F	T2D	NA	WHO guidelines FPG, 2h PP glucose, HbA1c	RT-PCR	plasma	miR-135a - upregulated
Decreased Plasma Level of TRIB3 is Associated with Circulating miR-124a in Patients with Type 2 Diabetes [226]	50 T2D 40 HC	T2D 54.92 (7.05) HC 52.82 (6.94)	M & F	T2D	NA	FBS OGTT HbA1c	RT-PCR	plasma	miR-124a - downregulated
Downregulation of miR-181a upregulates sirtuin-1 (SIRT1) and improves hepatic insulin sensitivity [227]	20 HC 20 diabetes	NA	M	T2D	NA	NA	RT-PCR	serum	miR-181a - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus [228]	15 control, 21 T2D in two batches (control a = 7, b =8; T2D a = 8, b =13) a = array b = PCR	control A 46.36 ± 7.5 B 43.36 ± 5.7 T2D A 46.76 ± 3.4 B 41.06 ± 12.1	M	T2D	NA	fasting glucose, WHO and IDF guidelines	microarray RT-PCR	serum	miR-144, miR-150, miR-192, miR-29a, miR-320 - upregulated miR-146a, miR-182, miR-30d - downregulated
Senescence-associated miR-34a and miR-126 in middle-aged Indians with type 2 diabetes [229]	30 controls 30 T2D	31-50	M & F	T2D	NA	ADA guidelines	RT-PCR	plasma	miR-34a - upregulated
Circulating MiRNAs of 'Asian Indian Phenotype' Identified in Subjects with Impaired Glucose Tolerance and Patients with Type 2 Diabetes [230]	49 NGT 49 T2D	NGT 44.3 ± 6.9 T2D 44.4 ± 8.1	M & F	T2D	NA	OGTT	microarray RT-PCR	serum	miR-130b-3p miR-374a-5p - upregulated
MicroRNAs 342 and 450 together with NOX-4 activity and their association with coronary artery disease in diabetes [231]	50 HC 50 T2D	HC 62.22 ± 0.69 T2D 62.06 ± 1.26	M & F	T2D	NA	NA	RT-PCR	serum	miR-342 - upregulated miR-450 - downregulated
MicroRNA-25 inhibits high glucose-induced apoptosis in renal tubular epithelial cells via PTEN/AKT pathway [232]	30 HC 30 DN	HC 53.35 ± 8.74 DN 51.65 ± 9.23	M & F	T2D	Nephropathy	OGTT	microarray RT-PCR	serum kidney samples	miR-25 - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Decreased Serum Level of miR-146a as Sign of Chronic Inflammation in Type 2 Diabetic Patients [233]	56 T2D 40 HC	54 T2D 64 HC	M & F	T2D	NA	fasting glucose, HbA1c	RT-PCR	serum	miR-146a - downregulated
Increased serum miR-7 is a promising biomarker for type 2 diabetes mellitus and its microvascular complications [234]	76 T2D microvascular complications (29 diabetic retinopathy, 28 with diabetic neuropathy, 6 with diabetic nephropathy, 2 with diabetic foot, others had a combination of complications) 76 T2DM 74 HC	HC 48.8 (15.2) T2D 48.5 (14.5) T2DMC 51.1 (13.6)	M & F	T2D	mixture of microvascular complications	fasting plasma glucose	RT-PCR	serum	miR-7 - upregulated further increase with microvascular complications
Altered immune metabolism at the interface of increased endoplasmic reticulum stress in patients with type 2 diabetes [235]	35 NGT 35 T2D miRNAs measured in n=15 x 2 groups only	NGT 44.7 ± 6.6 T2D 47.3 ± 6.7	M & F	T2D	NA	WHO guidelines	RT-PCR	PBMC	miR-146a - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Evaluation of Serum microRNAs in Patients with Diabetic Kidney Disease: A Nested Case Controlled Study and Bioinformatics Analysis [236]	42 DKD 50 T2D 25 HC	HC 47.6±7.9 T2D 50.3±10.3 DKD 51.5±9.3	M & F	T2D	nephropathy	NA	RT-PCR	serum	miR-99b, miR-122 - upregulated, increases with CKD miR-486 - downregulated, decreases with CKD miR-20a - downregulated only in CKD
Biomarkers Associated with Ischemic Stroke in Diabetes Mellitus Patients [237]	56 T2D 30 HC	HC 48.2 ± 8.9 T2D 49.8 ± 9.1	M & F	T2D	NA	WHO criteria	RT-PCR	leukocyte free platelets platelet poor plasma	in both sample types miR-144 - upregulated miR-223 - downregulated
Detection of platelet microRNA expression in patients with diabetes mellitus with or without ischemic stroke [238]	8 HC 7 DM	HC 43.13 ± 7.882 T2D 47.14 ± 9.155	M & F	T2D	NA	OGTT	RT-PCR	leukocyte free platelets platelet poor plasma	in both sample types miR-223 miR-146a - downregulated
Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes [239]	20 HC (11 lean + 9 with obesity) 17 T2D (2 lean + 15 with obesity	lean HC 32 (23, 67.5) T2D 41 (30.5, 51.5) obese HC 34 (19.4, 54) T2D 51 (36.4, 66.6) T2D 41-51	M & F	T2D	obesity	HbA1c	RT-PCR	plasma	miR-21, miR-24, miR-34a, miR-148a, miR-27a, miR-146a, miR-223 - upregulated
Correlation between miR-103 and miR-133a Expression and the Circulating ANGPTL8 in Type 2 Diabetic Patients and Healthy Control Subjects [240]	35 T2D 35 controls	T2D 59.7 (10.9) HC 58.9 (8.9)	M & F	T2D	NA	NA	RT-PCR	serum	miR-103, miR-133a - upregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Identification of circulating miR-101, miR-375 and miR-802 as biomarkers for type 2 diabetes [241]	49 NGT 155 T2D	NGT 46.0 ± 9.67 T2D 62.3 ± 13.2	NA	T2D	NA	NA	RT-PCR	serum	miR-101, miR-375, miR-802 - upregulated
Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study [139]	27 HC 31 T2D 16 T1D	HC 25.3±2.2 T2D 52.9±2.0 T1D 25.9±5.7	M & F	T1D T2D	NA	NA	RT-PCR	plasma	miR-30d, miR-148a, miR-21, miR-34a - upregulated in T2D miR-21, miR-375, miR-148a, miR-24 - upregulated in T1D
The changes in miR-130b levels in human serum and the correlation with the severity of diabetic nephropathy [242]	137 T2D 122 micoralbuminuria 68 macroalbuminuria 131 HC	HC 51 ± 11 T2D 50 ± 10 microalbuminuria 51 ± 13 macroalbuminuria 58 ± 9	M & F	T2D	nephropathy	HbA1c ADA criteria	RT-PCR	serum	miR-130b - downregulated
Clinical verification of a novel urinary microRNA panal: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis [243]	44 T2D macroalbuminuria 66 T2D micro-albuminuria 56 T2D 54 HC	T2D macroalbuminuria 59.6 (8.6) T2D microalbuminuria 60.6 (7.4) T2D 58.2 (8.0) HC 60 (6.5)	M & F	T2D	nephropathy	NA	RT-PCR	urine exosomes	miR-133b, miR-342, miR-30a - upregulated increase with nephropathy
Blood-based analysis of 84 microRNAs identifies molecules deregulated in individuals with type-2 diabetes, risk factors for the disease or metabolic syndrome [244]	16 CT (without Met-S or risk factors for T2D) 21 bearing Met-S and/or T2D-risk factors 40 T2D	Non-T2D 49 (19–69) CT 45 (19–52) MetS/T2D RF 51 (23–69) T2D 59 (35–75)	M & F	T2D	NA	NA	microarray and RT-PCR	whole blood	let-7f-5p miR-24-3p miR-214-3p - downregulated

Title and reference	Population size	Age (years)	Gender	Type of diabetes	Diabetic complication (if any)	Method of diabetes diagnosis	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Potential serum biomarkers for early detection of diabetic nephropathy [245]	25 T2DM normoalbuminuria 25 T2DM microalbuminuria 25 HC	T2D 48.76 ± 7.75 T2D microalbuminuria 50.52 ± 9.21 HC 45.8 ± 5.82	M & F	T2D	nephropathy	previous diagnosis but HbA1c, fasting plasma glucose, OGTT measured again	RT-PCR	serum	miR-130b - downregulated further decrease with albuminuria
Diagnostic and Prognostic Role of Serum miR-20b, miR-17-3p, HOTAIR, and MALAT1 in Diabetic Retinopathy [246]	81 HC 30 NDR 30 NPDR 20 PDR	HC 50.7 ± 7.7 NDR 51.0 ± 6.1 NPDR 53.9 ± 6.3 PDR 49.2 ± 4.9	M & F	T2D		ADA guidelines fasting glucose	RT-PCR	serum	miR-20b - downregulated, decrease with retinopathy - miR-17-3p - downregulated, only in retinopathy
Hyperglycaemia-induced reciprocal changes in miR-30c and PAI-1 expression in platelets [247]	50 HC 40 T2D	HC 52.2 ± 5.5 T2D 58.6 ± 6.2	M & F	T2D	NA	NA	RT-PCR	leukocyte depleted plasma	miR-30c - downregulated
Circulating microRNA-21 is an early predictor of ROS-mediated damage in subjects with high risk of developing diabetes and in drug-naïve T2D [248]	44 NGT 27 T2D	NGT 59.3 (9.82) T2D 61.69 (7.59)	M & F	T2D	NA	2h plasma glucose	RT-PCR	plasma	miR-21 - upregulated
Whole blood microRNA levels associate with glycemic status and correlate with target mRNAs in pathways important to type 2 diabetes [249]	534 NG 24 T2D	NG 41.9 (4.9) T2D 45.1 (3.5)	M & F	T2D	NA	NA	microarray	whole blood	miR-122-5p, miR-589-3p - upregulated miR-148a-3p, miR-144-5p, let-7a-5p - downregulated

Table 3-2. Dysregulated miRNAs and characteristics of eligible studies identified in search part 2 (abnormal bone phenotype).

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Circ-VANGL1 promotes the progression of osteoporosis by absorbing miRNA-217 to regulate RUNX2 expression [144]	15 OP 15 non OP	NA	NA	NA	RT-PCR	serum	miR-217 upregulated
Noncoding RNA miR-205-5p mediates osteoporosis pathogenesis and osteoblast differentiation by regulating RUNX2 [145]	33 PM OP 31 PM HC	PM OP - 62.70 ± 5.98 PM HC - 63.12 ± 4.78	F	NA	microarray and RT-PCR	bone from femoral neck	miR-205-5p, miR-27b-3p, miR-421, miR-320b - upregulated let-7g-3p, miR-6515-5p - downregulated
Medical examination powers miR-194-5p as a biomarker for postmenopausal osteoporosis [250]	Dataset 1 (N=230) - 92 normal bone mass, 79 osteopenia, and 59 OP Dataset 2 (N=30) - 12 normal bone mass, 8 osteopenia, and 10 OP All PM	48-66 (all PM)	F	LS T-score and BMD DXA	RT-PCR	whole blood	miR-194-5p - upregulated
MiR-128 inhibits the osteogenic differentiation in osteoporosis by down-regulating SIRT6 expression [251]	40 PM HC 40 PM OP	NA	F	T-score BMD (site not specified)	RT-PCR	femoral neck trabecular bone	miR-128 - upregulated
Serum Circulating MicroRNAs as Biomarkers of Osteoporotic Fracture [252]	1st study - 5 controls osteoarthritis with hip surgery 8 OP fracture 2nd study - validation 12 controls osteoarthritis with hip surgery 15 OP fracture	control - 65.4 ± 10.5 fracture - 81.0 ± 5.4	F	LS/FN BMD DXA	microarray and RT-PCR	serum	miR-122-5p, miR-125b-5p, miR-21-5p - upregulated

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	559 adults with fracture 123 adults without fracture	44-95	F	medical records, x-ray	RT-PCR	serum	miR-133a-3p, miR-20a-5p, miR-25-5p, miR-100-5p, miR-26a-5p, miR-103a-3p, miR-145-5p, miR-21-5p, miR-29a-3p, miR-106a-5p, miR-146a-5p, miR-221-5p, miR-122-5p, miR-148a-3p, miR-222-3p, miR-155-5p, miR-223-5p, miR-125b-5p, miR-17-5, miR-23a-3p, miR-24-3p, miR-93-5p, miR-16-5p - all miRNAs downregulated
The function of microRNA-211 expression in post-fracture bone cell apoptosis involving the transforming growth factor-β/ phosphoinositide 3-kinase signaling pathway [254]	41 OP fracture 40 HC	OP fracture - 44 (28 to 52) HC age not mentioned	OP fracture - 22 M, 19 F HC not mentioned	CT	RT-PCR	serum	miR-211 - upregulated
Serum miRNAs miR-140-3p and miR-23b-3p as potential biomarkers for osteoporosis and osteoporotic fracture in postmenopausal Mexican-Mestizo women [255]	preliminary set 20 PM OP 20 PM HC validation set 22 HC 28 osteopenia 26 OP 21 fracture	63 - 85	F	BMD measurement, T-score	microarray and RT-PCR	serum	miR-140-3p miR-23b-3p - upregulated
The Role of Serum Expression Levels of Microrna-21 on Bone Mineral Density in Hypostrogenic Postmenopausal Women with Osteoporosis: Study on Level of RANKL, OPG, TGFβ-1, Sclerostin, RANKL/OPG Ratio, and Physical Activity [256]	60 PM OP 60 PM HC	55-70	F	BMD measurement, site not mentioned, no clear diagnosis	RT-PCR	serum	miR-21 - upregulated

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
MiR-488 promotes fracture healing by targeting DKK1 [257]	OP and Controls (no clear description of controls)	NA	NA	T-score	RT-PCR	serum	miR-488 - downregulated
Characterization of Dysregulated lncRNA-Associated ceRNA Network Reveals Novel lncRNAs With ceRNA Activity as Epigenetic Diagnostic Biomarkers for Osteoporosis Risk [258]	OP datasets based on high and low BMD 1. GSE62589 - 5 high-BMD and 5 low-BMD 2. GSE56814 - 16 high-BMD and 15 low-BMD 3. GSE13850 - 10 high-BMD and 10 low-BMD	NA	2/3 data sets - F	BMD measurement	electronic validation - microarray	GSE62589 - no sample type mentioned GSE56814 - monocytes GSE13850 - B cells	miR-34a, miR-1308, miR-3124, miR-340, miR-589, miR-130b, miR-1260, miR-410, miR-30a, miR-194 - upregulated miR-574-5p - downregulated
Plasma miRNA levels correlate with sensitivity to bone mineral density in postmenopausal osteoporosis patients [259]	40 PM HC 40 PM osteopenia 40 PM OP	PM HC 56.5 ± 10.5 PM osteopenia 56.7 ± 10.7 PM OP 57.5 ± 11.3	F	BMD measurement DXA	RT-PCR	plasma	miR-133a - upregulated miR-21- downregulated
Abnormal expression of miR-135b-5p in bone tissue of patients with osteoporosis and its role and mechanism in osteoporosis progression [260]	30 OP 30 OA control	OP 52–73 years OA control 51-72 years	15 F and 15 M in each group	NA	RT-PCR	bone tissue	miR-135b-5p - upregulated
Circulating microRNAs as potential diagnostic biomarkers for osteoporosis [261]	12 Non-OP controls 61 osteopenia wo fracture 15 Osteopenia w fracture 33 OP without fracture 18 OP w fracture	Non OP controls - 67 ± 9.6 years Osteopenia wo fracture - 65.6 ± 9.5 years Osteopenia w fracture - 67 ± 9.5 years OP wo fracture - 68.6 ± 10 years OP w fracture - 70 ± 10 years	Non OP controls - 11 F and 1M Osteopenia wo fracture - 52 F and 9 M Osteopenia w fracture - 13 F and 2 M OP wo fracture - 27 F and 6 M OP w fracture - 16 F and 2M	BMD T-score; unclear how fracture history was obtained	microarray and RT-PCR	serum and plasma	miR-122-5p, miR-4516 - downregulated

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
LncRNA XIXT promotes osteogenic differentiation of bone mesenchymal stem cells and alleviates osteoporosis progression by targeting miRNA-30a-5p [151]	NA OP and Controls	NA	NA	NA	RT-PCR	serum	miR-30a-5p - upregulated
MicroRNA-19b-3p promotes cell proliferation and osteogenic differentiation of BMSCs by interacting with lncRNA H19 [152]	18 PM OP 12 PM HC	PM OP - 48–60 years PM HC - 46–58 years	F	NA	RT-PCR	serum	miR-19b-3p - upregulated
microRNAs in human circulating monocytes of postmenopausal osteoporotic Mexican-Mestizo women: A pilot study [262]	6 PM HC 6 PM OP	63-85 years	F	BMD T-score measurement	micorarray and RT-PCR	monocytes	miR-1270 - upregulated
Identification of suitable reference gene and biomarkers of serum miRNAs for osteoporosis [263]	19 HC 7 osteopenia 10 OP	HC 51.89 ± 2.56 osteopenia 72.86 ± 7.73 OP 77.4 ± 4.45	F	BMD measurement	RT-PCR	serum	miR-30b-5p, miR-103-3p, miR-142-3p, miR-328-3p - downregulated
Expression of microRNAs that regulate bone turnover in the serum of postmenopausal women with low bone mass and vertebral fractures [264]	35 PM OP/osteopenia wo fracture 35 PM OP/osteopenia w fracture 30 PM HC	PM HC 68 ± 5 PM OP/osteopenia wo fracture 68 ± 7 PM OP/osteopenia w fracture 71 ± 7	F	BMD measurements	RT-PCR	serum	miR-124-3p, miR-2861 - upregulated miR-21-5p, miR-23a-3p, miR-29a-3p - downregulated
MiR-133a in Human Circulating Monocytes: A Potential Biomarker Associated with Postmenopausal Osteoporosis [265]	10 high BMD 10 low BMD (all PM)	high BMD 63.6 ± 3.2 low BMD 61.6 ± 2.6	F	BMD measurement	micorarray and RT-PCR	monocytes	miR-133a - upregulated

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
circRNA_0016624 could sponge miR-98 to regulate BMP2 expression in postmenopausal osteoporosis [266]	20 PM OP 20 PM HC	PM OP 61.1 ± 8.5 PM HC 63.2 ± 7.9	F	LS BMD	RT-PCR	serum/plasma	miR-98 - upregulated
Correlation of plasma microRNA-21 expression and bone turnover markers in postmenopausal women [267]	N = 195 PM OP/osteopenia PM HC	population mean 59.79 ± 6.53	F	BMD measurement	RT-PCR	plasma	miR-21-5p - upregulated
MiRNA-365a-3p promotes the progression of osteoporosis by inhibiting osteogenic differentiation via targeting RUNX2 [153]	30 OP 30 HC	NA	NA	NA	RT-PCR	serum	miR-365a-3p - upregulated
MicroRNA-200a-3p accelerates the progression of osteoporosis by targeting glutaminase to inhibit osteogenic differentiation of bone marrow mesenchymal stem cells [154]	30 OP 30 HC	NA	NA	NA	RT-PCR	serum	miR-200a-3p - upregulated
MiRNA-19a-3p alleviates the progression of osteoporosis by targeting HDAC4 to promote the osteogenic differentiation of hMSCs [155]	42 OP 42 HC	NA	NA	NA	RT-PCR	serum	miR-19a-3p - downregulated
MiR-203 is essential for the shift from osteogenic differentiation to adipogenic differentiation of mesenchymal stem cells in postmenopausal osteoporosis [268]	60 PM OP 40 HC	PM OP 63.4 ± 2.4 HC 59.3 ± 3.2	NA	no clear description	RT-PCR	serum	miR-203 - downregulated

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
Circulating miR-374b-5p negatively regulates osteoblast differentiation in the progression of osteoporosis via targeting Wnt3 AND Runx2 [269]	85 OP 52 HC	50-90	F	BMD measurement	RT-PCR	serum	miR-374b-5p - upregulated
MiR-16-5p regulates postmenopausal osteoporosis by directly targeting VEGFA [156]	10 PM OP 10 PM HC	NA	F	NA	RT-PCR	serum exosomal miRNAs	miR-16-5p - upregulated
Evaluation of circulating miRNA-208a-3p, miRNA-155–5p and miRNA-637 as potential non-invasive biomarkers and the possible mechanistic insights into pre- and postmenopausal osteoporotic females [270]	70 OP 70 HC (pre-menopausal and PM)	Pre-menopausal HC 34.03 ± 5.72 OP 36.00 ± 7.15 PM HC 60.06 ± 6.57 OP 61.29 ± 7.69	F	BMD T-score	RT-PCR	serum	In pre-menopausal and PM-miR-208a-3p - upregulated in pre-menopausal - miR-155-5p - downregulated in PM - miR-155-5p, miR-637 - upregulated
MiR-373 promotes the osteogenic differentiation of BMSCs from the estrogen deficiency induced osteoporosis [271]	20 OP 20 HC	NA	F	BMD T-score	RT-PCR	serum and bone	miR-28, miR-373, miR-101 - downregulated In bone - miR-142 - downregulated
MiR-96 regulates bone metabolism by targeting osterix [272]	30 old 30 young 20 pre-menopausal 20 PM 20 elderly normal 20 elderly OP	old >70 young 20-40 premenopausal and PM OP - 45 to 60 normal elderly women and 20 elderly OP - 65 to 80	M & F	LS/FN BMD T-score	micorarray and RT-PCR	serum	Elderly OP vs elderly HC - miR-96 - upregulated
Circulating miR-338 Cluster activities on osteoblast differentiation: Potential Diagnostic and Therapeutic Targets for Postmenopausal Osteoporosis [273]	15 PM OP 15 PM HC	58-68	F	BMD T-score	RT-PCR	serum	miR-338, miR-3065-5p - upregulated

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
MiR-203 is involved in osteoporosis by regulating DKK1 and inhibiting osteogenic differentiation of MSCs [157]	60 OP 60 HC	NA	F	NA	RT-PCR	serum	miR-203 - downregulated
Identification of miR-708-5p in peripheral blood monocytes: Potential marker for postmenopausal osteoporosis in Mexican-Mestizo population [274]	7 PM OP 7 PM HC	PM HC 68.86 ± 0.89 PM OP 70.43 ± 3.20	F	BMD and BMD T-score	microarray and RT-PCR	monocytes	miR-708-5p - upregulated
Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	Case group 10 pre-menopausal F 10 PM F 16 M Control group 12 pre-menopausal F 11 PM F 16 M	Case group premenopausal F - 39.0. ± 8.6 PM F - 59.0 ± 11.4 M - 43.7 ± 11.1 controls premenopausal F - 42.5 ± 6.8 years PM F - 54.3 ± 7.4 F - 44.4 ± 9.6	F and M	BMD and x-ray	RT-PCR	serum	miR-152-3p, miR-335-5p, miR-320a - upregulated miR-30e-5p, miR-140-5p, miR-324-3p, miR-19b-3p, miR-19a-3p, miR-550a-3p, miR-186-5p , miR-532-5p, miR-93-5p, miR-378a-5p, miR-16-5p, miR-215-5p, miR-let-7b-5p, miR-29b-3p,miR-7-5p, miR-365a-3p - downregulated
miR-27a-3p negatively regulates osteogenic differentiation of MC3T3-E1 preosteoblasts by targeting osterix [276]	85 OP 52 HC	50–90	F	LS BMD and BMD T-score	RT-PCR	serum	miR-27a-3p - upregulated
MiRNA-133a is involved in the regulation of postmenopausal osteoporosis through promoting osteoclast differentiation [277]	10 PM OP 10 HC	PM OP - 59-80 HC - 62-75	F	BMD measurement Details on time fracture time not specified	RT-PCR	serum	miR-133a - upregulated

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
miR-144 promotes the proliferation and differentiation of bone mesenchymal stem cells by downregulating the expression of SFRP1 [146]	15 PM OP 15 PM HC	54 - 64	F	NA	RT-PCR	serum	miR-144 - upregulated
MicroRNA-579-3p promotes the progression of osteoporosis by inhibiting osteogenic differentiation of mesenchymal stem cells through regulating Sirt1 [147]	NA	NA	NA	NA	RT-PCR	serum	miR-579-3p - upregulated
MicroRNA-1297 promotes the progression of osteoporosis through regulation of osteogenesis of bone marrow mesenchymal stem cells by targeting WNT5A [148]	30 OP 30 HC	NA	NA	NA	RT-PCR	serum	miR-1297 - upregulated
Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	42 HC 39 low BMD 26 low BMD fracture 19 receiving a treatment for osteoporosis	HC - 68.8 (6.5) Low BMD - 67.9 (5.4) Low BMD fracture - 69.6 (7.0) Vertebral fractures /low BMD treatment 69.6 (6.4)	F (all PM)	BMD measurement	microarray and RT-PCR	serum	miR-375, miR-532-5p, miR-19b-3p, miR-152-3p, miR-23a-3p, miR-335-5p, miR-21-5p, miR-486-3p, miR-30e-5,p miR-127-3p, miR-214-3p, miR-106b-5p, miR-133b, miR-143-3p - upregulated
miR-342-3p promotes osteogenic differentiation via targeting ATF3 [149]	20 HC 20 OP	NA	NA	NA	RT-PCR	serum	miR-342-3p - downregulated
MiR-27a is Essential for the Shift from Osteogenic Differentiation to Adipogenic Differentiation of Mesenchymal Stem Cells in Postmenopausal Osteoporosis [279]	81 PM OP 74 HC premenopausal	PM OP - 51–62 HC premenopausal - 40 – 46	F	BMD measurement	microarray and RT-PCR	serum	miR-27a - downregulated

Title and reference	Population size	Age (years)	Gender	Diagnosis method	Method of miRNA quantification	Sample miRNA measured in	Dysregulated miRNAs and direction of expression compared to control group
MiR-422a as a Potential Cellular MicroRNA Biomarker for Postmenopausal Osteoporosis [280]	10 high BMD 10 low BMD	57-68	F (all PM)	BMD measurement	microarray and RT-PCR	monocytes	miR-422a - upregulated

Using the BioVenn online tool [110], 64 differentially expressed miRNAs were found to overlap between the two study searches. Table 3.3 lists these 64 overlapping miRNAs.

Table 3-3. Overlapping miRNAs between the two searches.

let-7b-5p	miR-96-5p	miR-186-5p
let-7g-5p	miR-98-5p	miR-194-5p
miR-7-5p	miR-100-5p	miR-203a-3p
miR-16-5p	miR-101-3p	miR-214-3p
miR-17-5p	miR-103a-3p	miR-217-5p
miR-19a-3p	miR-106b-5p	miR-221-3p
miR-20a-5p	miR-122-5p	miR-222-3p
miR-21-5p	miR-124-3p	miR-223-3p
miR-23a-3p	miR-125b-5p	miR-320a-3p
miR-24-3p	miR-128-3p	miR-320b
miR-25-3p	miR-130b-3p	miR-324-5p
miR-26a-5p	miR-133a-3p	miR-328-3p
miR-27a-3p	miR-133b	miR-335-5p
miR-27b-3p	miR-135b-5p	miR-338-5p
miR-29a-3p	miR-140-3p	miR-340-5p
miR-29b-3p	miR-142-5p	miR-342-3p
miR-30a-5p	miR-143-3p	miR-375-3p
miR-30b-5p	miR-144-5p	miR-486-5p
miR-30e-5p	miR-146a-5p	miR-574-3p
miR-34a-5p	miR-148a-3p	miR-589-5p
miR-34c-5p	miR-152-3p	
miR-93-5p	miR-155-5p	

3.3.4 Quality assessment and sensitivity analysis

Quality assessment of the eligible studies was done using the NOS tool and the 2 additional questions to capture the quality of the miRNA measurements, as described in Chapter 2. Each of the 64 miRNAs were then subject to a sensitivity analysis based on these 4 factors: NOS score, score from the 2 additional NOS questions, score for consistency in the direction of expression in diabetes and altered bone phenotype (as described in the eligible literature), and score for the miRNA of interest being described in multiple studies. Table 3.4 lists the scores (in descending order) from the sensitivity analysis for each overlapping miRNAs.

Table 3-4. List of the identified overlapping microRNAs and their respective scores (in decreasing order) based on the sensitivity analysis.

miRNA	NOS Score (out of 3)	Add. NOS Score (out of 2)	Score for direction of expression (out of 3)	Score for multiple studies (out of 5)	Total (out of 13)
miR-21	2.19	1.63	2	5	10.82
miR-335	2.5	1.5	3	3	10
miR-17	2.83	1.83	3	2	9.66
miR-375	2.42	2	3	2	9.42
miR-320a	2.625	1.63	3	2	9.255
miR-155	2.42	1.75	2	3	9.17
miR-124	2.83	1.83	2	2	8.66
miR-25	2.5	2	3	1	8.5
miR-133a	2.75	1.63	1	3	8.38
miR-223	2.43	1.79	2	2	8.22
miR-23a	2.25	1.75	0	4	8
miR-27a	2.25	1.75	1	3	8
miR-100	2.5	1.5	3	1	8
miR-106b	2	2	3	1	8
miR-128	2.5	1.5	3	1	8
miR-29a	2.75	2	0	3	7.75
miR-93	2.75	2	0	3	7.75
miR-125b	2	1.75	1	3	7.75
miR-338	1.75	1	3	2	7.75
miR-140	2.17	1.5	1	3	7.67
miR-30e	2.33	1.33	1	3	7.66
miR-122	1.63	1.88	0	4	7.51
miR-133b	2	1.5	3	1	7.5
let-7b	2.5	1.88	1	2	7.38
miR-146a	2.58	1.73	2	1	7.31
miR-486	2.38	1.88	1	2	7.26
miR-16	1.67	1.5	1	3	7.17
miR-24	2.42	1.75	1	2	7.17
miR-23b	2.5	1.5	1	2	7

miR-96	2.25	1.75	1	2	7
miR-217	1.5	1.5	3	1	7
miR-144	2.17	1.75	2	1	6.92
miR-19a	2	1.67	0	3	6.67
miR-142	2.13	1.38	0	3	6.51
miR-20a	2	1.5	1	2	6.5
miR-135b	1.5	1	3	1	6.5
miR-203a	1.25	1.25	1	3	6.5
miR-320b	2.5	1	1	2	6.5
miR-574	1.5	1	2	2	6.5
miR-34a	1.38	0.94	2	2	6.32
miR-7	2.5	1.5	0	2	6
miR-26a	2.17	1.83	0	2	6
miR-27b	2.25	0.75	1	2	6
miR-29b	2.33	1.67	0	2	6
miR-30a	1.25	0.75	1	3	6
miR-222	2.33	1.67	0	2	6
miR-101	2.25	1.5	0	2	5.75
miR-30b	1.83	1.83	0	2	5.66
miR-186	2.33	1.33	0	2	5.66
miR-103a	2.5	2	0	1	5.5
miR-152	2	1.5	0	2	5.5
miR-328	2.5	2	0	1	5.5
miR-589	1	0.5	3	1	5.5
miR-98	1.83	1.5	0	2	5.33
let-7g	2.25	1	0	2	5.25
miR-143	3	1	0	1	5
miR-148a	2.3	1.7	0	1	5
miR-340	1	0	3	1	5
miR-194	2	0.75	0	2	4.75
miR-342	1.21	1.43	0	2	4.64
miR-130b	1.63	1	0	2	4.63
miR-214	2	1.5	0	1	4.5
miR-221	2	1.5	0	1	4.5
miR-324	2	1	0	1	4

Amongst the top 10 scoring miRNAs from the sensitivity analysis, expression of miR-21, miR-335, miR-375, miR-320a and miR-124 were reported to be upregulated in studies related to diabetes and altered bone phenotype compared to a control (or) healthy population, while the expression of miR-17, miR-155, miR-25 and miR-223 were reported to be downregulated in studies related to diabetes and altered bone phenotype compared to a control (or) healthy population. However, mixed results in the expression of miR-133a were reported in the eligible studies (Table 3.5).

Table 3-5. List of the top 10 identified dysregulated microRNAs in diabetes and abnormal bone phenotype. (Identified from the sensitivity analysis).

miRNA	Direction of expression (compared to a control / healthy population)
miR-21	upregulated
miR-335	upregulated
miR-17	downregulated
miR-375	upregulated
miR-320a	upregulated
miR-155	downregulated
miR-124	upregulated
miR-25	downregulated
miR-133a	upregulated / downregulated ?
miR-223	downregulated

The entire list of direction of expression of the 64 miRNAs is listed in Appendix 1.

3.3.5 Bioinformatic analysis

The top 10 scoring miRNAs from the sensitivity analysis were used for a miRNA functional analysis. This was assessed using a network-based visual analytics platform called miRNet 2.0. Based on our interests in diabetes and bone, the following observations were made.

miR-17, miR-25, miR-223 and miR-124 were enriched in bone, bone marrow and pancreas. miR-155 was enriched in pancreas, adipose tissue and bone marrow, while miR-133a was enriched in bone marrow, pancreas and muscle tissue. miR-375 was enriched in bone and pancreas; miR-320a in pancreas and bone marrow and miR-335 in bone and bone marrow.

miR-17 was found to be important for the regulation of osteoclast differentiation and Akt signalling. This miRNA, along with miR-133a, was important in the progression of diabetes mellitus. miR-223 was found to play a role in obesity, diabetes mellitus and glucose metabolism. In contrast, miR-155 was found to play an important role in regulating the Akt signalling pathway and obesity. miR-124 was important in regulating osteoclast differentiation, glucose metabolism and regulation of the Wnt signalling pathway. Lastly, miR-335 was important in the context of obesity alone.

3.4 Discussion

3.4.1 Summary of main findings

I present a comprehensive systematic overview of 64 miRNAs in diabetes (T1D and T2D) and altered bone phenotype (fractures, BMD, bone turnover). These 64 miRNAs were subject to a sensitivity analysis. Amongst the top 10 scoring miRNAs from the sensitivity analysis, expression of miR-21, miR-335, miR-375, miR-320a and miR-124 were reported to be upregulated in studies related to diabetes and altered bone phenotype compared to a control (or) healthy population, while the expression of miR-17, miR-155, miR-25 and miR-223 were reported to be downregulated in studies related to diabetes and altered bone phenotype compared to a control (or) healthy population. However, mixed results in the expression of miR-133a were reported in the eligible studies. A miRNA functional analysis done using miRNet provided evidence for these miRNAs to play an important role in organs and processes related to diabetes and bone.

3.4.2 miR-21 in diabetes and abnormal bone phenotype

miR-21 was a top scoring miRNA. All the 5 identified studies related to diabetes reported an upregulation in its expression compared to a corresponding control population [139], [141], [142], [239], [248]. Amongst the 7 identified studies related to bone, an upregulation in the expression of miR-21 was reported in 4 studies [252], [267], [278], [281].

A study related to T1D done by Grieco and colleagues found the expression of miR-21 to be upregulated in serum of patients with T1D compared to the corresponding control population, and this increase in expression of miR-21 was also found to be negatively associated with bone mineral content at the femur. They also report the target genes of miR-21 to be involved in bone development and remodelling processes [25].

3.4.3 miR-335 in diabetes and abnormal bone phenotype

There was an agreement for miR-335 to be upregulated in all studies related to diabetes and bone [142], [173], [275], [278]. Kocijan and colleagues found an upregulation in expression of miR-335 in adults with idiopathic and postmenopausal low trauma osteoporotic fractures, but they found no correlation between this miRNA and BTMs or BMD. However, a study done using the MC3T3-E1 osteoblast cell line in a medium of high glucose found the opposite, where high glucose leads to a decrease in expression of miR-335. They reported an overexpression of miR-335 to inhibit high glucose-induced apoptosis by decreasing protein levels of DKK1 [282]. This difference in miR-335 expression could be due to the different sample types the miRNA was measured in.

3.4.4 miR-17 in diabetes and abnormal bone phenotype

Although I found only 1 study [253] on miR-17 in bone, the observation of a downregulation in expression of miR-17 in serum of postmenopausal women compared to premenopausal women was consistent with the findings of 3 studies mentioning about the downregulation of miR-17 in diabetes [136], [167], [246]. Feurer and colleagues reported no association between miR-17 and bone mass, bone turnover and previous fractures [253]. A very recent study in rats, found an environment of high glucose to reduce the expression of miR-17 in rat osteoblasts by targeting SMAD7 downstream. This decrease in miR-17 impaired the promotion of mesenchymal cells to osteoblasts, migration of osteoblasts and bone regeneration in vivo. Their results suggest the potential for miR-17/SMAD7 to be used as a promising therapeutic agent to treat diabetic bone disease [283].

3.4.5 miR-375 in diabetes and abnormal bone phenotype

I gathered strong evidence for miR-375 to be upregulated in diabetes [139], [185], [195], [210], [211], [241]. Although I found only one study on the expression of miR-375 in bone [278], the upregulation of miR-375 reported by the authors was in line with the evidence gathered for studies related to diabetes. A study published soon after the end of the literature search window for this systematic review reported a downregulation in expression of miR-375 in people with osteoporotic fractures without diabetes compared people with diabetes and an osteoporotic fracture, as well as with healthy controls [284]. The above-mentioned study contradicted the findings of Heilmeier et al., who reported the expression of miR-375 to be 1.3-fold higher in a diabetic control group compared to postmenopausal women with T2D and a previous fracture. Hence, additional evidence is required to understand the role of miR-375 in diabetic and bone [30].

3.4.6 miR-320a in diabetes and abnormal bone phenotype

Like miR-375, I gathered strong evidence for miR-320a to be upregulated in diabetes [135], [167], [173], [184]. Although I found only 1 study on miR-320a in bone, its expression was reported to be upregulated as well in people with low traumatic fractures when compared to controls without a fracture [275]. However, the authors found no correlation between miR-320a and BMD or BTMs. A study done in healthy baboons found increased expression of miR-320a to be negatively associated with BMD [285]. Additional studies are required to understand the potential role of miR-320a in diabetes and bone. It is worth mentioning that miR-320a is part of a commercially available miRNA biomarker kit called OsteomiR (TAmiRNA GmbH, Austria).

3.4.7 miR-155 in diabetes and abnormal bone phenotype

Mixed evidence was found on dysregulation of miR-155 in studies related to diabetes. The majority of the studies reported a downregulation in miR-155 in diabetes [134], [179], [209], [223] while 2 studies reported an upregulation in its expression [183], [204]. Samaloty and colleagues reported a negative association between miR-155 and insulin resistance and HbA1c [209]. Evidence found on miR-155 in bone suggests a downregulation in its expression in people with fractures compared to those without a fracture [253]. However, the authors found no association between miR-155 and BMD or BTMs. Another study reported a downregulation in miR-155 in premenopausal osteoporosis, while upregulated miRNA levels were found in people with postmenopausal osteoporosis. Further investigation found TNF-α and SOCS1 to be downstream targets of miR-155, important for osteogenic differentiation. These downstream targets functioned differently in pre and postmenopausal women [270]. Cumulatively, the downregulation of miR-155 in diabetes and osteoporosis and its negative association with insulin resistance make it a potential biomarker for diabetic bone disease.

3.4.8 miR-124 in diabetes and abnormal bone phenotype

Two studies reported an upregulation of miR-124 in diabetes [185], [190] where whereas Parsa and colleagues reported a downregulation in miR-124 in diabetes [226]. This difference may be because Kong et al. measured miR-124 in serum whereas Parsa et al. measured miR-124 in plasma. In evidence related to bone, Yavropoulou and colleagues reported an upregulation in the expression of miR-124 in postmenopausal women with low bone mass and fractures [264], also measured in serum. A study done in MLO-Y4 cells (murine osteocyte cell line) in high glucose conditions found an upregulation of miR-124 to disturb the protein expression of Gal-3. Gal-3 is a scavenging receptor of AGEs; and helps in maintaining bone homeostasis and regulating bone cell interactions [286]. These studies provide evidence for miR-124 to be a potential biomarker for diabetic bone disease.

3.4.9 miR-25 in diabetes and abnormal bone phenotype

Evidence for miR-25 as a potential biomarker for diabetic bone disease was gathered only from 1 study relevant to diabetes [232] and 1 study relevant to fractures [253]. The expression of miR-25 was reported to be downregulated in diabetes and fractures. Using human kidney (HK-2) cells, H. Li et al. show evidence for overexpression of miR-25 in high glucose conditions to suppress the production of reactive oxygen species, cell apoptosis and activity of caspase-3 [232]. However, a very recent study in patients with T2D and osteoporosis reported expression of miR-25 to be upregulated in the saliva of patients with T2D and osteoporosis compared to those with T2D alone as well as between patients with T2D alone and healthy

controls [287]. These conflicting results call for the need to validate the expression of miR-25 in larger cohort studies to better understand its role in diabetic bone disease and its potential to be used as a biomarker.

3.4.10 miR-133a in diabetes and abnormal bone phenotype

Mixed evidence was gathered on the expression of miR-133a in studies related to diabetes and bone. In studies related to diabetes, one study reported a downregulation of miR-133a [186], while another study reported an upregulation in this miRNA [240]. Kokkinopoulou et al. measured miR-25 in PAXgene blood while Ghasemi et al. used serum. In studies related to bone, all studies [259], [265], [277] reported an upregulation of miR-133a in osteoporosis while 1 study [253] reported a downregulation in expression of this miRNA. This may be due to the fact that Feurer et al. measured the expression of miR-133a in a cohort of people with previous incident fractures and the remaining studies measured miR-133a in a cohort of postmenopausal women with osteoporosis. This mixed evidence calls for the need to validate the expression of miR-133a in a cohort of patients with diabetes and osteoporosis (with and without fractures) to better understand its potential to be used as a biomarker for diabetic bone disease.

3.4.11 miR-223 in diabetes and abnormal bone phenotype

Strong evidence was gathered on the potential role of miR-223 in diabetes. However, 4 studies [137], [214], [237], [238] reported miR-223 to be downregulated in diabetes while 3 studies reported an upregulation in miR-223 in diabetes [207], [216], [239]. In the context of bone, only 1 study [253] was found, which reported a downregulation in miR-223 in those with a previous fracture. Going in the direction of majority, we can expect miR-223 to be downregulated in diabetes and bone. However, more evidence is required to understand its potential role in diabetic bone disease. A study done in MC3T3-E1 cells (osteoblast cell line) found expression of miR-223 to be upregulated with the treatment of advanced glycation end products, and this, in turn, decreased the expression of insulin-like growth factor 1 receptor in these cells and increased the apoptosis of these osteoblasts [288]. Advanced glycation end products are common markers of diabetes and there is growing evidence for them to be harmful to bone [289].

While performing the search for this systematic review, one very interesting study [290] had to be excluded because it fell within the exclusion criteria of this review. The authors compared patients with T2D with fractures with patients with T2D alone. Due to the absence of a non-diabetic control group, this study had to be excluded, in line with the exclusion criteria of this review. Nevertheless, the findings of the review were very interesting and I felt it could add a

lot of value to this thesis. The results of this systematic review did not capture the miRNAs described in this study. The authors found miR-550a to be upregulated and miR-382 to be downregulated in patients with T2D and fractures compared to those with T2D alone. Additionally, they reported miR-382 to enhance osteogenesis, miR-550a to inhibit osteogenesis and together, these 2 miRNAs to inhibit adipogenesis. Altogether, the authors suggest that these 2 miRNAs may be linked to fragility fractures in T2D.

The quality assessment done as part of this review showed that in miRNA studies the methods of normalisation of miRNA data and the use of spike in controls for normalisation are not standardised. This influences the readout of the miRNA expression. A candidate-driven approach and a high-throughput approach provide conflicting results at times, and the results of a high-throughput approach are not completely reproducible using RT-PCR. However, it is important to note that a high-throughput approach can comment on less popular miRNAs and in turn allow to explore new miRNA targets.

The search strategy of this review was kept broad to capture as much as information as possible on dysregulated miRNAs in diabetes and altered bone phenotype. During the data extraction phase of this review, I identified that our search strategy was very broad and should have been limited mainly to miRNAs dysregulated in serum and plasma; and miRNA expression validated by RT-PCR. The reasons for this are that (i) when miRNAs would be used as diagnostics in clinical practice, they would usually be measured in serum or plasma along with other routine clinical tests, and (ii) the gold standard method for measurement and validation of miRNAs is by using RT-PCR. Although I took these factors into account while performing the sensitivity analysis part of this review, it led to a collection of a large amount of information and I believe the inclusion of different validation methods and sample types the miRNAs were measured in contributed to the heterogeneity in the expression of a given miRNA identified in this review.

3.4.12 Strengths and limitations of this review

To our knowledge, this review is the first systematic review of dysregulated miRNAs in diabetes and abnormal bone phenotype. This review offers a collection of potential miRNAs as valuable biomarkers for diabetic bone disease. However, their role in diabetic bone disease needs to be well understood before they are used in clinical practice. Our next steps are to validate the findings from this systematic review in a population of patients with T1D and T2D and look for associations between the dysregulated miRNAs and markers related to bone such as BMD and bone turnover.

Several limitations were observed while performing this systematic review. Firstly, the decision to exclude those studies that did not report a statistically significant result in miRNA expression levels between the groups of interest, may allow for a bias to enter the analysis. Next, some of the included studies had a small sample size [138], [141], [143], [158], [172], [174], [177], [199], [238], [262], [274] and there was heterogeneity in the use of reference miRNAs across the study selection. Some studies did not validate their miRNA targets by RT-PCR and some studies that did look for associations between their miRNA targets and outcomes of interest (i.e. BMD, BTMs), did not report a positive outcome [253], [275]. The direction of expression of a given miRNA was also different between 2 studies, possibly due to the sample source used for the measurement. The quality and quantity of miRNAs can be affected by the sample source used to extract the RNA, normalisation methods used and miRNA quantification method. These factors were taken into consideration while building the quality assessment and sensitivity analysis methods used for this review. In this review, I gathered more robust evidence for miRNAs in T2D than for miRNAs in T1D and bone phenotype. Although there was an imbalance in evidence from literature for some miRNAs (miR-17, miR-375, miR-320a), they still scored high in the sensitivity analysis due to the use of well-regarded methods for miRNA measurements and well characterised study populations being compared.

3.4.13 Summary

64 dysregulated miRNAs relevant in diabetes (T1D and T2D) and altered bone phenotype (fractures, BMD and bone turnover) have been identified in this systematic review of the literature. Of these 64, a sensitivity analysis identified 10 dysregulated miRNAs to be promising biomarkers of diabetic bone disease. Unfortunately, due to the limitation of sample size and unstandardised methods, collaborative efforts on validating these miRNAs in large sample sizes are required to provide more information on the use of a panel of miRNAs for diabetic bone disease.

Chapter 4 - MicroRNAs in type 1 diabetes and the effect of prolonged duration of diabetes and neuropathy on type 1 diabetes and bone health

4.1 Introduction

4.1.1 Bone turnover in type 1 diabetes

Bone turnover is an important mechanism that underlies bone fragility. People with T1D exhibit a state of low bone turnover [76], [291]. In a recent meta-analysis done by Hygum and colleagues [82], levels of the IOF-recommended bone resorption marker for evaluation of bone turnover; CTX was found to be reduced in people with T1D type 1 diabetes compared to individuals without diabetes. However, the authors found no difference in levels of the IOF-recommended bone formation marker for evaluation of bone turnover, PINP in people with T1D type 1 diabetes compared to individuals without diabetes [82]. This may be due to the low number of studies (N = 2 for PINP) included in the analysis.

Diabetes-related complications such as neuropathy are likely to increase fracture risk and lower bone turnover. In a study investigating bone turnover in people with T1D type 1 diabetes with and without neuropathy, levels of PINP and CTX were found to be reduced in people with T1D type 1 diabetes with and without neuropathy when compared with healthy individuals. No difference in levels of PINP and CTX was found between people with type 1 diabetes with and without neuropathy [76]. This is a one-of-a-kind study that looked to study bone turnover in type 1 diabetes and neuropathy.

4.1.2 Bone mineral density type 1 diabetes

An explanation for the increased fracture risk and bone fragility in type 1 diabetes is decreased aBMD. A meta-analysis by Vestergaard and colleagues reported decreased aBMD at the lumbar spine and hip in patients with type 1 diabetes compared to individuals without diabetes, corresponding to Z-scores of -0.22 and -0.38, respectively [72]. Janghorbani and colleagues reported similar patterns of decreased aBMD in T1D type 1 diabetes [71]. This decreased aBMD in type 1 diabetes translates to a 1.42 relative risk of having a hip fracture [71]. A more recent meta-analysis by Vilaca and colleagues reports a 4.9-fold increase in the occurrence of a hip fracture in T1D due to decreased aBMD [68].

This displays the need for additional research to be done to provide a more accurate representation of the risk of hip fractures in type 1 diabetes.

4.1.3 Bone microarchitecture and bone strength in type 1 diabetes

Data available on bone microarchitecture in T1D is limited. A study done by Shanbhogue et al. which included 55 participants T1D (including 29 participants with microvascular complications and type 1 diabetes) and 55 individuals without diabetes reported decreased trabecular and cortical vBMD at the radius; and decreased vBMD and cortical thickness at the tibia in patients with T1D and microvascular complications when compared to individuals without diabetes. They reported no differences in cortical porosity. When comparing patients with T1D with and without microvascular complications, those with microvascular complications had lower total vBMD, trabecular vBMD and trabecular thickness at both the radius and tibia. They also reported lower cortical area, higher trabecular separation and higher trabecular network inhomogeneity at the tibia. However, the authors reported no significant differences in parameters of bone microarchitecture in patients with T1D without microvascular complications and those without diabetes [75].

A study done by our research group comparing patients with T1D and neuropathy (T1DN+, N=20), patients with T1D without neuropathy (T1DN-, N=20) and healthy controls (N=20) reported cortical porosity at the tibia to be 56% higher in T1DN+ patients when compared to the T1DN- group. This finding correlated positively with the severity of neuropathy measured by the Toronto Clinical Neuropathy Score and correlated negatively with nerve conduction amplitude and velocity [76].

On investigating parameters of bone strength (bone stiffness and failure load) in T1D, Shanbhogue et al. and Vilaca et al. reported no differences in bone stiffness and failure load in patients with T1D (with and without microvascular complications and neuropathy) and healthy controls [75], [76].

4.1.4 MicroRNAs in type 1 diabetes and bone health

The reasons for the increase in fracture risk in T1D is multifactorial and more research is required to understand the underlying mechanisms of bone fragility in T1D. After investigating bone turnover, BMD, bone microarchitecture and bone strength, I believe miRNAs could act as potential biomarkers to allow for early diagnosis of diabetes induced bone fragility.

So far, very few studies have investigated the role of miRNAs in T1D and bone metabolism and some of these studies have conflicting results. One of the first studies was published by Grieco et al. and they measured 6 microRNAs (miR-21, miR-24, miR-27a, miR-148a, miR-214, miR-375) in serum of 15 patients with T1D and compared them to 14 non-diabetic subjects. The authors reported increased expression levels of miR-148a and miR-21 in

patients with T1D when compared to the non-diabetic subjects. Correlation analysis of these two miRNAs showed no correlation with metabolic parameters such as BMI and HbA1c; however, miR-148a was found to be negatively correlated with total body aBMD and miR-21 was found to be negatively correlated with bone mineral content in the femur [25].

Although in the former study, the authors were only able to show a tendency for miR-214 to be increased in serum of patients with T1D compared to healthy controls [25], Wang et al. were able to find a significant increase in expression of miR-214 in bone in patients with T1D when compared to healthy controls [98]. This may be due to the different sample types (bone and serum) used to measure miR-214 in these two studies. It may also be possible that miR-214 is largely expressed in bone tissue and detection of its expression dilutes as it passes through circulation. These conflicting results suggest the need for additional research to be done for an informed decision on the use of miRNAs as promising biomarkers for diabetic bone disease.

4.1.5 Summary

Despite a difference in representation of fracture risk inT1D; conflicting evidence of bone microarchitecture and bone strength inT1D; and low evidence for the use of miRNAs as possible biomarkers for diabetic bone disease, several studies and meta-analysis in literature and previous chapters in this thesis provide evidence for low bone turnover, low aBMD and disturbed bone microarchitecture in T1D. In literature, there exists weak evidence for the use of miRNAs as suitable biomarkers for assessment of bone fragility in T1D.

Additionally, the studies mentioned above in this chapter provide evidence for microvascular complications such as neuropathy to weaken the bone in people with T1D. However, at the moment we have no evidence on whether the prolonged duration of diabetes and neuropathy weakens the bone further and increases bone fragility; and if T1D leads to bone loss over time.

Therefore, I performed a follow-up study (with a follow-up time of 4 years and 4 months) to a previous study called ‘An evaluation of bone microarchitecture in people with T1D with and without neuropathy’ (STH19719 (IRAS: 222726)) conducted by our research group. This follow-up study allowed us to investigate longitudinal changes in miRNA expression levels, bone turnover, aBMD, bone microarchitecture and bone strength in T1D and understand whether the prolonged duration of diabetes and neuropathy increased bone loss and bone fragility over time. This study also allowed to add value on the use of miRNAs as suitable biomarkers for diabetic bone disease.

4.1.6 Research questions

In comparison to healthy volunteers, does prolonged duration of T1D and neuropathy

1. Further decrease bone turnover and aBMD?
2. Worsen bone microarchitecture and bone strength?
3. If aBMD further decreases over time, do people with diabetes experience excessive bone loss?
4. Can we identify miRNAs as suitable biomarkers to better understand bone fragility in type 1 diabetes?

4.2 Methods

4.2.1 Participants

This study 'A longitudinal study of microRNA expression levels, bone mineral density, bone microarchitecture, bone strength and bone turnover markers in people with T1D with and without neuropathy and the comparison with healthy controls' (STH20992 (IRAS: 303770)), is a follow up study to a previous study conducted by our research group 'An evaluation of bone microarchitecture in people with T1D with and without neuropathy' (STH19719 (IRAS: 222726)). Hence, all participants who took part in the study at baseline and provided consent to be contacted for further research were invited to take part in this follow up study. All eligible study participants with T1D were evaluated for the presence of neuropathy using the Toronto Clinical Neuropathy Score and nerve conduction assessment using DPN Check. Study participants were accordingly classified into 2 groups, those with T1D and neuropathy (T1DN+) and those with T1D without neuropathy (T1DN-). Participants without T1D were classified as healthy controls. Between the baseline and follow up visit, care was taken to note if any participant had developed diabetes or neuropathy. Details on participant inclusion and exclusion criteria, and participant recruitment has been previously described in chapter 2.

4.2.2 Measurement of BTMs, aBMD, bone microarchitecture and miRNAs

Bone formation marker PINP and bone resorption marker CTX was measured in plasma using the IDS-iSYS Multidiscipline Automated System. aBMD was measured by DXA (Discovery A, Hologic Inc., Bedford, MA, USA) at the lumbar spine, total hip, and femoral neck. HR-pQCT scans at the standard site were performed to assess bone microarchitecture at the distal radius and tibia (XtremeCT I, Scanco Medical AG, Zurich, Switzerland) (chapter 2). HR-pQCT scans acquired at the baseline and follow up visit were 3D registered and transformed to measure longitudinal changes in bone microarchitecture. Micro finite element analysis was

used to estimate bone strength at the standard site at the distal radius and tibia (version 1.13; FE-solver included in the Image Processing Language, Scanco Medical AG, Zurich, Switzerland) (chapter 2). To measure longitudinal changes in bone strength, the HR-pQCT scans were 2D registered. miRNAs were measured in plasma at the baseline and follow up visit using the LC 480 Real-Time PCR system (Roche) (chapter 2).

4.2.3 Statistical analysis

In this chapter of the thesis, 64 miRNAs were measured in the sample selection. Hence, to allow for multiple comparisons and avoid the risk of false-positive errors, a Bonferroni correction was done. Statistical significance in miRNA expression levels between the study groups were analysed using this Bonferroni correction, which resulted in $p < 0.008$ to be considered as statistically significant. Normally distributed variables between the three study groups were compared using the one-way ANOVA test. For normally distributed variables relevant only to the respective groups, student's *t*-test was used. Results are represented as mean and standard deviation (SD), and as mean and 95% CI where appropriate. Pair-wise comparisons within a group were performed using the appropriate *t*-test. Variables that were not normally distributed, were analysed using the Mann-Whitney test and Kruskal-Wallis test where appropriate.

Method comparison of miRNA expression in serum and plasma was done using Passing and Bablok regression. Where appropriate, the strength of a relationship between two variables was assessed using Spearman's rank correlation. For all analyses, $p < 0.05$ was considered significant. Statistical analysis was done using GraphPad Prism (Version 9.3.1).

4.3 Results

4.3.1 Characteristics of study participants

All participants who took part in the study at baseline provided consent to be contacted for future research and were hence contacted to take part in this follow up study. Out of the 60 participants who took part in the study at baseline (20 T1DN+, 20 T1DN- and 20 healthy controls), 29 participants took part in the follow up study (14 T1DN+, 6 T1DN- and 9 healthy controls). The split of participants by gender is described in Table 4.1. During the follow up visit, 2 participants were lost because they were in the range of <5 years since menopause. One participant had eGFR <60 mL/min/1.73m^2. Three participants were on either osteoporosis related medications or on hormone replacement therapy. The remaining participants were not interested to participate in the study or had moved away from Sheffield. None of the participants in the healthy control group developed diabetes and none of the participants in the T1DN- group developed neuropathy. The average follow up time between the two visits was 4 years and 4 months. The characteristics of the study population are described in Table 4.1.

Table 4-1. Summary of characteristics of the study population who attended the follow-up visit.

	CTRL	T1DN-	T1DN+	p
N (M/F)	9 (5M / 4F)	6 (3M / 3F)	14 (9M / 5F)	
Age (years)	58.23 (11.91)	51.00 (15.02)	51.72 (11.93)	0.42
BMI (Kg/m^2)	24.67 (2.90)	24.81 (1.44)	26.83 (5.22)	0.40
HbA1c (mmol/mol)	36.71 (3.02)	62.11 (15.94)	65.14 (9.42)	<0.0001
Diabetes duration (years)	NA	24.21 (14.43)	27.86 (9.11)	0.11
TCNS	NA	2.71 (1.41)	13.12 (5.61)	<0.001
DPN sural right conduction velocity (m/s)	NA	48.31 (4.12)	31.11 (16.41)	<0.001
DPN sural right amplitude (µV)	NA	11.92 (5.93)	3.71 (2.21)	<0.001
DPN sural left conduction velocity (m/s)	NA	49.80 (5.20)	36.3 (23.1)	0.02
DPN sural left amplitude (µV)	NA	10.11 (4.80)	3.61 (3.92)	0.001

CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy, N = number of participants, BMI = body mass index, HbA1c = hemoglobin A1c, TCNS = toronto Clinical Neuropathy Score, all values except gender are represented as mean (SD).

Since only about half of the population who took part in the study at baseline, were eligible and consented to take part in this follow up study, the first step was to check if the population who took part in the study at follow up were representative of the population who part in the study at baseline. This was done by comparing those participants who took part in the follow up study and those who did not take part in the follow up study, within each of the three study groups. Within each of the three groups, no differences were observed in age, HbA1c, BMI, diabetes duration, TCNS and DPN sural conduction velocity and amplitude between those participants who took part in the follow up study compared to those who did not take part (Table 4.2). This suggests that the population who took part in the follow up study is representative of the initial population that was recruited at baseline.

All the study results from the baseline visit have been published [76] and were part of a PhD thesis submitted by Tatiane Vilaca from our research group.

Table 4-2. Summary of characteristics of participants who did and did not attend the follow-up visit.

	CTRL NFU	CTRL FU	p	T1DN- NFU	T1DN- FU	p	T1DN+ NFU	T1DN+ FU	p
N (M/F)	11 (7M,4F)	9 (5M, 4F)		14 (9M, 5F)	6 (3M, 3F)		6 (3M, 3F)	14 (9M, 5F)	
Age (years)	45 (16.21)	53.94 (18.51)	0.12	53.93 (38.46)	46.70 (33.47)	0.59	48.32 (18.11)	47.24 (13.67)	0.82
BMI (Kg/m^2)	24.32 (2.91)	24.21 (2.75)	0.76	24.92 (1.64)	24.74 (1.20)	0.82	25.96 (5.10)	25.24 (5.61)	0.71
HbA1c (mmol/mol)	33.55 (4.93)	36.10 (3.17)	0.19	59 (11.76)	61.54 (15.90)	0.89	75.03 (38.91)	68.07 (14.31)	0.53
Diabetes duration (years)	NA	NA		24.13 (14.60)	24.4 4 (13.95)	0.61	27.62 (8.91)	28.13 (9.04)	0.69
TCNS	NA	NA		2.40 (1.41)	2.75 (1.25)	0.69	13.01 (5.66)	13.33 (5.11)	0.72
DPN sural right conduction velocity (m/s)	NA	NA		47.93 (4.11)	48.23 (3.90)	0.71	31.01 (16.25)	31.10 (15.92)	0.84
DPN sural right amplitude (µV)	NA	NA		11.81 (5.92)	11.57 (5.73)	0.82	3.92 (2.11)	3.72 (2.44)	0.88
DPN sural left conduction velocity (m/s)	NA	NA		49.90 (5.32)	49.23 (5.10)	0.66	36.22 (23.51)	36.66 (22.92)	0.62
DPN sural left amplitude (µV)	NA	NA		10.22 (4.76)	10.41 (4.32)	0.79	3.51 (3.70)	3.60 (3.51)	0.91

CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy, N = number of participants, BMI = body mass index, HbA1c = hemoglobin A1c, NFU = participants who did not come for the follow-up visit, FU = participants who came for the follow up visit, N = number of participants, TCNS = toronto Clinical Neuropathy Score, all values except gender are represented as mean (SD).

4.3.2 Prolonged duration of type 1 diabetes and neuropathy doesn't worsen bone turnover

At baseline, PINP was not different between T1DN+, T1DN- and healthy controls. No difference in PINP was found between the two diabetic groups (T1DN+ and T1DN-) either (Figure 4.1A). This was contradicting to the results found at baseline containing all the study participants (N = 60) where PINP was found to be 34% lower in T1DN- and 28% lower in T1DN+ in comparison to healthy controls. At follow up, PINP was found to be 31% lower in T1DN+ (p = 0.002) when compared to healthy controls. No difference in PINP was found between the two diabetic groups (T1DN+ and T1DN-) (Figure 4.1B). Between the baseline and follow up visit, PINP was 7% lower in T1DN+, 4% higher in T1DN- and 3% higher in the healthy controls (Figure 4.1C). However, no significant difference in the change of PINP between the two visits was observed across the three groups. I observed one person in the T1DN+ to have a steep increase in PINP of about 60 units between the two study visits. This

is because the person had gained weight over the study period and now fell within a range or normal BMI compared to being underweight at the baseline visit.

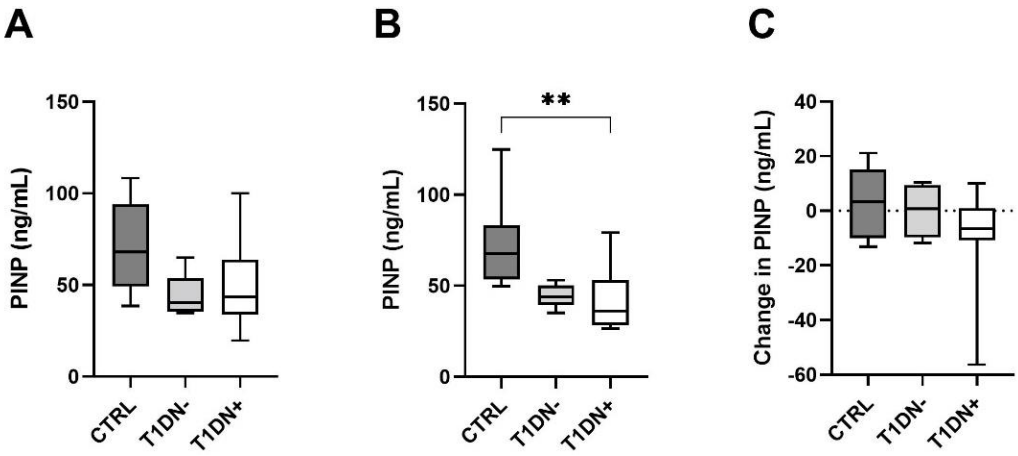

Figure 4-1. Plasma levels of PINP in the three study groups.

(A) At the baseline visit, (B) At the follow up visit, (C) Change in levels of PINP during the two study visits. CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy,

At baseline, CTX was 35% lower (p = 0.02) in T1DN+ compared to healthy controls. No difference in CTX was found between the two diabetic groups (T1DN+ and T1DN-) (Figure 4.2A). This result is in line with the findings found at baseline containing all the study participants (N = 60) [76]. However, I was not able to replicate the findings of CTX being 90% lower in T1DN- compared to healthy controls at baseline containing all the study participants (N = 60). In the entire study population at baseline (N = 60), I observed CTX to be 44% lower in T1DN- (p = 0.01) when compared to healthy controls. This difference may be due to the fact that Vilaca et al. measured CTX in serum, while in this study I repeated all measurements in plasma. At follow up, CTX was found to be 43% lower in T1DN+ (p = 0.003) when compared to healthy controls (Figure 4.2B). No difference in CTX was found between the two diabetic groups (T1DN+ and T1DN-). Between the baseline and follow up visit, CTX was 8% lower in T1DN+, 4% lower in T1DN- and 1% lower in the healthy controls (Figure 4.2C). However, no significant difference in the change of CTX between the two visits was observed across the three groups. The same person in the T1DN+ who had a steep increase in PINP, also had an increase in CTX of about 0.7 units.

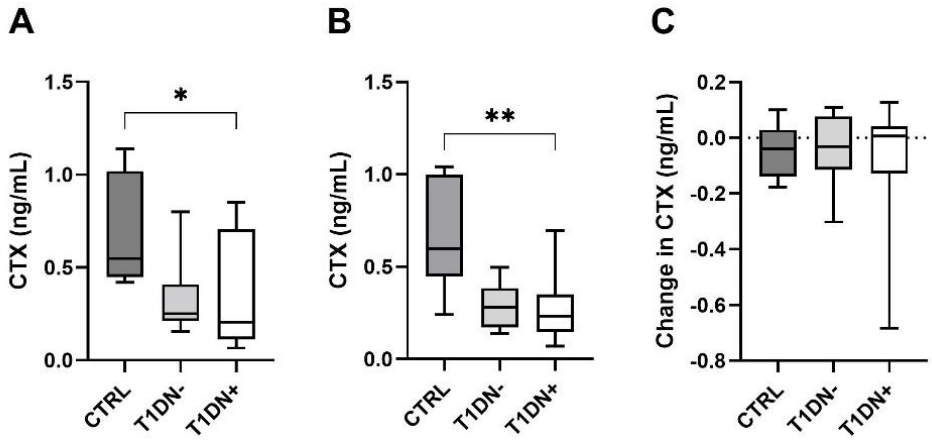

Figure 4-2. Plasma levels of CTX in the three study groups.

(A) At the baseline visit, (B) At the follow up visit, (C) Change in levels of CTX during the two study visits. CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy,

4.3.3 Prolonged duration of type 1 diabetes and neuropathy doesn't lead to excessive bone loss

At baseline and at follow up, aBMD at the lumbar spine (LS), femoral neck (FN) and total hip (TH) was not different between the three groups (Table 4.3).

Table 4-3. Summary of aBMD measured in the three study groups at the baseline and follow-up visit.

		CTRL	T1DN-	T1DN+	p
Baseline visit	LS aBMD (g/cm^2)	0.93 (0.79 to 1.08)	0.98 (0.80 to 1.16)	0.99 (0.91 to 1.10)	0.67
	LS aBMD T-score	-1.25 (-2.47 to -0.03)	-0.81 (-2.30 to 0.67)	-0.70 (-1.51 to 0.12)	0.68
	FN aBMD (g/cm^2)	0.72 (0.65 to 0.79)	0.75 (0.58 to 0.92)	0.74 (0.67 to 0.82)	0.88
	FN aBMD T-score	-1.43 (-1.92 to -0.94)	-1.17 (-2.39 to 0.04)	-1.25 (-1.84 to 0.67)	0.86
	TH aBMD (g/cm^2)	0.91 (0.81 to 1.01)	0.95 (0.76 to 1.14)	0.92 (0.84 to 1.01)	0.87
	TH aBMD T-score	-0.66 (-1.19 to -0.13)	-0.33 (-1.50 to 0.84)	-0.54 (-1.17 to 0.10)	0.82
Follow up visit	LS aBMD (g/cm^2)	0.93 (0.79 to 1.08)	0.97 (0.79 to 1.14)	1.01 (0.92 to 1.10)	0.54
	LS aBMD T-score	-1.26 (-2.48 to -0.05)	-0.91 (-2.34 to 0.52)	-0.57 (-1.37 to 0.23)	0.54
	FN aBMD (g/cm^2)	0.71 (0.63 to 0.78)	0.74 (0.57 to 0.91)	0.74 (0.67 to 0.81)	0.83
	FN aBMD T-score	-1.53 (-2.04 to -1.02)	-1.27 (-2.47 to -0.07)	-1.30 (-1.82 to -0.78)	0.80
	TH aBMD (g/cm^2)	0.89 (0.79 to 0.99)	0.93 (0.75 to 1.10)	0.93 (0.84 to 1.01)	0.83

	TH aBMD T-score	-0.79 (-1.37 to -0.22)	-0.51 (-1.56 to 0.54)	-0.50 (-1.11 to 0.10)	0.76

All values represented as mean (95% CI). CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy, LS = lumbar spine, FN = femoral neck, TH = total hip, aBMD = areal bone mineral density.

The % change in aBMD per year at the lumbar spine, femoral neck and total hip was <1% in all the 3 study groups is listed in Table 4.4. In the CTRL group, at the total hip, I observed a 0.42% significant decrease in aBMD between the two study visits (p = 0.04).

Table 4-4. Percent change in aBMD per year, measured at the lumbar spine, total hip and femoral neck in the three study groups.

		Baseline visit	Follow up visit	Change in aBMD per year	p
LS aBMD (g/cm^2)	CTRL	0.93 (0.79 to 1.08)	0.93 (0.79 to 1.08)	-0.05% (-0.76% to 0.66%)	0.91
	T1DN-	0.98 (0.80 to 1.16)	0.97 (0.79 to 1.14)	-0.23% (-0.92% to 0.45%)	0.89
	T1DN+	0.99 (0.91 to 1.10)	1.01 (0.92 to 1.10)	0.29% (-0.32% to 0.90%)	0.82
FN aBMD (g/cm^2)	CTRL	0.72 (0.65 to 0.79)	0.71 (0.63 to 0.78)	-0.32% (-0.82% to 0.17%)	0.17
	T1DN-	0.75 (0.58 to 0.92)	0.74 (0.57 to 0.91)	-0.25% (-0.74% to 0.24%)	0.24
	T1DN+	0.74 (0.67 to 0.82)	0.74 (0.67 to 0.81)	-0.12% (-0.62% to 0.38%)	0.67
TH aBMD (g/cm^2)	CTRL	0.91 (0.81 to 1.01)	0.89 (0.79 to 0.99)	-0.42% (-0.01% to -0.82%)	0.04
	T1DN-	0.95 (0.76 to 1.14)	0.93 (0.75 to 1.10)	-0.55% (-1.26% to 0.16%)	0.10
	T1DN+	0.92 (0.84 to 1.01)	0.93 (0.84 to 1.01)	0.12% (-0.23% to 0.47%)	0.45

All values represented as mean (95% CI). CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy, LS = lumbar spine, FN = femoral neck, TH = total hip, aBMD = areal bone mineral density.

4.3.4 Outcomes of measurement of bone microarchitecture at the radius

Three scans at the radius were excluded (1 in the T1DN- group and 2 in the T1DN+ group) due to movement artefacts. The HR-pQCT scan measurement at the follow up visit was done using the position of the radius at the baseline visit as a marker. The outcomes of the HR-pQCT measurements (except stiffness and estimated failure load) at follow up were measured using the 3D registration method described earlier in Chapter 2. At follow up, stiffness and failure load were measured using the 2D registration method described earlier in Chapter 2.

Table 4.5 lists the data from the outcomes of HR-pQCT scan measurements at the standard site at the radius.

At the baseline visit at the standard site at the radius, none of the outcomes from the HR-pQCT scan measurements were significantly different between the three study groups. This was in agreement to the findings at baseline when all the study participants were included (N = 60) [76].

At the follow up visit at the standard site at the radius, Ct. vBMD was significantly increased by 9.4% in T1DN+ when compared to healthy controls (p = 0.03), -75.06 (-145.80 to -4.33), (mean difference (95% CI of difference) (Table 4.5). However, the change in Ct. vBMD at the standard site at the radius was not significantly different been the 3 study groups (CTRL vs T1DN-, p = 0.14, -44.83 (-102.0 to 12.31) and CTRL vs T1DN+, p = 0.11, -37.71 (-82.88 to 7.47) (mean difference (95% CI of difference)) (Figure 4.3). In the T1DN+ group, Ct. vBMD between the baseline and follow up visit was also not significant (p = 0.09) (Figure 4.4).

Table 4-5. Outcome of HR-pQCT measurements at the radius in the three study groups at baseline and follow up.

		CTRL	T1DN-	T1DN+	p
Baseline visit	Tt. vBMD (mg HA/cm^3)	305.31 (270.42 to 340.20)	304.11 (253.62 to 354.63)	330.32 (299.51 to 361.11)	0.39
	Tb. vBMD (mg HA/cm^3)	183.04 (155.02 to 211.12)	161.60 (136.16 to 187.14)	187.67 (152.34 to 222.91)	0.55
	Ct. vBMD (mg HA/cm^3)	862.66 (820.32 to 950.24)	892.60 (829.23 to 956.21)	900.03 (878.71 to 921.11)	0.18
	BV/TV (%)	15.25 (12.92 to 17.59)	13.47 (11.34 to 15.60)	15.64 (12.69 to 18.58)	0.55
	Ct. Th. (µm)	0.76 (0.69 to 0.83)	0.85 (0.63 to 1.07)	0.89 (0.80 το 0.99)	0.12
	Tb. N (mm^{-1})	2.12 (1.95 to 2.29)	2.01 (1.76 to 2.25)	1.95 (1.72 το 2.18)	0.43
	Tb. Th. (µm)	0.07 (0.06 to 0.08)	0.07 (0.06 to 0.08)	0.08 (0.07 το 0.09)	0.20
	Tb. Sp. (µm)	0.40 (0.36 to 0.44)	0.44 (0.38 to 0.49)	0.46 (0.36 το 0.56)	0.58
	Ct. Po. (%)	2.64 (1.52 to 3.76)	1.44 (-0.04 to 2.91)	2.43 (1.87 to 2.99)	0.18
	Stiffness (kN/mm)	89.77 (62.21 to 117.3)	84.11 (57.66 to 110.6)	105.00 (87.25 to 122.7)	0.34
	Est. failure load (kN)	4.58 (3.17 to 5.99)	4.20 (2.93 to 5.47)	5.27 (4.37 to 6.17)	0.37
Follow up visit	Tt. vBMD (mg HA/cm^3)	294.92 (257.22 to 332.51)	309.00 (260.21 to 357.87)	325.74 (293.54 to 357.81)	0.36
	Tb. vBMD (mg HA/cm^3)	184.12 (154.61 to 213.50)	169.88 (148.62 to 190.91)	186.72 (152.00 to 221.41)	0.77

	Ct. vBMD (mg HA/cm^3)	795.23 (737.31 to 853.22)	870.02 (800.31 to 939.82)	870.34 (833.80 to 906.83)	**0.03**
	BV/TV (%)	15.34 (12.89 to 17.79)	14.15 (12.38 to 15.91)	15 59 (12.67 to 18.45)	0.77
	Ct. Th. (µm)	0.74 (0.68 to 0.81)	0.82 (0.60 to 1.04)	0.87 (0.78 to 0.96)	0.12
	Tb. N (mm^{-1})	2.29 (2.15 to 2.43)	2.17 (1.93 to 2.41)	2.07 (1.83 to 2.31)	0.25
	Tb. Th. (µm)	0.07 (0.06 to 0.08)	0.07 (0.06 to 0.08)	0.07 (0.07 to 0.08)	0.24
	Tb. Sp. (µm)	0.37 (0.34 to 0.41)	0.40 (0.35 to 0.45)	0.43 (0.34 to 0.53)	0.47
	Ct. Po. (%)	2.37 (1.44 to 3.29)	1.31 (-0.04 to 2.66)	2.31 (1.76 to 2.86)	0.16
	Stiffness (kN/mm)	83.51 (55.05 to 111.12)	81.30 (54.95 to 107.65)	97.27 (80.08 to 114.47)	0.47
	Est. failure load (kN)	4.31 (2.88 to 5.74)	4.07 (2.82 to 5.32)	4.91 (4.04 to 5.77)	0.51

All values represented as mean (95% CI). CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy, Tt. vBMD = total volumetric BMD, Tb. vBMD = trabecular volumetric BMD, Ct. vBMD = cortical volumetric BMD, BV/TV = trabecular bone volume fraction, Ct. Th. = cortical thickness, Tb. N = trabecular number, Tb. Th. = trabecular thickness, Tb. Sp. = trabecular separation, Ct. Po. = cortical porosity, Est. failure load = estimated failure load.

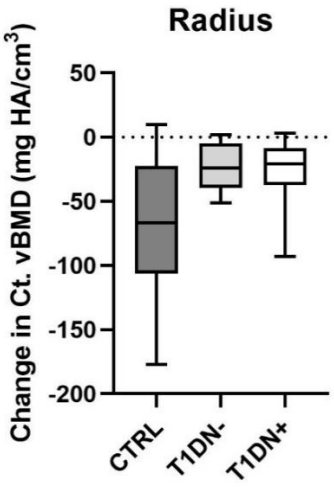

Figure 4-3. Change in cortical volumetric BMD (Ct. vBMD) at the radius in the three study groups during the two study visits.

CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy.

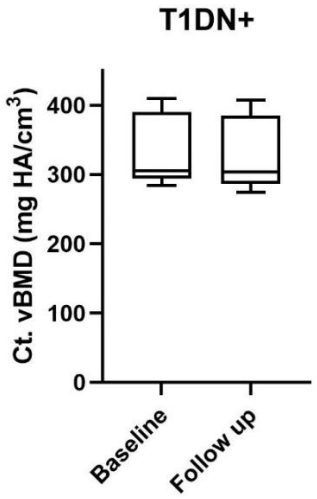

Figure 4-4. Comparison of the measurement of cortical volumetric BMD (Ct. vBMD) at the radius in patients with type 1 diabetes and neuropathy at the baseline and follow up visit.

CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy.

4.3.5 Outcomes of measurement of bone microarchitecture at the tibia

One scan at the tibia was excluded (1 in the CTRL group) due to movement artefacts. The HR-pQCT scan measurement at the follow up visit was done using the position of the tibia at the baseline visit as a marker. The outcomes of the HR-pQCT measurements (except stiffness and failure load) at follow up were measured using the 3D registration method described earlier in Chapter 2. At follow up, stiffness and failure load were measured using the 2D registration method described earlier in Chapter 2.

Table 5.6 lists the outcomes from the HR-pQCT scan measurements at the standard site at the tibia. At the baseline visit at the standard site at the tibia, only cortical porosity was found to be 14.8% higher in the T1DN+ group when compared to the T1DN- group (p = 0.04), -3.24 (-6.40 to -0.08), mean difference (95% CI of difference). This was in agreement to the finding at baseline when all the study participants were included (N = 60) [76]. At the follow up visit at the standard site at the tibia, none of the outcomes from the HR-pQCT scan measurements were significantly different between the three study groups. During follow up, cortical porosity at the standard site at the tibia was not significantly different between CTRL and T1DN+ (p = 0.12), -2.60 (-5.62 to 0.43), mean difference (95% CI of difference) (Table 5.6). Cortical porosity at the standard site at the tibia was also not significantly different in the T1DN+ group between the 2 study visits (p = 0.19) (Figure 4.5).

Table 4-6. Outcomes of HR-pQCT measurements at the tibia in the three study groups at baseline and follow up.

		CTRL	T1DN-	T1DN+	p
Baseline visit	Tt. vBMD (mg HA/cm^3)	284.91 (250.94 to 318.83)	293.41 (221.66 to 365.23)	305.34 (269.56 to 341.03)	0.72
	Tb. vBMD (mg HA/cm^3)	173.17 (140.89 to 205.52)	185.53 (145.44 to 225.62)	197.02 (168.32 to 225.86)	0.49
	Ct. vBMD (mg HA/cm^3)	864.33 (820.37 to 908.36)	878.21 (770.44 to 985.91)	863.00 (833.21 to 892.87)	0.89
	BV/TV (%)	14.43 (11.73 to 17.12)	15.46 (12.11 to 18.80)	16.42 (14.03 to 18.81)	0.49
	Ct. Th. (µm)	1.11 (0.98 to 1.24)	1.11 (0.62 to 1.60)	1.18 (1.01 to 1.35)	0.84
	Tb. N (mm^{-1})	1.82 (1.57 to 2.07)	1.91 (1.56 to 2.26)	2.00 (1.72 to 2.28)	0.61
	Tb. Th. (µm)	0.08 (0.07 to 0.09)	0.08 (0.07 to 0.09)	0.08 (0.07 to 0.09)	0.82
	Tb. Sp. (µm)	0.48 (0.41 to 0.56)	0.44 (0.35 to 0.54)	0.45 (0.37 to 0.53)	0.76
	Ct. Po. (%)	6.56 (4.62 to 8.51)	4.29 (2.66 to 5.92)	7.53 (5.78 to 9.28)	**0.04**
	Stiffness (kN/mm)	229.71 (174.98 to 284.64)	247.02 (143.61 to 350.32)	245.32 (208.41 to 281.76)	0.85
	Est. failure load (kN)	11.52 (8.79 to 14.25)	12.41 (7.23 to 17.60)	12.32 (10.44 to 14.20)	0.84
Follow up visit	Tt. vBMD (mg HA/cm^3)	278.77 (240.82 to 316.63)	295.43 (216.32 to 374.51)	302.91 (265.44 to 340.43)	0.69
	Tb. vBMD (mg HA/cm^3)	169.34 (140.01 to 198.67)	186.82 (145.91 to 227.77)	196.2 (166.12 to 226.31)	0.42
	Ct. vBMD (mg HA/cm^3)	843.02 (793.32 to 892.81)	862.2 (737.94 to 986.55)	847.42 (817.56 to 877.22)	0.88
	BV/TV (%)	14.11 (11.66 to 16.55)	15.57 (12.16 to 18.98)	16.35 (13.84 to 18.86)	0.42
	Ct. Th. (µm)	1.15 (1.02 to 1.29)	1.18 (0.68 to 1.68)	1.22 (1.02 to 1.41)	0.90
	Tb. N (mm^{-1})	1.89 (1.57 to 2.20)	1.98 (1.57 to 2.38)	1.98 (1.66 to 2.30)	0.90
	Tb. Th. (µm)	0.07 (0.06 to 0.08)	0.08 (0.06 to 0.09)	0.08 (0.07 to 0.09)	0.46
	Tb. Sp. (µm)	0.47 (0.40 to 0.54)	0.44 (0.34 to 0.54)	0.47 (0.36 to 0.57)	0.93
	Ct. Po. (%)	6.56 (4.64 to 8.47)	4.63 (2.47 to 6.79)	7.23 (5.65 to 8.81)	0.12
	Stiffness (kN/mm)	220.30 (169.60 to 270.00)	246.05 (139.96 to 352.15)	242.59 (204.84 to 280.33)	0.71
	Est. failure load (kN)	11.11 (8.57 to 13.65)	12.32 (7.07 to 17.58)	12.19 (10.30 to 14.11)	0.73

All values represented as mean (95% CI).

CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy, Tt. vBMD = total volumetric BMD, Tb. vBMD = trabecular volumetric BMD, Ct. vBMD = cortical volumetric BMD, BV/TV = trabecular bone volume fraction, Ct. Th. = cortical thickness, Tb. N = trabecular number, Tb. Th. = trabecular thickness, Tb. Sp. = trabecular separation, Ct. Po. = cortical porosity, Est. failure load = estimated failure load.

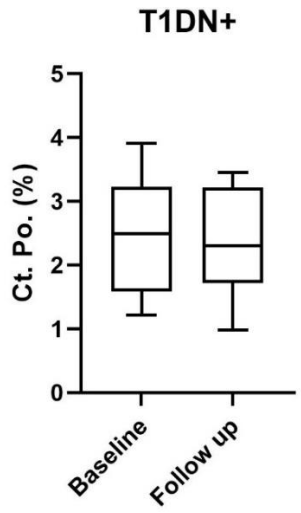

Figure 4-5. Comparison of cortical porosity at the tibia in patients with type 1 diabetes and neuropathy at the baseline and follow up visit.

At the radius, the cortical porosity at the standard site decreased by 0.027% in the CTRL group, 0.13% in the T1DN- group and 0.12% in the T1DN+ group between the two study visits. This change in cortical porosity was not significantly different between the 3 study groups (Figure 4.6A).

At the tibia, the cortical porosity at the standard site increased by 0.01% in the CTRL group and 0.3% in the T1DN- group; and decreased by 0.34% in the T1DN+ group between the two study visits. This change in cortical porosity was not significantly different between the 3 study groups (Figure 4.6B).

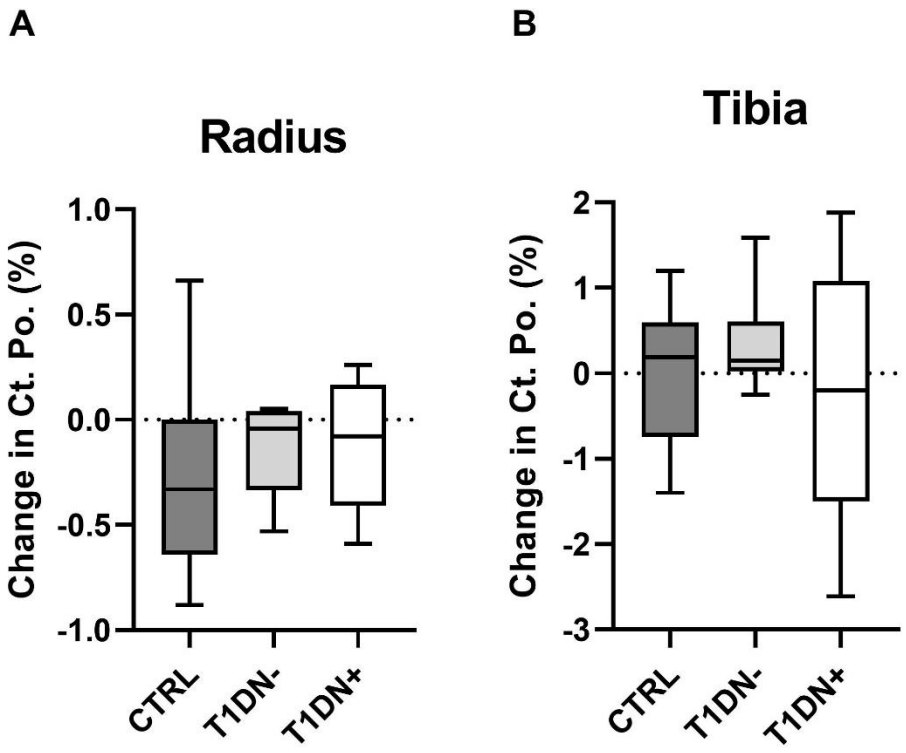

Figure 4-6. Change in cortical porosity at the radius (A) and tibia (B) in the three study groups during the two study visits.

CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy.

4.3.6 MicroRNAs in type 1 diabetes

At the baseline visit, the set of 61 specifically selected endogenous miRNAs, including 2 miRNAs for monitoring haemolysis and 3 spike in controls (UniSp4, UniSp3 and Cel-miR-39-3p) were measured in plasma of the entire study population (N = 60). All samples passed the data quality check derived from the spike-in controls. None of the samples were haemolysed. I observed an unexpected result where within a given miRNA and within a given experimental group, I observed a heterogeneous distribution in the expression levels of all the endogenous miRNAs measured. The data looked like as if within a given experimental group, a given miRNA was highly expressed in some samples of a given experimental group and lowly expressed in some samples of the same experimental group. The same results were observed when the miRNAs were measured in serum as well. Figure 4.7 explains the above observation for miR-21-5p.

A Passing and Bablok regression was performed to compare baseline miRNA expression levels in serum and plasma. For all the miRNAs measured, I observed that those miRNAs that were less abundant in serum were less abundant in plasma as well. Figure 4.8 explains this observation for miR-21-5p.

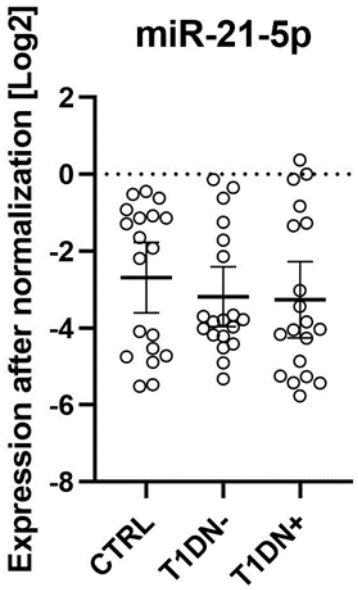

Figure 4-7. Level of expression of miR-21-5p in the three study groups measured at baseline.

Expression of miR-21-5p is normalised to UniSp4. CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy.

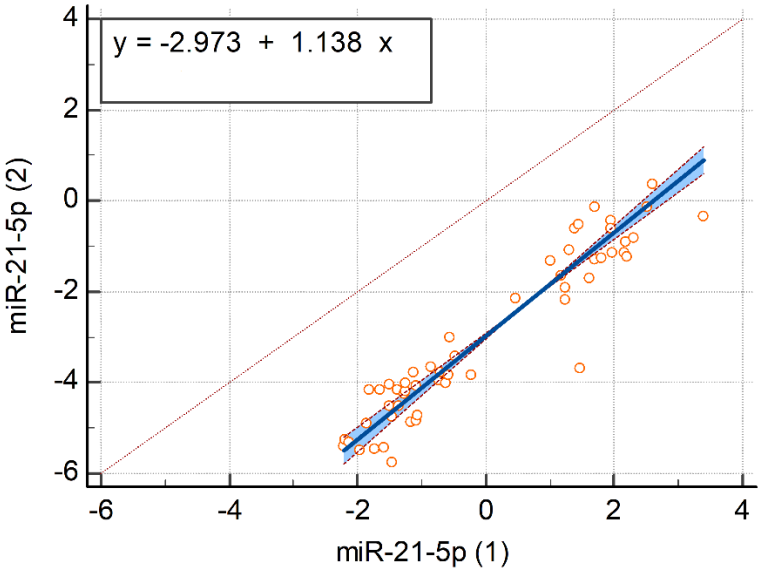

Figure 4-8. Representation of a Passing and Bablok regression for miR-21-5p measured at baseline.

Expression of miR-21-5p is normalised to UniSp4. x-axis - miR-21-5p (1) = serum expression of miR-21-5p, y-axis miR-21-5p (2) = plasma expression of miR-21.

I tried to find out the reasons for the above observation by investigating: (i) the season during which the blood sample was collected, (ii) the time duration within which serum and plasma samples were separated and processed and (iii) the medications the participants were on. None of these factors helped to understand why I observed the above unexpected

observation. Due to this observation, I was unable to look for longitudinal changes in miRNA expression levels in the study population.

At the follow up visit, the set of 61 specifically selected endogenous miRNAs, including 2 miRNAs for monitoring haemolysis and 3 spike in controls (UniSp4, UniSp3 and Cel-miR-39-3p) were measured in plasma. All samples passed the data quality check derived from the spike-in controls. None of the samples were haemolysed. The following microRNAs were excluded from the analysis: miR-124-3p, miR-217-5p, miR-135b-5p and miR-203a-3p. This is because their detection in plasma was below the level of detection in about 1/3 of all the high quality samples.

Figure 4.9 contains plots of plasma expression (at the follow up visit) of the 9 top ranked miRNAs from the systematic review and sensitivity analysis done in Chapter 3. None of these 9 microRNAs were found to be significantly upregulated or downregulated ($p < 0.05$) between the 3 study groups. Neither did any of the measured miRNAs have a p-value < 0.008 (Bonferroni correction).

Although not statistically significant ($p < 0.05$ / $p < 0.008$), miR-133a-3p showed a tendency to be downregulated in T1DN+ and T1DN- when compared to the CTRL group ($p = 0.057$ and $p = 0.058$, respectively) (Figure 4.9H).

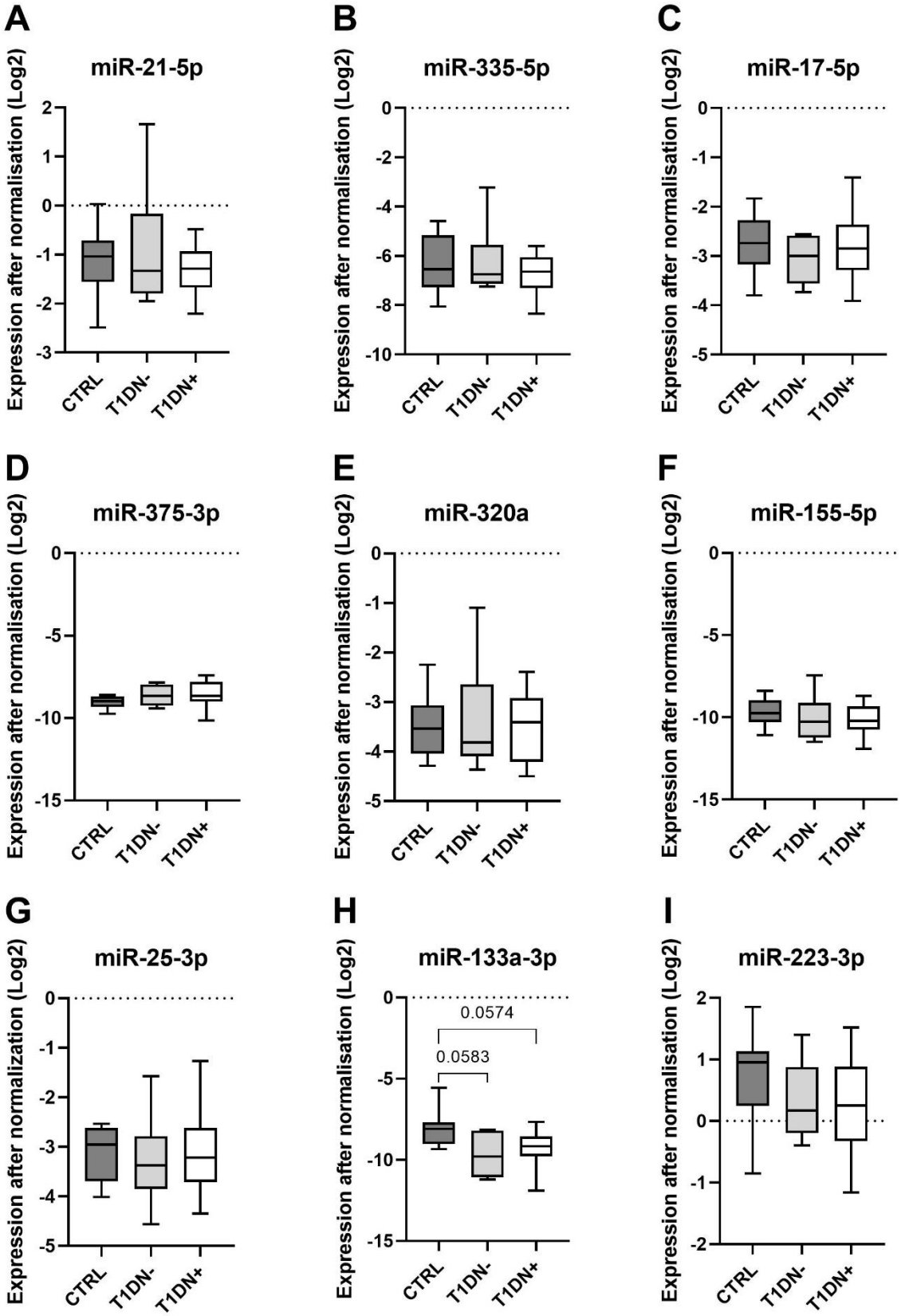

Figure 4-9. Plasma expression of miRNAs measured at the follow up visit in the study population.

Expression of the miRNAs is normalised to UniSp4. CTRL = healthy controls, T1DN- = type 1 diabetes without neuropathy, T1DN+ = type 1 diabetes with neuropathy.

Since the expression of miR-133a-3p showed a tendency to be lower in T1DN+ and T1DN- compared to CTRL, I wondered what the expression of this miRNA would be in T1D in general. Due to our small study sample size, I combined the two T1D (T1DN- and T1DN+) groups together. After combining the two groups of T1D (T1DN- and T1DN+), plasma expression of miR-133a-3p was ~1.4 Cq lower in T1D when compared to CTRL. This difference was statistically significant ($p < 0.05$) (Figure 4.10).

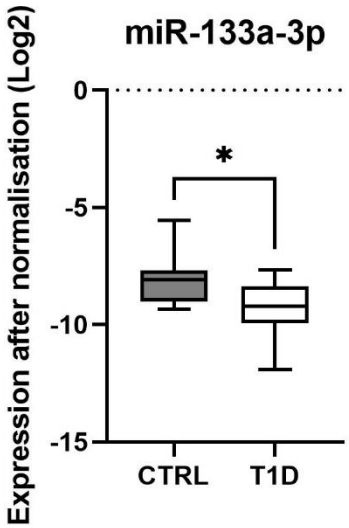

Figure 4-10. Plasma expression of miR-133a-3p measured at the follow up visit in all patients with type 1 diabetes and healthy controls.

Expression of miR-133a-3p is normalised to UniSp4. CTRL = healthy controls, T1D = all patients with type 1 diabetes.

A Spearman's rank correlation was performed between miR-133a-3p and HbA1c to understand if the decrease in expression of miR-133a-3p may be a reason of the increase in HbA1c levels in T1D. I observed no significant negative correlation between miR-133a-3p and HbA1c in T1D ($r = 0.03$, $p = 0.90$). I next performed a Spearman's rank correlation between miR-133a-3p, PINP and CTX to understand if the decrease in expression of miR-133a-3p in T1D may be a reason for the observed low bone turnover. I observed no significant association between miR-133a-3p and PINP ($r = 0.08$, $p = 0.74$) and between miR-133a-3p and CTX ($r = 0.13$, $p = 0.59$) (Table 4.7).

Table 4-7. Spearman's rank correlation and p value of association between miR-133a-3p, HbA1c, PINP and CTX in the study population with type 1 diabetes.

	HbA1c		PINP		CTX	
	r	p	r	p	r	p
miR-133a-3p	0.03	0.90	0.08	0.74	0.13	0.59

4.4 Discussion

4.4.1 Summary of main findings

The average follow up time between the two study visits was 4 years and 4 months. During the study's follow up visit, almost half of the study population was lost because some participants were ineligible, some were not interested to take part anymore and some participants had moved away from Sheffield. This resulted in a total population of 29 participants. The population who took part in the study at follow up were representative of the initial population who took part in the study at baseline.

During the follow up visit, plasma PINP was 31% and plasma CTX was 43% lower in T1DN+ when compared to the CTRL group and no difference in plasma PINP or plasma CTX was observed between the two diabetic groups (T1DN- and T1DN+). I found no significant change in plasma PINP or plasma CTX between the two visits in all 3 study groups. aBMD measured by DXA at the lumbar spine, femoral neck and total hip was not significantly different between the 3 groups during the follow up visit and no differences in aBMD were found between the two diabetic groups (T1DN+ and T1DN-). The average percent change in BMD per year at all the 3 sites measured was less than 1%. At the standard site at the radius, during the follow up visit, I found only cortical vBMD to be increased in T1DN+ when compared to the CTRL group. The change in cortical vBMD at the standard site at the radius between the 2 visits was not significant in the 3 study groups. At the standard site at the tibia, during the follow up visit, I observed no differences in any of the outcomes of HR-pQCT scan measurements (including cortical porosity) between the 3 study groups. The average change in cortical porosity at the standard site at the radius and tibia was less than 1% during the two study visits. From all the miRNAs measured in plasma at the follow up visit, I found expression of miR-133a-3p was found to be downregulated in T1D (T1DN+ and T1DN- combined) when compared to the CTRL group. However, this miRNA did not correlate with bone turnover.

4.4.2 Bone turnover in type 1 diabetes and neuropathy

During the baseline visit containing the entire study population (N=60), PINP was found to be significantly reduced by 34% in T1DN- in comparison to healthy controls while CTX was found to be ~90% reduced in both the diabetic groups (T1DN+ and T1DN-) when compared to healthy controls [76]. In this follow up study (N=29), I was unable to replicate the above baseline findings. One reason for this could be the different sample type PINP and CTX were measured in. At baseline (including the entire population of 60) [76], PINP and CTX were measured in serum and in this study, PINP and CTX were re-measured in plasma, as CTX is thought to be more stable in plasma [292]. During follow up, I found PINP to be 31% lower ($p = 0.002$) and CTX to be 43% lower ($p = 0.003$) in T1DN+ in comparison to the CTRL group. No significant differences in the change in plasma PINP and CTX during the two study visits was observed between the three groups. Moreover, I found no significant differences in plasma PINP and CTX between the baseline and follow up visit in the two diabetic groups (T1DN+ and T1DN-). This finding of CTX not being statistically significant in T1D (in both men and women) between a baseline and follow up visit (a follow up time of 5 years and 10 years) is in line with Hamilton et al. [88], [293]. However, Hamilton and colleagues have reported a 15% significant decrease in serum PINP in only men with T1D after a mean follow up period of 10 years [88]. Our results support literature evidence for a steady state of low bone turnover in T1D [293], [294], the only difference being, in our study PINP was measured in plasma and the mean follow up time was 4 years and 4 months. At present there are no other longitudinal studies on bone turnover in type 1 diabetes and neuropathy. Our results suggest that the decrease in bone turnover occurs early on in T1D and neuropathy and does not reduce further with a prolonged duration of the disease.

4.4.3 Bone mineral density in type 1 diabetes and neuropathy

During the baseline and follow up visit, no differences in aBMD were observed at the lumbar spine, femoral neck and total hip in the T1DN- and T1DN+ group compared to controls. This finding observed in the representative population of the study is in line with the finding when the entire baseline study population was included [76]. However, this finding is conflicting to what is observed in literature of an overall decrease in aBMD in T1D [72], [295]. During the course of this study, I observed a significant 0.42% decrease in aBMD in the CTRL group. I believe this may be due to age related bone loss [296].

BMD relates to age, gender, height and BMI. It is important to note that at the baseline visit of this study [76], efforts were taken to match the study groups for age, gender and BMI. However, the matching was not fully successful. It is possible that the attempt to match the

study groups could have potentially influenced the differences observed between the study groups.

Additionally, I found no significant differences in the rate of change in aBMD per year at the lumbar spine, femoral neck and total neck between the three study groups, as well as between the diabetic groups (T1DN+ and T1DN-) during the two study visits. The yearly rate of change in aBMD was <1% between the three study groups at all the three sites measured, suggesting that the prolonged duration of T1D and neuropathy does not lead to accelerated bone loss. There have been other longitudinal studies of BMD in T1D, but our study is the first to study longitudinal changes in aBMD in T1D and neuropathy. Miazgowski and Czekalski [87] found no difference in aBMD during a 2-year follow period at the lumbar spine and whole body in a population of 54 adults with T1D. Hamilton et al. also found no change in aBMD at the total hip and femoral neck after adjusting for age and BMI, and no increased risk of progression to osteopenia or osteoporosis after a mean follow up time of 10 years [88]. Another study of only 7 participants found a very small but significant 0.5% decrease in bone mineral content in the trabecular bone in insulin dependent diabetic patients after a follow up time of ~11 years [89]. The results of these various studies do not contradict our findings, all suggesting that there is possibly no clinically alarming sustained adverse effect of prolonged duration of T1D on aBMD.

Literature provides support for the early onset of T1D and poor metabolic control during the growing phase to have a negative impact on aBMD; and for this negative effect to stabilise with better control of T1D. In our study, 17 (7 T1DN-/10 T1DN+) participants had an early onset of T1D (earlier than 18 years old). However, I did not have access to their metabolic control profile during their growing period and hence, I am unable to comment on the potential impact of metabolic control on aBMD during early onset of the disease. In a study of 41 young children (aged between 7-12 years) with T1D, Bechtold et al. have reported that these children experienced a deficit in bone size during their first examination and this normalised during their second examination 5.5 years later [297]. Further, a recent meta-analysis done by Loxton et al. show evidence for young adults with T1D (mean ± SD age = 12.6 ± 2.3 years) to have low BMD and poor bone development throughout childhood when compared with healthy children [298].

Although the sample size of this study was small (N = 29), our results suggest that the prolonged duration of T1D and neuropathy doesn't lead to significant bone loss. However, these results should be taken with caution because to our knowledge, ours is the first study to assess longitudinal changes in aBMD in patients with T1D and neuropathy.

4.4.4 Outcomes of HR-pQCT scan measurements in type 1 diabetes and neuropathy

T1D is associated with a significant increase in fracture risk, particularly after the age of 40 years [71], [72], [299]. Based on the age of the population recruited for the baseline study (35 – 65 years) [76], we hypothesised that aBMD would rapidly decline in T1D and more so during the ~4 year follow up period analysed as part of this thesis. The results by Vilaca et al. and of this chapter did not support the above hypothesis. There is evidence in literature for the estimated hip fracture risk based on aBMD to be poorly associated with fractures documented in patients with T1D [72]. This suggests that assessment of bone quality may be important, and it prompted us to investigate structural changes in the bone microarchitecture using HR-pQCT scan measurements.

In an extended analysis using the same study population and longitudinal HR-pQCT imaging to assess dynamic bone remodelling and markers of mechanoregulation in patients with T1DN- and T1DN+, we have been able to provide initial evidence *in vivo* that participants with T1D show lower bone remodelling and impaired adaptation to mechanical loading. This may result in weakening of the bone microarchitecture, especially in T1DN+ [300].

During the baseline and follow up visit of this study, I found no significant differences in the outcomes of HR-pQCT scan measurements between the CTRL and T1DN- group at the radius and tibia. Our results are in agreement with the study done including all 60 participants at baseline [76] and Shanbhogue and colleagues [75].

4.4.5 Outcomes of HR-pQCT scan measurements at the radius in type 1 diabetes and neuropathy

During the baseline visit, at the standard site at the radius, I found no significant differences in any of the outcomes from the HR-pQCT scan measurements between the three groups, in agreement with Vilaca and colleagues [76]. During the follow up visit, at the standard site at the radius, I found a 9.4% increase in cortical vBMD in T1DN+ in comparison to the CTRL group. Although I observed quite a large increase, this finding is in agreement with Verroken and colleagues where they found a 2.1% increase in cortical vBMD at the radial shaft in 64 adults with T1D [301] using a peripheral quantitative tomography device, while I used a high resolution quantitative tomography device. Of note, literature evidence on cortical vBMD at the radius in T1D is contradicting as Shanbhogue and colleagues report a decrease in cortical vBMD at the radius in patients with T1D with and without microvascular complications [75]. At present there exists very little research on bone microarchitecture in T1D and microvascular complications including neuropathy, and hence, more evidence is required to comment on this finding.

In our study, I also found that the change in cortical vBMD over an average follow up time of 4 years and 4 months was not statistically significant between the three groups. Additionally, the increase in cortical vBMD between the baseline and follow up visit in T1DN+ was also not statistically significant. Our results suggest that the prolonged duration of T1D and neuropathy doesn't lead to a faster decay in bone microarchitecture and bone quality. To our knowledge, this is the first study that has assessed longitudinal changes in bone microarchitecture in T1D and neuropathy. Hence, additional research is required to validate our finding.

4.4.6 Outcomes of HR-pQCT scan measurements at the tibia in type 1 diabetes and neuropathy

In the representative population at baseline, I was able to reproduce the finding of increased cortical porosity at the tibia in T1DN+ in comparison to the T1DN- group. In the representative population I reported a 28.7% increase in cortical porosity, while Vilaca and colleagues reported an increase of 56% [76]. This difference may be due to the smaller number of patients in the representative population. After 4 years and 4 months, I found no further significant increase in cortical porosity in T1DN+. Between the two study visits, the change in cortical porosity was <1% and moreover, the change was not significantly different in the T1DN+ group. The same was observed at the radius. This suggests that changes in bone microarchitecture attenuate with time and do not worsen with prolonged duration of T1D and neuropathy. These patients with T1D may also have a better metabolic control which may support this finding. However, in this study I did not have access to their complete metabolic control profiles and did not investigate their diabetes treatment plans. It is important to note that additional longitudinal studies need to be done to validate this finding.

In the representative population at baseline and follow up, at the standard site at the tibia, I found no significant differences in the other outcomes of HR-pQCT scan measurements. Although in T2D, a 5-year longitudinal study assessing 32 postmenopausal women (10 T2D without history of fractures at baseline (T2D), 10 T2D with a positive history of fracture prior to enrolment (T2DFX), 12 non diabetic without history of fractures at baseline (CTRL)), at baseline at the tibia, the T2DFX group had the highest porosity amongst the three study groups (66.3% greater cortical porosity and 71.9% higher cortical porosity volume, when compared with T2D). Longitudinally, cortical porosity increased over time in all the three study groups and at similar annual rates, while T2DFX had the highest annual decreases in bone strength indices (4.7x and 6.7x greater decrease in failure load and stiffness respectively, when compared to T2D; and 14.1x and 22.2x greater decrease in failure load and stiffness when compared to CTRL) [90].

To our knowledge, ours is the first study that assessed longitudinal changes in outcomes of HR-pQCT scan measurements in T1D and neuropathy. Our study had a small sample size. Hence, our results need to be taken with caution and validated by additional and longer studies in larger cohorts of patients with T1D and microvascular complications.

4.4.7 MicroRNAs in type 1 diabetes and bone heath

In addition to investigating structural changes in bone microarchitecture using HRpQCT, in this study I was also interested in identifying miRNAs as suitable biomarkers using RT-qPCR to understand the reasons for bone fragility and increased fracture risk in T1D. During the analysis of the miRNA measurements at baseline in serum and plasma, I observed an unfavourable finding of heterogeneity in miRNA expression levels within a given group. Due to this the data from miRNA measurements at baseline could not be used. The reasons for this unfavourable finding still remain unexplained. Due to this, I was not able to analyse any longitudinal changes in miRNA expression levels in this study.

Nevertheless, I measured miRNAs in plasma at the follow up visit. In all the 59 identified endogenous miRNAs measured, the following 4 miRNAs (miR-124-3p, miR-217-5p, miR-135b-5p and miR-203a-3p) were excluded from the analysis because their detection in plasma was below the level of detection in 1/3 of all the high quality samples. The reason for this could be the fact that results from the systematic review done as part of this thesis (Chapter 3) show that the above miRNAs were chosen due to their significant expression patterns in patients with T2D.

Amongst the remaining miRNAs measured and detected, I found no significant differences in miRNA expression levels between the three study groups. Although not significant, I observed a tendency for the expression of miR-133a-3p to be downregulated in T1DN- ($p = 0.058$) and T1DN+ ($p = 0.057$), in comparison to the CTRL group. On combining both the diabetic groups (T1DN+ and T1DN-) together (namely, T1D), I observed miR-133a-3p to be significantly downregulated by ~1.4 Cq in T1D in comparison to the CTRL group ($p < 0.05$). A study done in miR-133a overexpressed Akita (genetic mouse model of T1D relevant to humans) mice, prevented diabetes–related cardiac fibrosis, hypertrophy and impaired contractility, suggesting that increased levels of miR-133a in a diabetic heart could prevent cardiac remodelling [302]. Although only in mice, at present, the above mentioned study is the only study that provides evidence for downregulation of miR-133a in T1D. Up to now, there is no evidence for miR-133a to play a role in T1D and neuropathy in humans. However, there is evidence for miR-133a to be upregulated in adults with pre-diabetes and T2D, and cardiovascular related diseases [303], [304]. The lack of literature support makes it difficult to comment on the potential use of miR-133a-3p as a suitable biomarker for T1D. Due to the

lack of literature support, one may question the inclusion of miR-133a in the systematic review. It is important to note that the miRNAs chosen from the systematic review were based on literature evidence in either T1D or T2D.

Moreover, in our analysis, I found no association between miR-133a-3p and BTMs (PINP and CTX). A study comparing miR-133a expression in circulating monocytes in 20 postmenopausal women with high and low BMD reported the expression of miR-133a to be lower in the postmenopausal women with high BMD when compared to those with low BMD [265]. Another study comparing ten postmenopausal women with osteoporosis and ten healthy controls, found serum miR-133a to be significantly upregulated and negatively correlated with lumbar spine BMD in the postmenopausal women with osteoporosis [277]. Since the direction of expression of miR-133a is opposite in T1D and in markers of bone health (aBMD), additional functional analysis is required to understand the role miR-133a plays in T1D and bone metabolism.

4.4.8 Strengths and limitations

This study has its strengths and limitations. One of the strength of this study is that, to our knowledge, this is the first study that assessed longitudinal changes in the outcomes of HR-pQCT scan measurements in T1D and neuropathy and the expression of miR-133a in T1D. Although we lost half of our study population during the follow up visit, the participants who took part in the study at follow up were representative of the population who took part in the initial study at baseline. Next, the same technician performed the DXA and HR-pQCT scan measurements at both the study visits and this technician was an experienced person. This allowed to maintain accuracy while aligning the position of the scan sites during the follow up visit. Another is the use of 3D image registration to compare longitudinal changes in the outcomes of HR-pQCT scan measurements in T1D and neuropathy. The 3D image registration was also done by an experienced person.

However, this study has several limitations. During the initial baseline study visit involving all 60 study participants, best efforts were taken to match the participants in the three groups for several variables. For example, age and weight / BMI could act as confounders and affect the bone microarchitecture. However, recruitment was challenging and the participants were not fully matched individually between the three groups. Of note, the distribution of these variables within the 3 study groups were similar.

Almost half of the study population was lost during follow up. The study was underpowered and the sample size of the follow up visit (N = 29) was lesser than the required number of 32 identified from the sample size power calculation (Chapter 2.3.1). I speculate that no

significant longitudinal changes in bone turnover, BMD or outcomes of HR-pQCT scan measurements; and miRNA measurements were observed due to the small sample size. This also made it difficult to comment on bone loss due to the prolonged duration of T1D. During the baseline study involving all 60 study participants, the first-generation HR-pQCT scanner was used and hence, the same was used during the follow up visit to adhere with consistency in the measurements and direct comparability. The resolution of this first-generation scanner is limited to a voxel size of 82 µm. This means that only pores greater than 82 µm would be detected and over 60% of pores in the cortical bone are less than 100 µm in diameter [305]. A newer generation of the HR-pQCT scanner is now available with a higher resolution of 60.7 µm [306] and using this, may change our study findings.

The small sample size of the study did not allow us to replicate any of the findings on miRNA expression reported in the systematic review (Chapter 3) which made it difficult to identify potential miRNAs as biomarkers for T1D and bone fragility. Even after multiple attempts, we were unable to find a reason for the observed heterogeneous distribution of miRNA expression at baseline in both serum and plasma. This made it unsuitable to use this data, and hence, I could not address one of our aims, which was to assess longitudinal changes in miRNA expression in T1D and neuropathy.

In all, our findings suggest that either ~4 years is not a long enough time to notice changes in bone turnover, aBMD, bone quality and structural changes in bone in T1D and neuropathy, and that the study participants need to be followed for longer to understand the impact of prolonged duration of T1D and neuropathy on bone; or the prolonged duration of T1D and neuropathy does not weaken bone quality further and rather an early onset of T1D and neuropathy may have a negative impact on bone. Additional studies with larger sample sizes and longer follow up periods are needed to validate the findings of this chapter.

4.4.9 Summary

To our knowledge, this is the first study that reported longitudinal changes in bone turnover, aBMD and bone microarchitecture in T1D and neuropathy. After a follow up visit of 4 years and 4 months, no longitudinal changes in bone turnover, BMD or outcomes of HR-pQCT scan measurements were observed in T1DN+, T1DN- and healthy controls. No excessive bone loss was observed and cortical porosity at the tibia did not worsen with prolonged duration of T1D and neuropathy. I provide preliminary evidence for the use of miR-133a-3p as a potential biomarker for T1D.

Chapter 5 - MicroRNAs and bone mineral density in type 2 diabetes: relationship to bone turnover and bone balance

5.1 Introduction

5.1.1 Bone mineral density and fracture risk in type 2 diabetes

Just like type 1 diabetes, type 2 diabetes is also associated with increased risk of fractures [68]. It is important to note that, although the relative risk of fractures is higher in type 1 diabetes than in type 2 diabetes, both populations are at an increased risk. A meta-analysis done by Vestergaard and colleagues reported increased BMD at the lumbar spine and hip in patients with type 2 diabetes compared to individuals without diabetes. This corresponds to a Z-score (mean ± SEM) of 0.41 ± 0.01 at the lumbar spine and 0.27 ± 0.01 at the hip. A similar pattern of increased BMD in type 2 diabetes has been reported by Janghorbani and colleagues [71]. This observation of increased BMD in type 2 diabetes translated to a 0.77 relative risk of having a hip fracture [71]. A more recent meta-analysis done by our research group, reported a 1.3 fold increase in the occurrence of a hip fracture in type 2 diabetes despite of increased BMD [68]. This statement of increased fracture risk in type 2 diabetes may seem paradoxical as BMD is increased in people with type 2 diabetes. This suggests that directly or indirectly several factors such as increased hyperglycaemia, oxidative stress, adipokine alterations, increased marrow fat, prolonged duration of T2D, insulin treatment, T2D-related micro-vascular complications and impaired bone microarchitecture may be associated with fragility fractures in T2D regardless of BMD [84], [307], [308]. This calls for the need to assess the bone microstructure in type 2 diabetes to better understand the increased fracture risk.

5.1.2 Bone turnover in type 2 diabetes

Just like type 1 diabetes, type 2 diabetes is also associated with low bone turnover. A meta-analysis done by Linde and colleagues found levels of the bone resorption marker CTX and bone formation marker OC to be significantly lower in patients with T2D compared to non-diabetic patients [83]. However, they reported no difference in levels of PINP (IOF recommended bone formation marker). This may be due to the heterogeneity in the study populations, such as age, ethnicity, disease duration and metabolic status included in the analysis. In another study carried out by the same authors comparing patients with T2D with age and gender matched controls, they found serum PINP and CTX to be significantly lower in T2D. They have also reported serum CTX to be negatively associated with the mean amplitude of glycaemic excursions (MAGE) and the dawn glucose levels, whereas serum

PINP was only negatively associated with the dawn glucose levels and borderline significantly associated with MAGE ($p = 0.05$) [84]. There is also evidence for increased hyperglycaemia to be associated with low bone turnover [307].

One can hypothesise that the low bone turnover observed in T2D may be responsible for the increased fragility fracture risk, supporting the use of bone turnover markers to predict fractures in T2D. However, this hypothesis was proved null by Napoli and colleagues in the Health, Ageing, and Body Composition (Health ABC) Study. The Health ABC study, which compared older adults aged 70 – 79 years, found fracture risk to be increased with higher CTX in non-diabetics (HR=1.10, 95% CI: 1.01 to 1.20), whereas fracture risk was not increased in T2D (HR=0.92, 95% CI: 0.81 to 1.04). Similarly, they found PINP and OC to be associated with increased fracture risk in non-diabetics but not in T2D ($p = 0.109$ and $p = 0.078$, respectively) [133]. This suggests that BTMs do not predict fracture risk in T2D and there is a need to identify other biomarkers to predict the increased fracture risk in T2D.

This also suggests that BTMs are possibly not very good predictors of bone loss in T2D. Women with postmenopausal osteoporosis have a negative balance between bone resorption and bone formation. It may be this negative balance that is associated with fracture risk. In T2D, the high BMD is associated with low bone turnover. However, the extent of imbalance in bone formation and bone resorption is yet to be investigated.

Methods have previously been described to calculate bone balance from bone turnover: (i) One study suggests calculating the ratio between bone resorption and bone formation [309]; (ii) Another study suggests calculating the Z-scores for postmenopausal women relative to an age-matched group and then subtracting them for bone formation and bone resorption; (iii) A third study uses the multiple of medians to calculate bone turnover and bone balance [310]. However, each of these methods have their own limitations. Methods (i) and (iii) assume BTMs to be specific to bone and all BTMs to have the same relative distribution. Method (ii) only evaluated bone balance and not the overall bone turnover. In this chapter I use a method called the T-score bone marker plot that evaluates bone turnover and bone balance together by combining methods (i) and (ii). This T-score bone marker plot has been previously described by our research group [128].

5.1.3 MicroRNAs in type 2 diabetes

During the course of this thesis, we have come to understand that in T2D, BMD and BTMs are not suitable markers of increased fragility fracture risk. This calls for the need to identify other biomarkers to better predict fracture risk in T2D. In this context, microRNAs are of interest.

There is evidence for miRNAs to act as promising biomarkers for fragility fractures in T2D and osteoporosis. A miRNA-qPCT array experiment performed by Heilmeier et al. identified 48 miRNAs to be differentially expressed in serum between fractured and non-fractured individuals with T2D and 23 miRNAs to be differentially expressed in serum of non-diabetic individuals with an osteoporotic background with and without fractures. Of these, miR-550a-5p and miR-382-3p was identified as being the most promising circulating miRNA for diabetic bone disease; and miR-382-3p and 188-3p were identified to be the most promising miRNAs for osteoporosis. Further, using in vitro functional studies, these miRNAs have shown to play a role in bone metabolism. miR-382-3p significantly increased osteogenic differentiation, while miR-550a-5p inhibited the process. miR-382-3p and miR-550a-5p impaired adipogenic differentiation, while miR-188-3p did not show an effect on osteogenesis or adipogenesis [311].

Another study done by Heilmeier et al. supports the use of miRNA signatures to improve fracture risk prediction in T2D [99]. The authors identified 3 miRNAs to be indicative of diabetic fragility fractures in postmenopausal women from the AGES-Reykjavik cohort. In the analysis, miR-19b-1-5p was found to be associated with lower incident diabetic fragility fractures, while in postmenopausal women with higher circulating levels of miR-203a and miR-31-5p, an increase risk for fractures was observed. Next, a fracture risk prediction model was built with covariates (age, BMI, BMD, clinical FRAX and FRAX with BMD) and all 3 miRNAs combined. This combined model predicted fractures better than the fracture assessment using BMD and FRAX alone, supporting the use of miRNAs as promising biomarkers to improve fracture risk prediction in T2D [99].

5.1.4 Summary

BMD is higher in patients with T2D. However, this finding is paradoxical as patients with T2D are also prone to an increased risk of fragility fractures. Irrespective of the low bone turnover in T2D, BTMs are not capable of predicting fracture risk in T2D. This suggests that there may be alterations in the bone structure that makes the bone weak and more prone to fractures, as well as dysregulation in other modulators such as hormones like PTH and Sclerostin regulating bone remodelling. At present, the extent of imbalance in bone formation and bone resorption in T2D is not known. Literature provides promising evidence for the use of miRNAs as promising biomarkers for fracture risk prediction in T2D.

Therefore, I performed an observational, case-controlled study to evaluate bone turnover, BMD, other modulators of bone remodelling and miRNAs in patients with T2D. This study also allowed to understand a plausible relationship between bone turnover, bone mineral density and bone balance.

Research questions

In comparison to healthy controls,

1. Is bone turnover decreased in T2D?
2. Is BMD increased in T2D?
3. Does BMD in T2D continue to be higher in T2D after adjusting for confounders known to alter BTMs?
4. If BMD is increased and bone turnover in decreased, is bone positive in T2D?
5. Are other modulators and hormones important for bone remodelling dysregulated in T2D?
6. Are miRNA expression levels different in T2D?

5.2 Methods

5.2.1 Participants

This study is an observational, case-controlled study. The participants were classified into 2 groups: patients with T2D and healthy controls. The patients with type 2 diabetes recruited as part of the DiabOsteo Study in Odense, Denmark were included in this analysis. Healthy controls recruited as part of the XtremeCT study, and the Fat and Bone Study in Sheffield, UK were included in this analysis. Best efforts were taken to match the study participants based on gender, age (± 3 years), BMI (± 3 kg/m^2) and height (± 10 cm). Details on participant inclusion and exclusion criteria and participant recruitment have been previously described in Chapter 2.

5.2.2 Measurement of BTMs, aBMD and miRNAs

Bone formation markers PINP, BAP and OC; and bone resorption markers CTX and TRAcP5b were measured in serum using the IDS-iSYS Multidiscipline Automated System (IDS, Boldon, UK). Other modulators and hormones important for bone remodelling such as PTH and vitamin D were also measured using the IDS-iSYS Multidiscipline Automated System (IDS, Boldon, UK). Sclerostin was measured using a sandwich enzyme immunoassay or ELISA (Biomedica Medizinprodukte GmbH. Austria). At the Odense site, BMD at the lumbar spine, total hip and femoral neck was measured by DXA using a Discovery A densitometer (Hologic Inc., Bedford, MA, USA) along with the appropriate Hologic software. At the Sheffield site, BMD was measured at the lumbar spine, total hip and femoral neck using a Discovery A densitometer and Horizon A densitometer (Hologic Inc., Bedford, MA, USA) along with the appropriate Hologic software. Quality control of the densitometers at both sites was done by measuring

the respective anthropometric bone phantoms during the entire study period. miRNAs were measured in serum using the LC 480 Real-Time PCR system (Roche) as described in Chapter 2.

5.2.3 Statistical analysis

Normally distributed variables between the two study groups were compared using the Student's *t*-test. Variables that were not normally distributed were compared using the Welch's *t*-test and Mann- Whitney test where appropriate. Multiple linear regression models were used to assess the impact of covariates on the variables of interest. The T-score bone marker plot was used to measure overall bone turnover and bone balance (represented as T-scores). Where appropriate, the strength of a relationship between two variables was assessed using Spearman's rank correlation. In this chapter of the thesis, the strength of the relationship between 43 miRNAs and markers of bone health were assessed using Spearman's rank correlation. To allow for multiple comparisons and avoid the risk of false-positive errors, a Bonferroni correction was done. Based on this, a p values of association < 0.001 between a miRNA and marker of bone health was considered to be statistically significant. Statistical analysis was done using GraphPad Prism (Version 9.3.1). The T-Score bone marker plot was created using Excel 2016.

5.3 Results

5.3.1 Characteristics of study participants

173 patients with T2D (70 females and 103 males) and 115 healthy controls (49 females and 66 males) were included in this analysis. The patients with T2D had an average fasting plasma glucose of 143 mg/dL (9.7) and eGFR of 70.81 mL/min/1.73m^2 (21.30), mean (SD), suggesting that these participants had early-stage renal impairment. Their BMI was significantly higher when compared to the matched CTRL group. Table 5.1 summarizes the characteristics of the study participants included in this analysis.

Table 5-1. Summary of characteristics of the study participants in the two study groups.

	CTRL	T2D	p
N (F/M)	115 (49 / 66)	173 (70 / 103)	
Age (years)	67.63 (9.85)	69.50 (11.21)	0.14
BMI (Kg/m^2)	30.73 (5.42)	32.32 (5.41)	0.02
Fasting plasma glucose (mg/dL)	-	143 (9.71)	
eGFR ($mL/min/1.73m^2$)	-	70.81 (21.30)	

CTRL = healthy controls, T2D = patients with type 2 diabetes, N = number of participants, BMI = body mass index, all values except gender are represented as mean (SD).

5.3.2 Low bone turnover in type 2 diabetes

Serum levels of the bone formation markers PINP and OC were significantly reduced by 18% ($p < 0.05$) and 19% ($p < 0.0001$) respectively, in patients with T2D compared to their matched CTRL group (figure 5.1A and B). Serum levels of the bone formation marker bone ALP was not significantly different in patients with T2D when compared to their matched CTRLs (figure 5.1C).

Serum levels of the bone resorption markers CTX and TRAcP 5b were also significantly reduced by 14% and 19% respectively, ($p < 0.0001$), in patients with T2D when compared to their matched CTRL group (figure 5.1D and E).

In an age-, gender and BMI-adjusted multiple linear regression model (Table 5.2, Model 2), serum levels of PINP, OC and TRAcP 5b remained significantly low in T2D. However, after adjusting for the covariates of age, gender and BMI, serum levels of CTX was not found to be statistically different between the two study groups. Serum levels of BAP did not change after adjusting for covariates such as age, gender and BMI (Table 5.2, Model 2). I observed no associations between BTMs (PINP and CTX) and HbA1c in T2D (Table 5.3).

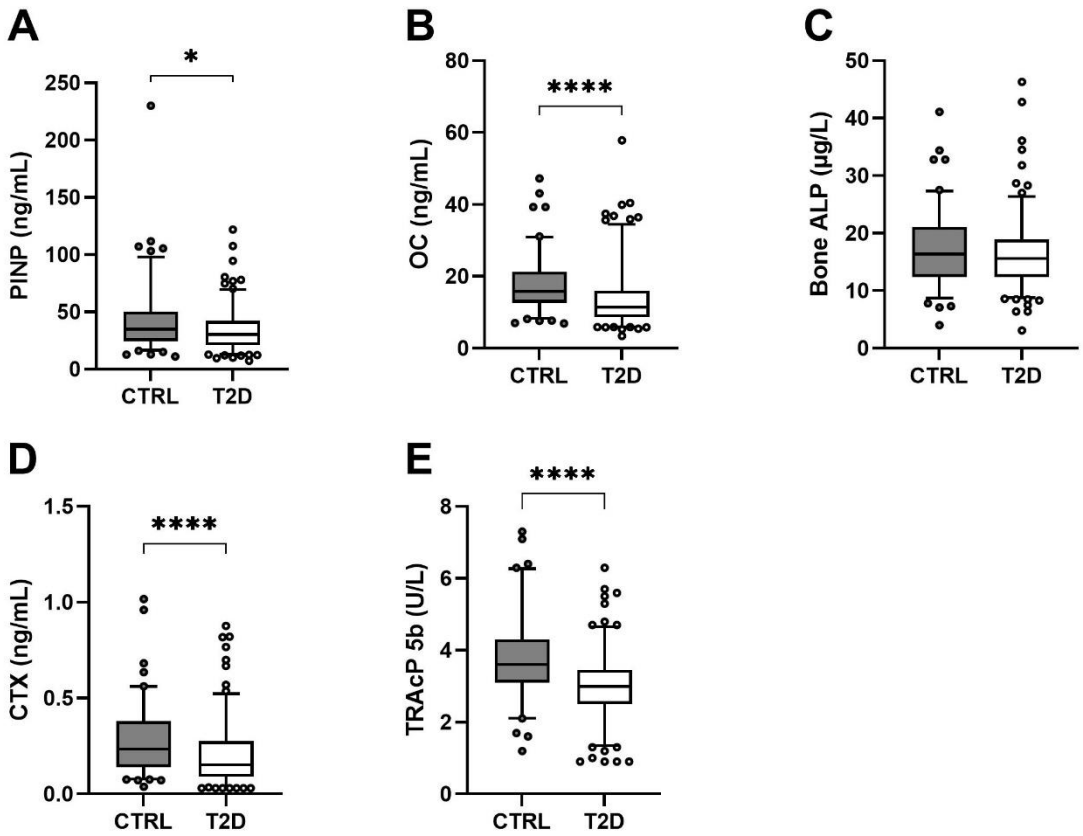

Figure 5-1. Serum levels of the measured bone turnover markers in the study population.

CTRL = healthy controls, T2D = patients with type 2 diabetes.
(A) PINP, (B) OC, (C) Bone ALP, (D) CTX and (E) TRAcP 5b.
Table 5-2. Serum levels of the measured bone turnover markers in the study population.

Serum BTMs	Model 1			Model 2	
	CTRL	T2D	p	β (95% CI)	p
PINP	42.11 (36.90 to 47.32)	34.63 (31.82 to 37.44)	0.011	7.57 (1.82 to 13.31)	0.01
OC	17.11 (16.33 to 19.21)	13.83 (12.57 to 15.08)	<0.0001	3.64 (1.59 to 5.69)	<0.001
bone ALP	17.04 (15.83 to 18.26)	16.35 (15.43 to 17.28)	0.33	0.73 (-0.84 to 2.30)	0.36
CTX	0.28 (0.24 to 0.31)	0.24 (0.16 to 0.31)	<0.0001	0.04 (-0.06 to 0.14)	0.45
TRAcP 5b	3.78 (3.53 to 4.02)	3.06 (2.91 to 3.2)	<0.0001	0.74 (0.46 to 1.01)	<0.0001

CTRL = healthy controls, T2D = patients with type 2 diabetes. Values represented as mean (95% CI) and beta estimate (95% CI) where appropriate
Model 1: unadjusted model, Model 2: adjusted for age, gender and BMI

Table 5-3. Spearman's rank correlation and p value of association between HbA1c, PINP and CTX in T2D.

	HbA1c	
	r	p
PINP	0.04	0.67
CTX	-0.07	0.48

5.3.3 Bone turnover and bone balance in type 2 diabetes: T-score bone marker plot

Using the T-score bone marker plot, I found bone turnover to be significantly decreased in patients with T2D compared to their matched CTRL group. However, bone balance was found to be significantly positive in these patients with T2D when compared to their matched CTRLs (Figure 5.2). Figure 5.3 represents the above information in a T-Score bone marker plot.

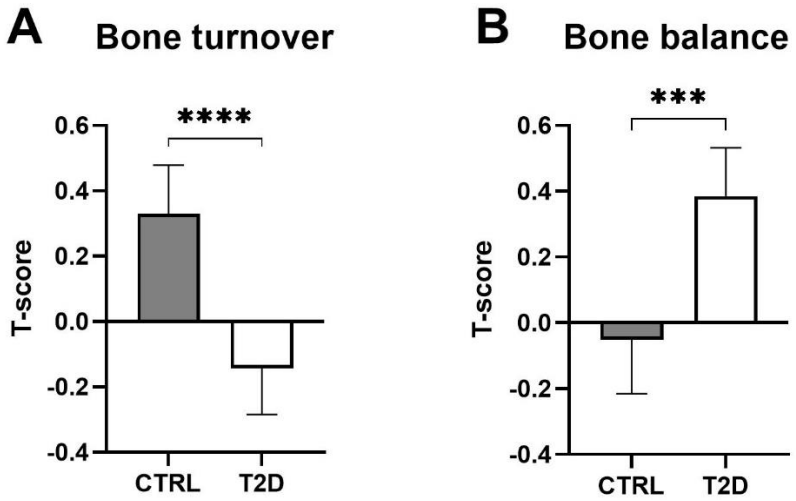

Figure 5-2. Comparison of Bone turnover and bone balance in the study population.

CTRL = healthy controls, T2D = patients with type 2 diabetes.

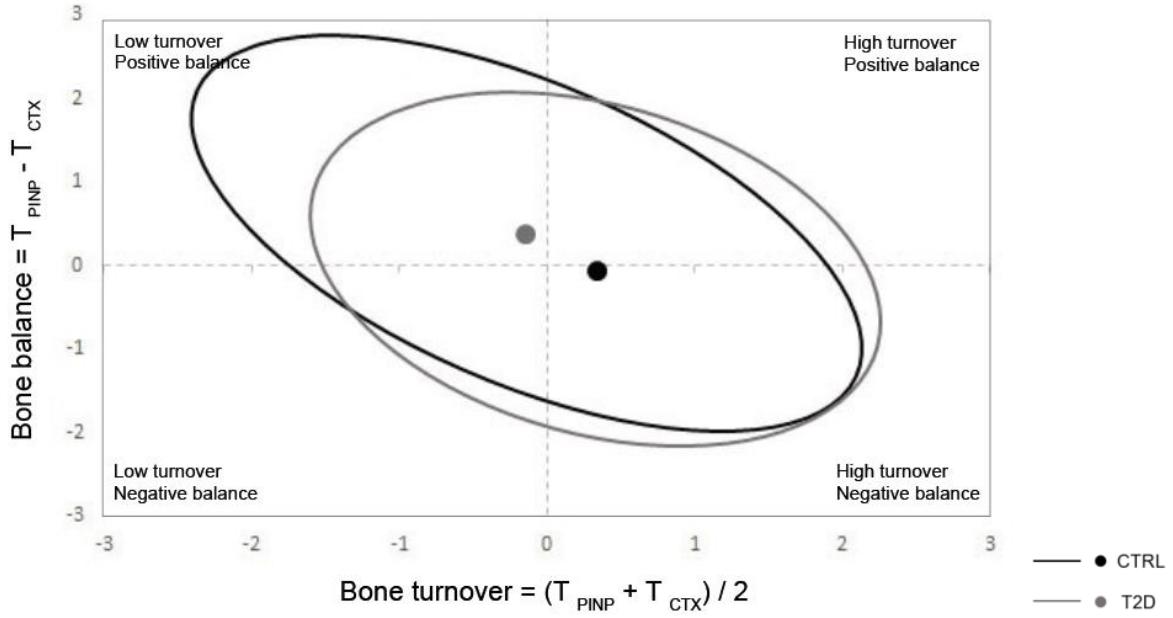

Figure 5-3. Bone turnover and bone balance in the study population, illustrated using the T-score bone marker plot.

5.3.4 Low bone mineral density in T2D

In order to understand if the observed positive bone balance in patients with T2D can be explained by aBMD, I compared BMD at the lumbar spine, total hip and femoral neck in patients with T2D with their matched CTRLs.

I found no differences in aBMD at the lumbar spine, total hip and femoral neck in people with T2D when compared with their matched CTRL group. No differences in aBMD at all the three measured sites were observed even after adjusting for age and gender (Table 5.4, Model 2). After adjusting for age, gender and BMI, I found aBMD at the total hip to be significantly reduced ($p = 0.02$) in patients with T2D when compared to their matched CTRLs (Table 5.4, Model 3). This suggests that BMI is a strong determinant of aBMD at the total hip.

Further, after adjusting for bone turnover and bone balance, I found aBMD at the total hip and femoral neck to be significantly reduced ($p < 0.05$) in T2D when compared to their matched CTRLs (Table 5.4 model 4). aBMD at the lumbar spine was not significantly different between the two study groups even after adjusting for variables such as age, gender, BMI, bone turnover and bone balance, which we believe to be important determinants of aBMD.

Table 5-4. Summary of bone mineral density measured at the total hip, femoral neck and lumbar spine in the study population.

	Model 1			Model 2		Model 3		Model 4	
BMD (g/cm^2)	CTRL	T2D	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
Total hip BMD	0.99 (0.96 to 1.02)	0.98 (0.95 to 1.00)	0.4	0.03 (-0.01 to 0.06)	0.12	0.04 (0.01 to 0.07)	0.02	0.05 (0.02 to 0.08)	0.001
Femoral neck BMD	0.80 (0.78 to 0.83)	0.79 (0.77 to 0.82)	0.5	0.02 (-0.01 to 0.06)	0.19	0.03 (-0.00 to 0.06)	0.09	0.04 (0.01 to 0.07)	0.02
Lumbar spine BMD	1.06 (1.03 to 1.1)	1.06 (1.01 to 1.10)	0.84	0.01 (-0.04 to 0.06)	0.67	0.02 (-0.03 to 0.07)	0.39	0.04 (-0.01 to 0.09)	0.13

CTRL = healthy controls, T2D = patients with type 2 diabetes. Values represented as mean (95% CI) and beta estimate (95% CI) where appropriate.
Model 1: unadjusted model, Model 2: adjusted for age and gender, Model 3: adjusted for age, gender and BMI, Model 4: adjusted for age, gender, BMI, bone turnover and bone balance

5.3.5 PTH, vitamin D and sclerostin in T2D

In order to understand if the increased risk for fractures, low bone turnover and low BMD in T2D could be explained by other hormones important for bone remodelling such as PTH, vitamin D and sclerostin, I measured them in serum in patients with T2D and compared their expression against their matched CTRL group.

Serum PTH was not different between patients with T2D and their matched CTRLs (Figure 5.4A). After adjusting for covariates such as age, gender and BMI that may determine the PTH expression, PTH still remained to be at similar levels between the two study groups (Table 5.5, Model 2).

On the other hand, serum 25-hydroxy vitamin D was found to be significantly higher and 1,25-dihydroxy vitamin D were found to be significantly lower ($p < 0.01$), in patients with T2D when compared to their matched CTRLs (Figure 5.4C and D). These levels of vitamin D continued to be significantly different between the two study groups even after adjusting for age, gender and BMI (Table 5.5, Model 2).

I found serum sclerostin to be significantly higher in patients with T2D when compared to their matched CTRL group. The increase in sclerostin in T2D remained significantly high even after adjusting for age, gender and BMI (Figure 5.4B, Table 5.5).

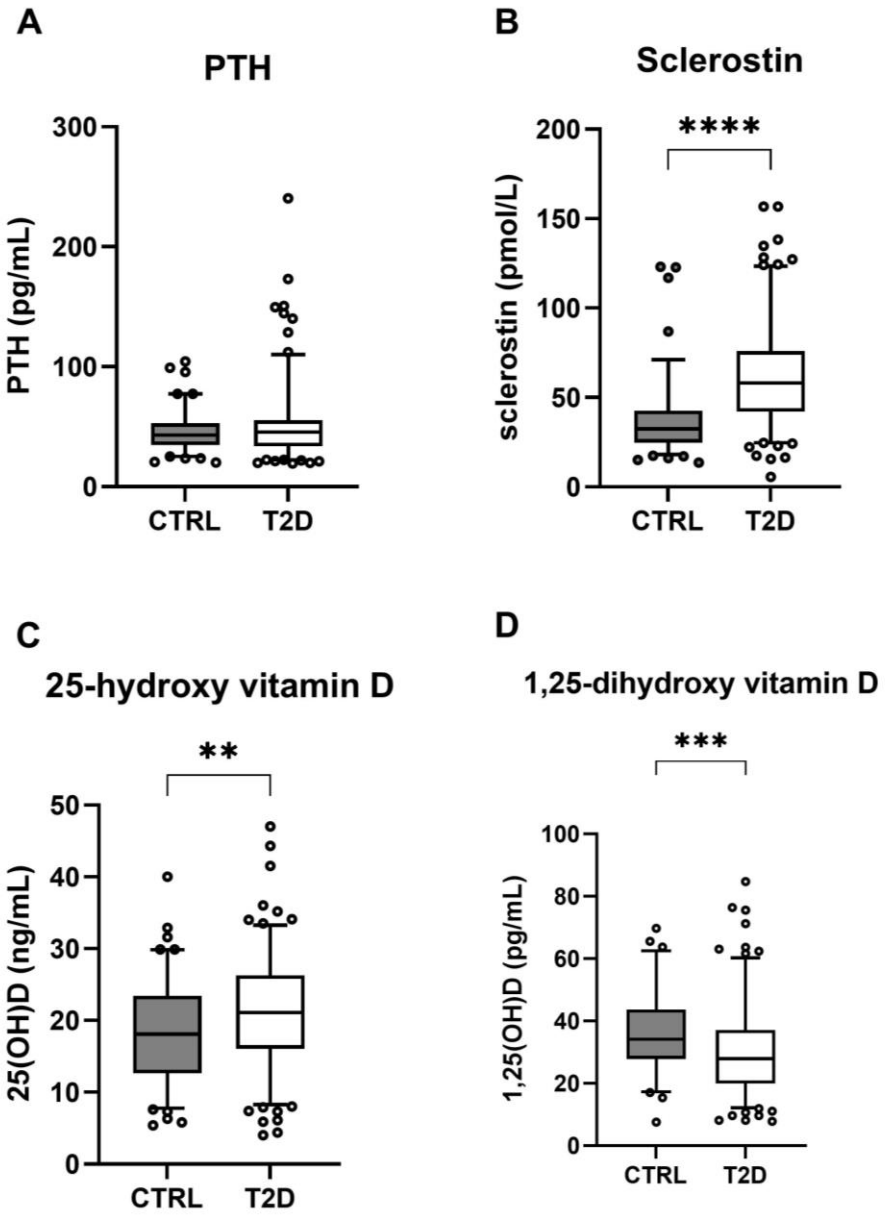

Figure 5-4. Serum levels of hormones important for bone remodelling measured in the study population. (A) PTH, (B) sclerostin, (C) 25-hydroxy vitamin D and (D) 1,25-dihydroxy vitamin D. CTRL = healthy controls, T2D = patients with type 2 diabetes.

Table 5-5. Serum levels of hormones important for bone remodelling measured in the study population.

	Model 1			Model 2	
	CTRL	T2D	p	β (95% CI)	p
PTH	45.98 (38.50 to 46.30)	51.46 (40.40 to 47.30)	0.56	3.59 (-3.106 to 10.29)	0.29
Sclerostin	36.82 (33.26 to 40.38)	62.71 (58.36 to 67.06)	<0.0001	26.50 (19.58 to 33.42)	<0.0001
25 vit D	18.58 (15.90 to 20.41)	21.24 (19.73 to 23.22)	0.005	2.73 (0.9397 to 4.525)	0.003
1,25 vit D	36.00 (32.40 to 36.77)	30.32 (25.13 to 29.80)	0.0001	-5.22 (-9.069 to -1.369)	0.008

CTRL = healthy controls, T2D = patients with type 2 diabetes. Values represented as mean (95% CI) and beta estimate (95% CI) where appropriate. Model 1: unadjusted model, Model 2: adjusted for age, gender and BMI.

Using Spearman's rank correlation, I observed a negative correlation between 25-hydroxy vitamin D and HbA1c; and a positive correlation between 1,25-dihydroxy vitamin D, and CTX and eGFR (Table 5.6 and 5.7). However, I observed no association between vitamin D and bone turnover (T-score), and bone balance (T-score) (Table 5.7).

Table 5-6. Spearman's rank correlation and p value of association between sclerostin, Vitamin D, HbA1c, PINP and CTX in the type 2 diabetic population.

	HbA1c		PINP		CTX	
	r	p	r	p	r	p
Sclerostin	0.13	0.22	0.03	0.74	0.02	0.84
25-hydroxy vitamin D	-0.36	**<0.01**	-0.03	0.81	0.02	0.84
1,25-dihydroxy vitamin D	-0.16	0.11	0.07	0.50	0.28	**<0.01**

Table 5-7. Spearman's rank correlation and p value of association between vitamin D, eGFR, bone turnover and bone balance in the type 2 diabetic population.

	eGFR		Bone turnover (T-score)		Bone balance (T-score)	
	r	p	r	p	r	p
25-hydroxy vitamin D	-0.15	0.15	-0.15	0.06	0.12	0.13
1,25-dihydroxy vitamin D	0.25	**0.01**	0.09	0.26	-0.07	0.37

5.3.6 MiRNAs in type 2 diabetes and their association with bone biomarkers

The set of 61 specifically selected endogenous microRNAs, including 2 microRNAs for the monitoring of haemolysis and 3 spike in controls (UniSp4, UniSp3 and Cel-miR-39-3p) were measured in serum in entire study population. Three samples were excluded from the analysis as they were haemolysed (2 in the CTRL group and 1 in the T2D group). To my surprise, I found the Cq values for UniSp4 (used for miRNA expression normalisation) to be extremely high (in the range of 28-31) for a large number of samples, particularly in the CTRL group. These samples with very high Cq for UniSp4 were excluded because they were within limit unacceptable in our laboratory (55 in the CTRL group and 12 in the T2D group). A total number of 218 samples were included in the analysis (58 in the CTRL group and 160 in the T2D group).
The cDNA conversion kit containing the spike in controls (TAmiRNA GmbH, Austria) used for the measurement of miRNAs in this study allows for 64 individuals measurements. Since I had 218 samples to measure miRNAs in, 3 such kits were used. The 1 kit out of the three belonged to a different lot. To avoid a systemic bias, the aliquots of the spike in controls of all the three kits were pooled together prior to using them for miRNA measurements. Smaller aliquots were made from the pooled aliquot of the spike in controls to avoid several freeze thaw cycles. Despite the care taken, I observed very high Cq values for UniSp4 in almost half of the CTRL group. I checked if the kits I used for miRNA measurements were in date, and indeed they were. The reasons for the above observation are still unknown.

By excluding the CTRL samples with high Cq values for UniSp4, I believed that I would be subjecting the CTRL group population to a negative bias and by leaving in the samples with acceptable Cq values for UniSp4, I would be subjecting the remaining samples to a positive bias. Since the T2D population were not subject to this bias, we felt it systematically incorrect to compare miRNA expression between the two study groups. Hence, I had to leave out the complete set of data on miRNA expression in the CTRL group and couldn't analyse the data further to identify dysregulated miRNA expression between T2D and CTRLs in this study.

Of the 59 selected endogenous miRNAs measured, serum expression of 8 miRNAs, namely: miR-124-3p, miR-338-5p, miR-133b, miR-96-5p, miR-217-5p, miR-135b-5p, miR-203a-3p and miR-7-5p was below the level of detection in 1/3 of the high quality samples in the study population. Hence, these miRNAs were excluded from further analyses explained below.

In order to understand if any of the remaining 51 selected miRNAs may have a potential role to play in T2D and bone fragility or fracture risk, I performed Spearman's rank correlation to

assess if any of the 51 selected miRNAs associated with bone turnover (PINP and CTX) or sclerostin. Results of the associations between the miRNAs and PINP, CTX and sclerostin are listed in Table 5.8.

I observed a negative correlation between sclerostin and miR-30e-5p, and miR-148a-3p. Four miRNAs, namely: miR-24-3p, miR-23b-3p, miR-574-3p, miR-328-3p positively correlated with PINP. I observed no correlation between the measured miRNAs and CTX. However, using the Bonferroni correction for multiple comparisons, the above associations were no longer statistically significant because their p value of association was not < 0.001.

Table 5-8. Spearman's rank correlation and p value of association between miRNAs, PINP, CTX and sclerostin in the type 2 diabetic study population.

miRNA	PINP		CTX		Sclerostin	
	r	p	r	p	r	p
miR-21-5p	0.10	0.27	0.05	0.57	-0.15	0.08
miR-335-5p	0.08	0.37	0.08	0.35	-0.12	0.19
miR-17-5p	0.10	0.27	0.03	0.77	-0.11	0.23
miR-375-3p	0.11	0.21	0.11	0.21	-0.04	0.66
miR-155-5p	0.06	0.53	0.08	0.36	-0.12	0.19
miR-25-3p	0.16	0.08	0.03	0.70	-0.13	0.15
miR-133a-3p	0.13	0.13	0.13	0.14	-0.10	0.26
miR-223-3p	-0.04	0.65	0.03	0.73	-0.08	0.36
miR-27a-3p	0.14	0.11	0.12	0.19	-0.11	0.22
miR-100-5p	0.00	0.97	0.00	0.99	-0.16	0.07
miR-106b-5p	0.08	0.37	0.00	0.99	-0.11	0.23
miR-128-3p	0.12	0.17	0.03	0.71	-0.16	0.08
miR-29a-3p	0.07	0.44	0.02	0.87	-0.13	0.16
miR-93-5p	0.13	0.16	0.02	0.85	-0.10	0.27
miR-125b-5p	-0.01	0.93	0.04	0.67	-0.17	0.05
miR-140-3p	0.13	0.13	0.03	0.77	-0.10	0.25
miR-30e-5p	0.02	0.83	-0.04	0.69	-0.20	**0.02**
miR-122-5p	0.15	0.09	0.09	0.30	-0.12	0.20
let-7b-5p	0.08	0.37	0.00	1.00	-0.16	0.07
miR-146a-5p	0.09	0.31	0.09	0.34	-0.13	0.15
miR-16-5p	0.13	0.16	0.01	0.88	-0.13	0.14
miR-24-3p	0.18	**0.04**	0.12	0.18	-0.13	0.15
miR-23b-3p	0.19	**0.03**	0.16	0.08	-0.15	0.10
miR-144-5p	0.05	0.55	-0.05	0.61	-0.16	0.07
miR-19a-3p	0.16	0.08	0.03	0.72	-0.10	0.28
miR-142-5p	0.06	0.52	-0.03	0.76	-0.09	0.34

miR-20a-5p	0.08	0.38	0.02	0.83	-0.10	0.24
miR-574-3p	0.19	**0.04**	0.11	0.23	-0.14	0.12
miR-34a-5p	0.06	0.49	-0.03	0.70	-0.14	0.11
miR-26a-5p	0.14	0.12	0.14	0.13	-0.13	0.13
miR-27b-3p	0.12	0.18	0.10	0.26	-0.13	0.15
miR-29b-3p	-0.11	0.23	-0.10	0.26	-0.09	0.31
miR-30a-5p	0.10	0.25	0.09	0.33	-0.17	0.06
miR-222-3p	0.12	0.17	0.05	0.61	-0.11	0.24
miR-101-3p	0.06	0.48	-0.01	0.93	-0.12	0.20
miR-30b-5p	0.15	0.10	0.08	0.36	-0.13	0.15
miR-186-5p	0.10	0.27	0.01	0.90	-0.15	0.10
miR-103a-3p	0.14	0.12	0.06	0.52	-0.10	0.25
miR-152-3p	0.12	0.18	0.06	0.51	-0.16	0.07
miR-328-3p	0.23	**0.01**	0.17	0.06	-0.06	0.50
miR-98-5p	0.06	0.51	0.00	0.97	-0.12	0.18
let-7g-5p	0.03	0.71	-0.03	0.75	-0.14	0.11
miR-148a-3p	0.13	0.16	0.03	0.78	-0.19	**0.03**
miR-340-5p	0.10	0.26	0.03	0.73	-0.13	0.15
miR-194-5p	0.17	0.06	0.07	0.43	-0.13	0.14
miR-342-3p	0.09	0.32	0.07	0.42	-0.12	0.17

5.4 Discussion

5.4.1 Summary of main findings

Best efforts were taken to match the patients with T2D from the DiabOsteo Study with healthy controls chosen from the XtremeCT Study and Fat and Bone Study. The participants were matched based on gender, age (±3 years), height (±10 cm) and BMI (±3 kg/m^2). Serum bone formation markers, PINP and OC were 18% and 19% lower in patients with T2D compared to their matched healthy controls. Serum bone resorption markers, CTX and TRAcP 5b were 14% and 19% lower respectively, in patients with T2D compared to their matched CTRLs. Serum BAP was not different in T2D compared to their matched healthy controls. After adjusting for age, gender and BMI, the following BTMs, PINP, OC and TRAcP5b) were significantly lower in T2D. Using the T-score bone marker plot, I found the patients with T2D to represent a state of low bone turnover and a positive bone balance. No differences in aBMD at the lumbar spine, total hip and femoral neck were found between the patients with T2D and their matched healthy controls. However, I found adjusted (age, gender, BMI, bone turnover and bone balance) aBMD at the total hip and femoral neck to be lower in people with T2D. I further found serum levels of sclerostin and 25-hydroxy vitamin D to be higher and 1,25-

dihydroxy vitamin D to be lower in T2D, as well as 1,25-dihydroxy vitamin D to be positively associated with CTX. Measurement of miRNAs in serum reported a positive association between PINP and 4 miRNAs, namely: miR-24-3p, miR-23b-3p, miR-574-3p and miR-328-3p; and a negative association between sclerostin, and miR-30e-5p and miR-148a-3p.

5.4.2 Low bone turnover and positive bone balance in type 2 diabetes

I observed an overall significant decrease of 57 T-score units in patients with T2D, confirming evidence reported in literature [82], [84]. In this study, serum levels of PINP (-7.48 ng/mL (-9.88 to -5.08)), OC (-3.28 ng/mL (-4.13 to -3.76)), CTX (-0.04 ng/mL (-0.08 to 0)) and TRAcP5b (-0.72 U/L (-0.82 to -0.62)) were lower in patients with T2D when compared to their matched CTRLs. A meta-analysis done by Hygum et al. also reported serum levels of PINP (-10.80 ng/mL (-12.83, -8.77)), OC (-2.51 ng/mL (-3.01, -2.01)), CTX (-0.10 ng/mL (-0.12, -0.08)) and TRAcP5b (-0.31 U/L (-0.56, -0.05)) to be lower in T2D. The decrease in bone formation markers (PINP and OC) and bone resorption markers (CTX and TRAcP5b) in T2D compared to their matched CTRLs observed in this study were within similar ranges reported in Hygum et al.'s meta-analysis.

Estimates of bone marker balance has been calculated previously in the context of menopause using different approaches: (i) By calculating the Z-score for postmenopausal women relative to an age-matched control group [312], [313], (ii) By using the multiple of medians of the respective BTMs to calculate bone turnover and bone balance [310] and (iii) By calculating the bone balance index for postmenopausal women based on the relationship between urinary N-telopeptide (NTX) and serum osteocalcin in women more than 5 years before their final menstrual period [314]. The method used to calculate bone balance in this study has been previously published by our research group [128]. The advantages of this model over the other 3 described above is that: (i) it relates bone balance in the group of interest to a healthy young women (T-score), (ii) it allows for the skewed distribution of PINP and CTX (by log transformation) and (iii) the data is standardized (by using standard deviation units), overall allowing for the calculation of overall bone turnover and bone balance separately [128].

In this study, I calculated bone turnover and bone balance using the T-score bone marker plot in the context of T2D. Using this approach, I further confirmed a state of low bone turnover and positive bone balance in our T2D study population. I observed bone balance (bone balance represented as a T-score) to be increased by 43.5 T-score units in T2D when compared to their matched CTRLs, and this difference was significant. To our knowledge, we are the first to report bone balance in T2D using this method.

In this study, I observed bone turnover (0.33 (0.18 to 0.48)) and bone balance (-0.05 (-0.22 to 0.11)) (represented as a T-score) in the healthy CTRLs to be within similar ranges of healthy pre-menopausal women (aged 20-29 years, bone turnover 0.44 (0.30 to 0.58) and bone balance −0.06 (−0.25 to 0.12) as reported by [128]. In the T2D group, the observed low bone turnover (-0.14 (-0.29 to -0.002)) and positive bone balance (0.39 (0.24 to 0.53)) (represented as a T-score) was opposite to that observed in postmenopausal women 21 years and more since menopause (mean age = 72.6 years). The mean age of the T2D population assessed in this study was 69.50 years.

An important confounding factor in the evaluation of T2D on bone health is obesity [84]. In our cohort, although both populations were in the obese range, I observed the patients with T2D to have a significantly higher BMI compared to their matched healthy controls. Some studies suggest that obese subjects have a relative lower risk of proximal femur and vertebral fractures compared to people with normal BMI. However, the risk of fracture in obese adults is variable at different skeletal sites and depends on the impact of a fall. In particular, the risk of a proximal femur, upper leg and ankle fracture is higher in obese adults compared to adults with normal BMI [315]. Additionally, increased fat mass can act negatively on bone by increasing the production of adipokines which is known to affect bone turnover [316]. For this reason, in this study, best efforts were taken to match the study subjects for BMI as well as for age and gender. However, BMI was still heavier in the patients with T2D. After adjusting for age, gender and BMI, the following bone turnover markers, PINP, OC, CTX and TRAcP5b continued to remain significantly low in people with T2D compared to their matched healthy controls. One of the main sources of alkaline phosphatase is the liver [83]. Hence, in this study bone specific alkaline phosphatase (BAP) was measured and I found no difference in its serum expression between T2D and matched healthy controls. After adjusting for age, gender and BMI, BAP was significantly lower in T2D compared to their matched healthy controls. Linde and colleagues have also shown that in a combined population of T2D and controls, serum CTX and serum PINP was negatively associated with HbA1c, however, this association was lost upon adjusting for diabetes status and was not present in stratified analyses of controls and T2D [84]. Similar to Starup-Linde et al.'s research, in our study I found no association between HbA1c and serum PINP and serum CTX in the patients with T2D. This suggests that the low bone turnover in T2D is not related to the diabetes status.

5.4.3 Low bone mineral density in T2D

In T2D, obesity is strongly associated with higher aBMD [317]. I would like to remind you that in our study population, although we tried our best to match for BMI, patients with T2D had a significantly higher BMI compared to their matched healthy controls. It may be possible that,

the higher BMI in patients with T2D could explain the observed positive bone balance. In order to test this hypothesis, I compared aBMD at the lumbar spine, femoral neck and total hip between our study groups. I found no difference in aBMD at the lumbar spine, femoral neck and total hip between our study groups. I was not surprised by this finding as other authors have also reported the same [318], [319]. However, a meta-analysis done by Ma and colleagues reported BMD to be higher at the lumbar spine, femoral neck and total hip in T2D [320].

aBMD is strongly linked to age, gender and BMI. Since we were not able to match our study population completely for these well-known confounders, I adjusted aBMD based on these confounders. In our study, after adjusting for age, gender and BMI, I found BMD at the total hip to be significantly lower in patients with T2D compared to their matched healthy controls. Since low bone turnover is associated with T2D, it may be a mediator of the effect of diabetes on BMD. Hence, in our analysis, I tested the effect on bone turnover on aBMD in T2D. After adjusting for bone turnover and bone balance, aBMD at the total hip continued to remain significantly low in T2D. I also found aBMD at the femoral neck to be significantly lower in T2D after this adjustment. This observation of aBMD being lower in T2D is in agreement with the findings of previous studies [321], [322]. Yaturu and colleagues reported BMD at the hip to continue to be lower in T2D compared to controls without diabetes after adjusting for age and BMI.

Taken together, the observed low bone turnover and positive bone balance does not explain the observed low aBMD at the hip in this T2D population. While the low aBMD may be a risk factor for the increased incidence of hip fractures and osteoporosis in T2D, it also calls for the need to evaluate bone quality in T2D. In Chapter 1, I have shown evidence for increased cortical porosity in T2D.

5.4.4 Hormones regulating bone remodelling in T2D

Osteocytes are known to be key mediators of PTH signalling. PTH promotes a balance in bone formation and bone resorption via osteocyte signalling [323], [324]. Several studies have reported a decrease in serum PTH in T2D [325]–[327]. However, in this study, I found no difference in serum PTH between the two study groups even after adjusting for age, gender and BMI and hence, I was unable to comment on PTH being a determinant of the low bone turnover in T2D. Research done by Dobnig et al. have shown that decreased levels of PTH and increased levels of glycaemia independently contributed to a state of low bone turnover in elderly patients with T2D in a nursing home setting [325].

Wnt signalling is an important regulator of bone remodelling. Impairment of Wnt signalling can affect bone quality in T2D. Sclerostin is a marker of mature osteocytes and usually detected in osteocytes surrounded by mineralized bone. It has been demonstrated that mechanical loading decreases the expression of sclerostin in osteocytes. sclerostin is an antagonist to Wnt-signalling, meaning it binds to the Wnt co-receptors LRP5/6 downstream and disrupts bone remodelling. An increase in sclerostin leads to lesser bone formation and increased bone resorption [96].

In our study population, I found increased serum sclerostin levels in patients with T2D compared to their matched healthy controls. This finding is in line with the findings of other authors [328], [329]. However, I found no correlation between serum sclerostin and HbA1c in our T2D population, as reported by García-Martín and colleagues. One could speculate that the low bone formation and increase in bone resorption in T2D could be due to the increase in sclerostin, eventually leading to poor bone quality and increase in bone fragility. However, in our study I found no association between serum sclerostin, and PINP and CTX. This suggests that there may be other factors that may be responsible for the increase in bone fragility in T2D.

There are 2 forms of vitamin D present in blood. 25-hydroxy Vitamin D is the form of vitamin D that is used as a measure active form of vitamin D and is used as a measure of vitamin D deficiency and 1,25-dihydroxy vitamin D is the most potent metabolite of vitamin D and is produced by the kidneys. Vitamin D supplements contain 25-hydroxy vitamin D . This 25-hydroxy vitamin D is activated by the kidneys to make 1,25 dihydroxy Vitamin D. 1,25-dihydroxy vitamin D is believed to maintain the body's sensitivity to insulin and hence reduce insulin resistance, which is an important characteristic of T2D [330]. Individuals with vitamin D deficiency are at a higher risk of developing osteoporosis [331]. In this study, I measured both forms of vitamin D. I found increasing levels of 25-hydroxy vitamin D in T2D compared to the CTRL group. It may be possible that these people were prescribed Vitamin D supplements for general wellbeing. However, I did not have access to this information to explore further. On the other hand, I found decreasing levels of serum 1,25-dihydroxy vitamin D in T2D when compared to the CTRL group. I also found 1,25-dihydroxy vitamin D to be positively associated with eGFR. Of note, the T2D population included those with eGFR in the range of 30-60 mL/min/1.73m^2, suggesting that some of the T2D participants had mild renal failure. If the T2D participants were on vitamin D supplements, it could mean that due to mild renal failure, their capacity to convert 25-hydroxy vitamin D to 1,25-dihydroxy vitamin D decreases, explaining the low levels of 1,25-dihydroxy vitamin D observed [332].

5.4.5 miRNAs in type 2 diabetes and bone metabolism

While measuring miRNAs in the study population, particularly in the group of healthy controls, I observed an analytical error where in the Cq values of UniSp4 (used to monitor RNA isolation and normalisation of data) were beyond expected levels and the measured miRNAs has very poor recovery. The reasons for this are unknown. This meant that the samples of healthy controls with very high UniSp4 Cqs needed to be excluded from the analysis. By removing these samples, we would be subjecting this population of healthy controls to a negative bias and by leaving in those that had accepted Cqs of Unisp4, we would be subjecting them to a positive bias. There is a possibility that the healthy control samples being included have Cq values lower than they should actually be. Since the population of T2D were not subject to this bias, we believed it would be incorrect for us to compare expression of miRNAs between the two study groups. Due to this reason, I was unable to identify dysregulated miRNAs in T2D.

Nevertheless, in T2D, I observed a significant positive association between serum PINP and serum expression of miR-24-3p, miR-23b-3p, miR-574-3p and miR-328-3p; and a negative correlation between serum sclerostin and miR-30e-5p and miR-148a-3p. However, after adjusting for multiple comparisons using the Bonferroni correction, the above mentioned assoications were no longer statistically significant because their p value of association was not <0.001. Hence, these finsings are described as ‘chnace findings’ below in this section.

<u>miR-24</u>

In this study I found serum PINP to be decreased in T2D and a positive association between miR-24 and PINP. Literature provides coflicting evidence for the expression of miR-24-3p in T2D. This may be due to the different sample types the miRNA was measured in. Avgeris et al. measured miR-24-3p in peripheral blood and found its expression to be decreased in T2D. Their finding was in agreement with Kokkinopoulou and colleagues who also measured miR-24-3p in peripheral blood. Prabu and colleagues also reported a downregulation in expression of miR-24-3p in T2D and they measured the expression of this miRNA in urinary extracellualr vesicles of patients with T2D and nephropathy. However, Wang et al. and Lopez. et al. reported an upregulation in expression of miR-24-3p in T2D and both the studies measured the miRNA expression in plasma [184], [239]. Literature also provides evidence for miR-24-3p to have an effect on osteogenesis. Using rat adipose derived mesenchymal stromal cells, Liu et al. show that an overexpresion in miR-24 has a negative effect on osteogenesis mediated by HOXB7 and β-catenin [333] and Zhao et al. show the same using MC3T3 cells, an osteoblast cell line [334]. They report overexpression of miR-24 to significantly inhibit osteoblast differentiation, which in turn decreased alkaline phosphatase activity, matrix mineralisation and the expression of osteogenic differentiation markers via post transcription

of T-cell factor-1. In the OFELY cohort, Feurer et al. reported a decrease in serum expression of miR-24 in individuals with a previous fracture compared to those without a fracture, although they found no association between this miRNA and bone mass or bone turnover [253].

Although the findings provide evidence for miR-24-3p to be a promising biomarker for T2D and bone disease, due to the conflicting findings mentioned above, the expression of miR-24 in a given sample and population (T2D with and without osteoporosis / fractures) needs to be standardized and validated before it can be developed as a potential biomarker for T2D bone disease.

miR-23b

In this study, I found a positive association between miR-23b and PINP and decreasing levels of PINP in T2D. Evidence from literature suggests plasma miR-23b-3p to be upregulated in children with T1D and positively correlated with OPG. Ramirez-Salazar et al. have also shown miR-23b to be upregulated in adults with osteopenia, osteoporosis and fractures. However, this evidence doesn't support our finding of a positive correlation between miR-23b and PINP. The evidence gathered thus far from literature suggests that our finding of a positive association between miR-23b and PINP is a chance finding.

miR-328

In this study, I found a positive association between miR-328 and PINP and decreasing levels of PINP in T2D. Research by chen et al. suggests that the downregulation of miR-328 in osteoporosis inhibits osteoblast cell differentiation and maturation, decreases ALP activity and supresses bone mineral deposition [263]. However, research on miR-328 in diabetes reports an upregulation [173], [335]. Abundant expression of miR-328 altered the expression of extracellular signal-regulated kinase (ERK) signalling pathway, increased insulin resistance and accelerated the apoptosis of islet cells in diabetic mice on a high fat diet [335]. Taken together, the different directions of expression of miR-328 in diabetes and osteoporosis does not make it a suitable biomarker for diabetic bone disease. However, this needs to be validated with further research.

miR-574

In this study, I found a positive association between miR-574 and PINP and decreasing levels of PINP in T2D. Research suggests the expression of miR-574 to be downregulated in T2D [140], [218] and in osteoporosis [258]. Although very little is known on the role of miR-574 in diabetes and bone, this miRNA has potential to be promising biomarker for diabetic bone disease.

miR-148a

In this study I found serum sclerostin to be higher in T2D compared to matched healthy controls and a negative correlation between serum sclerostin and miR-148a-3p. This would mean that in T2D, the increase in sclerostin may lead to a potential decrease in expression of miR-148a. However, several studies in T2D have reported an increase in expression of miR-148a [139], [142], [174], [239] and I found evidence for miR-148a to be reduced in T2D in only 1 study [249]. Several studies on miR-148a and osteoporosis and postmenopausal osteoporosis have also reported an increase in its expression [336]–[339]. Research suggests that the increase in expression of miR-148a in osteoporosis negatively regulates the Wnt/β-catenin signalling pathway, increasing sclerostin and DKK1 which in turn poses a negative effect on bone metabolism by decreasing osteoblast differentiation and increasing osteoclast maturation. The Wnt/ β-catenin signalling pathway is a downstream target of miR-148a [336], [339]. Although in T1D, Grieco and colleagues have reported a negative association between miR-148a and total body BMD, meaning the increase in miR-148a was associated with a decrease in total body BMD [25]. This evidence is suggestive of our finding of a negative association between sclerostin and miR-148a to be a chance finding.

miR-30e

In this study I also found serum sclerostin to be higher in T2D compared to matched healthy controls and a negative correlation between serum Sclerostin and hsa-miR-30e-5p. Just like miR-148a, several studies in T2D have reported an increase in expression of miR-30e [183], [194]. Takahashi and colleagues have reported an increase in expression of miR-30e in T2D [142]. In the bone field, Zarecki and colleagues have reported an overexpression of miR-30e in people with low BMD and vertebral fractures compared to healthy controls [278]. However, Kocijan et al. have reported a downregulation in miR-30e in people with osteoporotic fractures [275]. Support from literature suggests that miR-30e inhibits osteogenesis of mesenchymal stronmal cells by tareting Igf2 and Runx2 and thereby increasing adipogenesis [340]. The evidence gathered thus far from literature suggests that our finding of a negative association between sclerostin and miR-30e is a chance finding.

5.4.6 Limitations of the study

This study has its limitations. The population of T2D were recruited from Denmark and the popualtion of health controls were recrutied from UK. To balance for this, best efforts were taken to match the two study populations for age, gender and BMI as close as possible. We were unable to cross caliberate the different DXA scanners used at the two sites. The reasons for the low recovery of UniSP4 in the control group are unknown. This made it impossible for

us to compare miRNA expression levels between T2D and controls, which is the biggest limitation of this study.

5.4.7 Summary

I was able to confirm the evidence of low bone turnover in T2D. Patients with T2D experience a state of low bone turnover and a positive bone balance. However, this positive bone balance observed is not explained by aBMD as I observed BMD at the total hip to be reduced in T2D after adjusting for age, gender and BMI. I provide preliminary evidence for miR-24-3p and miR-574-3p to be promising biomarker candidates for T2D bone disease.

Chapter 6 - Capability of the ‘OsteomiR kit’ in predicting incident vertebral and non-vertebral fracture risk in the OPUS population

6.1 Introduction

So far in this thesis, I have not been able to demonstrate strong evidence for the use of miRNAs as suitable biomarkers for bone fragility and fracture risk in patients with type 1 diabetes and type 2 diabetes. This could be due to several reasons. The small sample size of the study populations (especially for the study on type 1 diabetes an neuropathy, N=29) could be an underlying reason. The miRNA signature panel chosen to be analysed as part of this thesis could have been chosen with validation from next generation sequencing in the chosen population of interests rather than just from findings of a systematic review. Differences in sample processing, various technical differences in the isolation of RNA and measurement of miRNAs, and diversity in the demographic characteristics of the different sample cohorts’ part of the systematic review from which the miRNA signature panel was chosen, may have contributed to the non-significant results observed so far in this thesis. In addition, intrinsic biological variability of the miRNAs could also be a contributing factor.

A high degree of variability and signal-to-noise ratio in miRNA measurements is a concern. Physiological variations occur in the body over time due to changes in circadian rhythm and intake of food. Such variations affect the levels of different biological molecules, especially when the level of the molecule is low. miRNAs are one such type of biological molecules which are affected by physiological changes in the body. If the baseline level of a miRNA is affected by physiological, environmental or biochemical changes over a period of time, this disturbance can be seen in the form of a difference in the expected miRNA expression level and could potentially contribute to the noise in the measurement [341]. Although the literature supports the use of miRNAs as potential biomarkers for disease diagnosis, at present, very little is known on inter- and intra-individual variability of miRNA expression levels, and if circadian rhythm, feeding or fasting have an impact on miRNA expression levels. At present, the limited data available suggests there to be large variability in miRNA measurements [342], [343]. A lot more research is required to understand the stability of miRNAs.

This chapter aimed to validate commercially-available microRNA biomarker kit called ‘OsteomiR’ to understand the general usability of microRNAs as suitable biomarkers for osteoporosis. For this purpose, the OPUS population was chosen. The OPUS population was selected to validate the ‘OsteomiR Kit’ because of its large sample size, its prospective design

and well characterised clinical outcomes including incident vertebral, rate of bone loss, and non-vertebral fractures.

6.1.1 OsteomiR – microRNA biomarkers of bone quality

The 'OsteomiR kit' developed by TamiRNA GmbH, Austria allows for simple and standardized analysis of specific circulating microRNAs in human serum and plasma which reflect bone quality. The osteomiRs (validated bone biomarkers) have been chosen carefully based on a series of clinical studies on the basis of their – (i) association with bone turnover, bone microstructure and histomorphometry; (ii) Significant dysregulation in patient with osteoporotic fractures and other bone diseases, (iii) association with bone loss and osteoporotic treatment response; (iv) literature support to regulate bone formation and bone resorption, as well as calcification; and (v) literature support to be BMD independent risk factors for osteoporotic fractures. A summary of evidence for the miRNAs included in the 'OsteomiR kit' is detailed in Figure 6.1.

miRNA ID	Bone Turnover	Microstructure and Histomor-phometry	Osteoporosis & other bone diseases	Bone Loss & Treatment Response	Calcification	Therapeutic Activity	Mechanism of action
let-7b-5p	•	•	•				VEGF, HMGA2
miR-127-3p			•				S1PR3
miR-133b	•		•	•			Runx2/FOXC1
miR-141-3p			•		•		WNT
miR-143-3p			•				17β-estradiol, osterix
miR-144-5p	•						RANK
miR-152-3p			•				
miR-17-5p				•	•		Smad5, BMP2
miR-188-5p			•	•		•	PPARγ via HDAC9/RICTOR
miR-19b-3p	•		•	•			
miR-203a			•	•	•		Runx2, Dlx5, 17β-estradiol
miR-214-3p					•	•	ATF4
miR-29b-3p	•	•	•		•		HDAC4, TGFβ3, CTNNBIP1
miR-31-5p			•	•	•	•	WNT via FZD3
miR-320a			•		•		HOXA10
miR-335-5p	•	•	•	•	•		WNT via DKK1
miR-375			•		•	•	WNT via LRP5 and β-catenin
miR-550a-3p	•	•	•		•		
miR-582-5p			•				

Figure 6-1. Summary of evidence of microRNA bone biomarkers included in the 'OsteomiR kit.

(Source: OsteomiR handbook, TAmiRNA GmbH, Austria).

Research question

Are miRNAs listed in the 'OsteomiR kit' developed by TAmiRNA GmbH (Austria) capable of predicting incident vertebral and non-vertebral fractures in the OPUS population?

6.2 Methods

6.2.1 Participants

The study participants chosen for this analysis were from the Osteoporosis and Ultrasound (OPUS) study [115]. OPUS was a population based study involving participants from five European centres: Aberdeen (UK), Berlin (Germany), Kiel (Germany), Paris (France) and Sheffield (UK). Study participants were recruited at random from government provided registers, population health registers and general practices during the period of April 1999 to April 2001. The study had a follow up period of 6 years and was coordinated by the "Medizinische Physik" in Kiel. Written informed consent was collected from each of the study participants.

For the purpose of this analysis, 1329 women aged 55 – 79 years from the OPUS study population were included. These participants were chosen as they attended the follow-up visit and had a serum sample available to measure miRNAs. The use of samples in this study was approved by the South Yorkshire North Derbyshire Musculoskeletal Biobank.

6.2.2 Fracture assessment

At the baseline visit, prevalent vertebral fractures (i.e. osteoporotic fractures and deformities at the spine) were assessed by quantitative morphometry. A threshold of vertebral height ratio of <0.80 was used and this was followed by visual radiological differential diagnosis. At the follow up visit, incident vertebral fractures were assessed using a threshold ratio of <0.80 of the baseline vertebral height and quantitative morphometry. For consistency, all spine radiographs were read by an experienced radiologist at the Berlin Centre.

At the baseline and 6-year follow-up visit, participants were required to fill out the OPUS Risk Factor Questionnaire which captured information on previous fractures. Prevalent non-vertebral fractures (i.e. hip, shoulder and wrist) were captured through this questionnaire filled at baseline. Incident non-vertebral fractures were captured through self-report and questions detailed in the OPUS Risk Factor Questionnaire filled at the follow-up visit. This information was then verified by radiographs or medical records.

The fracture information collected during the study was used to analyse if the 'OsteomiR kit' had the potential to predict fracture risk and comment on bone loss and bone fragility in the OPUS population.

6.2.3 Measurement of aBMD, BTMs and miRNAs

At the baseline visit, bone formation marker PINP and bone resorption marker CTX were measured in serum in the non-fasted state using the IDS-iSYS Multidiscipline Automated System (Immunodiagnostic Systems, Boldon, United Kingdom). aBMD at the proximal femur was measured using DXA in the posterior-anterior projection (Hologic QDR-4500; Hologic, Bedford, MA, USA in the Kiel, Sheffield and Paris sites) and in the anterior-posterior projection (Lunar Expert devices; GE Lunar, Madison, WI, USA in the Berlin and Aberdeen sites). Well-defined and established quality assurance concepts, quality control procedures [344], [345] and standard operating procedures based on the manufacturers' specifications were followed for the measurement of bone density at all five study centres. miRNAs present in the 'OsteomiR kit' were measured in non-fasted serum samples at the baseline visit using the LC 480 Real Time PCR System (Roche). The 'OsteomiR kit' contains 19 such miRNA bone biomarkers and 5 control miRNAs. These control miRNAs are synthetic miRNA spike-in controls (UniSp4, cel-miR-39-3p and UniSp3) and endogenous miR-23a-3p and miR-451a (to check for the presence of haemolysis), as described in Chapter 2. Baseline measurements of PINP, CTX and miRNAs were used in this analysis.

Expression level of the miRNAs were normalised using global mean normalisation.

$$\text{Global mean normalised Cq} = \text{Raw Cq}_{\text{chosen miRNA}} - \text{mean Cq}_{\text{all measured miRNAs}}$$

6.2.4 Statistical analysis

The global mean normalised Cq values of the measured miRNAs were used to build the statistical models described below. The capability of the OsteomiRs to predict vertebral fractures in the chosen OPUS population was assessed using the multiple logistic regression model and its result is expressed as an odds ratio. The capability of the OsteomiRs to predict non-vertebral fractures in the chosen OPUS study population was assessed using the Cox regression model and its result is expressed as a hazard ratio. The reason for using these different time series models is that we do not know when the vertebral fracture had occurred, therefore a multiple logistic regression model is suitable; and for non-vertebral fractures we do know when they occurred, therefore a Cox regression model is suitable. In the backward rejection regression models, the risk of a having an incident vertebral or non-vertebral fracture is represented as a Z-score, which is the number of standard deviations from the mean. The capability of the OsteomiRs to predict the rate of bone loss in the chosen OPUS population was assessed using the multiple linear regression model and its result is expressed as a beta-estimate. Where appropriate, the strength of a relationship between two variables was

assessed using Spearman's rank correlation. Statistical analysis was done using GraphPad Prism (Version 9.3.1).

6.3 Results

6.3.1 Characteristics of study participants

1329 women of the OPUS population were included in this analysis. They were between the age group of 55 – 79 years and had an average follow up time of 5 years and 8 months. Within this selected study population, 58 incident vertebral fractures and 157 incident non vertebral fractures were recorded throughout the study period. This translated to 4.4% of the chosen population suffered an incident vertebral fracture and 7.5% of the chosen population suffered an incident non-vertebral fracture. Measurement of the bone formation marker PINP and bone resorption marker CTX in serum, as well as BMD at the total hip has been previously published by our research group [128]. Results of PINP and CTX at the baseline visit, and results of aBMD at the total hip at the baseline and follow up visit were used for the purpose of this analysis. The characteristics of the study population at the baseline visit are listed in Table 6.1.

Table 6-1. Summary of characteristics of the chosen OPUS population at baseline.

N	1329
Age (years)	65.66 (6.68)
BMI (kg/m^2)	26.45 (4.40)
Number of incident vertebral fractures (N, %)	58 (4.4%)
Number of incident non-vertebral fractures (N, %)	157 (7.5%)
Baseline visit – TH aBMD (g/cm^2)	0.69 (0.12)
Follow up visit – TH aBMD (g/cm^2)	0.67 (0.12)
Rate of bone loss from the total hip (%)	-3.45% (8.20)
Serum PINP (ng/mL)	50.80 (21.79)
Serum CTX (ng/mL)	0.22 (0.17)

N = number of participants, BMI = body mass index, TH aBMD = total hip areal BMD, all values except the number of fractures are represented as mean (SD). Fractures are represented as mean (%).

6.3.2 Expression of OsteomiRs in the OPUS population

All the 19 miRNAs in the 'OsteomiR kit' were measured in the serum sample collected at the baseline visit. miRNA measurements were normalized using the global mean normalization approach. This is because, 2 different batches of RNA isolation and cDNA conversion kits were used for the study analysis, which lead to heterogeneity in the Cq values of the spike-in

controls, mainly UniSp4. Mean Cq values (SD) (non-normalized) for each of the 19 OsteomiRs are listed in Table 6.2. A higher Cq value of the miRNA represents a lower concentration of the respective miRNA in the sample measured. Based on the guidance received from the miRNA expert team at TAmiRNA, a Cq value < 30 was set as a cut off for a miRNA to be highly concentrated in a given sample. Hence, in this study, I found miR-17-5p and miR-19b-3p to be upregulated in serum sample of the chosen OPUS population and the remaining miRNAs measured were downregulated (Table 6.2).

Table 6-2. Cq values of the measured OsteomiRs in the chosen OPUS population.

OsteomiR	Cq value
let-7b-5p	31.44 (2.41)
miR-127-3p	36.65 (2.81)
miR-133b	36.46 (2.53)
miR-141-3p	36.42 (2.78)
miR-143-3p	31.96 (2.02)
miR-144-5p	33.10 (1.72)
miR-17-5p	29.65 (1.99)
miR-188-5p	35.31 (2.73)
miR-19b-3p	27.13 (2.04)
miR-203a-3p	37.96 (5.04)
miR-214-3p	36.14 (1.98)
miR-152-3p	32.68 (1.37)
miR-29b-3p	32.27 (2.00)
miR-31-5p	38.07 (3.84)
miR-335-5p	34.83 (2.37)
miR-375	34.23 (2.06)
miR-582-5p	35.36 (3.40)
miR-320a	30.18 (2.10)
miR-550a-3p	36.47 (3.13)

All values represented as Mean (SD). Higher Cq value indicates lower miRNA concentration.

6.3.3 Capability of OsteomiRs to predict incident vertebral fracture risk in the OPUS population

The multiple logistic regression model was used to analyse the capability of OsteomiRs to predict incident vertebral fractures in the OPUS population. miR-214-3p returned an odds ratio of < 1 (0.76 to 0.99) and a significant p value of 0.03. This suggests that a 1 Cq unit decrease

in the expression of miR-214-3p possibly reduces the risk of an incident vertebral fracture by up to 24%, and may be capable in predicting incident vertebral fractures in the OPUS population. On the other hand, miR-31-5p also returned an odds ratio of <1 (0.85 to 0.99). However, this relationship was only border line significant (p = 0.052). In summary, the downregulation of miR-214-3p and miR-31-5p may possibly lead to fewer vertebral fractures. Table 6.3 lists the predictive capability of the OsteomiRs as an odds ratio.

Table 6-3. Ability of OsteomiRs in predicting incident vertebral fracture risk in the chosen OPUS population, represented as an odds ratio.

OsteomiR	Logistic model β1 (95% CI)	Odds ratio	p
let-7b-5p	-0.27 to 0.09	0.76 to 1.10	0.33
miR-127-3p	-0.11 to 0.097	0.89 to 1.10	0.84
miR-133b	-0.12 to 0.17	0.89 to 1.19	0.77
miR-141-3p	-0.12 to 0.11	0.89 to 1.1	0.89
miR-143-3p	-0.14 to 0.33	0.87 to 1.34	0.47
miR-144-5p	-0.06 to 0.34	0.94 to 1.41	0.16
miR-17-5p	-0.18 to 0.40	0.84 to 1.50	0.49
miR-188-5p	-0.06 to 0.13	0.94 to 1.13	0.45
miR-19b-3p	-0.17 to 0.31	0.84 to 1.37	0.59
miR-203a-3p	-0.05 to 0.06	0.95 to 1.06	0.80
miR-214-3p	**-0.28 to -0.005**	**0.76 to 0.99**	**0.03**
miR-152-3p	-0.42 to 0.12	0.66 to 1.23	0.46
miR-29b-3p	-0.23 to 0.30	0.80 to 1.35	0.87
miR-31-5p	-0.16 to -0.001	0.85 to 0.99	0.05
miR-335-5p	-0.15 to 0.15	0.86 to 1.16	0.94
miR-375	-0.28 to 0.02	0.76 to 1.02	0.09
miR-582-5p	-0.01 to 0.12	0.99 to 1.13	0.09
miR-320a	-0.16 to 0.15	0.85 to 1.16	0.95
miR-550a-3p	-0.09 to 0.10	0.91 to 1.11	0.86

6.3.4 Capability of miR-214-3p in predicting incident vertebral fracture risk in the OPUS population

In order to assess the robustness in the capability of miR-214-3p to predict incident vertebral fractures, a logistic regression model with backwards rejection was built. Expression of miR-214-3p and other variables which we believe to be determinants of incident vertebral fractures

(age, BMI, PINP, CTX, total hip aBMD (baseline visit)) were included in the model. The results of the logistic regression with backwards rejection are displayed in Table 6.4 – 6.8.

Table 6-4. Results from a logistic regression analysis assessing the capability of miR-214-3p in predicting incident vertebral fracture risk in the chosen OPUS population (complete model).

Variable	|Z|	p
intercept	1.57	0.12
Age	2.29	0.02
BMI	0.30	0.76
PINP	2.42	0.02
CTX	1.85	0.06
Baseline visit TH aBMD	3.17	0.002
miR-214-3p	1.85	0.06

Table 6-5. Results from a logistic regression analysis assessing the capability of miR-214-3p in predicting incident vertebral fracture risk in the chosen OPUS population (BMI rejected from the model).

Variable	|Z|	p
intercept	1.54	0.12
Age	2.34	0.02
PINP	2.41	0.02
CTX	1.84	0.07
Baseline visit - TH aBMD	3.31	0.001
miR-214-3p	1.83	0.07

Table 6-6. Results from a logistic regression analysis assessing the capability of miR-214-3p in predicting incident vertebral fracture risk in the chosen OPUS population (miR-214-3p rejected from the model).

Variable	|Z|	p
intercept	1.31	0.1915
Age	2.32	0.02
PINP	2.41	0.02
CTX	1.78	0.07
Baseline visit TH aBMD	3.30	0.001

Table 6-7. Results from a logistic regression analysis assessing the capability of miR-214-3p in predicting incident vertebral fracture risk in the chosen OPUS population (CTX rejected from the model).

Variable	|Z|	p
intercept	1.50	0.13
Age	2.47	0.01
PINP	1.79	0.07
Baseline visit TH aBMD	3.34	0.0008

Table 6-8. Results from a logistic regression analysis assessing the capability of miR-214-3p in predicting incident vertebral fracture risk in the chosen OPUS population (PINP rejected from the model).

Variable	|Z|	p
intercept	2.1	0.04
Age	2.56	0.01
Baseline visit TH aBMD	3.18	0.002

Since miR-214-3p dropped out from the logistic regression model, its expression was added back into the model along with the significant determinants of incident vertebral fractures identified (age and baseline visit - total hip aBMD) and the analysis was repeated (Table 6.9).

Table 6-9. Results from a logistic regression analysis assessing the capability of miR-214-3p in predicting incident vertebral fracture risk in the chosen OPUS population (miR-214-3p added back into the model).

Variable	|Z|	p
intercept	2.31	0.02
Age	2.582	0.01
Baseline visit TH aBMD	3.20	0.001
miR-214-3p	1.83	0.07

Results from this section show that miR-214-3p is not capable of predicting incident vertebral fractures in the OPUS population after adjusting for age and total hip aBMD. The results suggest age and total hip BMD to be strong determinants of incident vertebral fractures (Table 6.9).

6.3.5 Capability of miR-31-5p in predicting incident vertebral fracture risk in the OPUS population

In order to assess the robustness in the capability of miR-31-5p to predict incident vertebral fractures, a similar logistic regression model with backwards rejection was built. Expression of miR-214-3p and other variables which we believe to be determinants of incident vertebral fractures (age, BMI, PINP, CTX, total hip aBMD (baseline visit)) were included in the model. The results of the logistic regression with backwards rejection are displayed in Table 6.10 – 6.13.

Table 6-10. Results from a logistic regression analysis assessing the capability of miR-31-5p in predicting incident vertebral fracture risk in the chosen OPUS population (complete model).

Variable	|Z|	p
intercept	1.48	0.14
miR-31-5p	1.82	0.07
Age	2.22	0.03
BMI	0.02	0.82
PINP	2.43	0.02
CTX	1.83	0.07
Baseline visit TH aBMD	3.15	0.002

Table 6-11. Results from a logistic regression analysis assessing the capability of miR-31-5p in predicting incident vertebral fracture risk in the chosen OPUS population (BMI rejected from the model).

Variable	|Z|	p
intercept	1.46	0.14
miR-31-5p	1.82	0.07
Age	2.27	0.02
PINP	2.42	0.02
CTX	1.82	0.07
Baseline visit TH aBMD	3.31	<0.001

Table 6-12. Results from a logistic regression analysis assessing the capability of miR-31-5p in predicting incident vertebral fracture risk in the chosen OPUS population (CTX rejected from the model).

Variable	|Z|	p
intercept	1.65	0.10
miR-31-5p	1.79	0.07
Age	2.43	0.02
PINP	1.77	0.08
Baseline visit TH aBMD	3.35	<0.001

Table 6-13. Results from a logistic regression analysis assessing the capability of miR-31-5p in predicting incident vertebral fracture risk in the chosen OPUS population (PINP rejected from the model).

Variable	|Z|	p
intercept	2.29	0.02
miR-31-5p	2.00	0.045
Age	2.51	0.01
Baseline visit TH aBMD	3.16	0.002

Results from this section show that miR-31-5p is capable of predicting incident vertebral fractures in the OPUS population after adjusting for age and total hip BMD. The low expression of hsa-miR-31-5p leads to fewer vertebral fractures in the OPUS population. However, age and total hip BMD are stronger determinants of incident vertebral fractures (Table 6.13).

6.3.6 Combined capability of miR-214-3p and miR-31-5p in predicting incident vertebral fracture risk in the OPUS population

I next wondered if the predictive capability of incident vertebral fractures by the OsteomiRs would increase by combining the expression of miR-214-3p and miR-31-5p. In order to assess this, a logistic regression with backwards rejection was performed. Expression of miR-214-3p and miR-31-5p; and other variables which we believed to be determinants of incident vertebral fractures (age, BMI, PINP, CTX, total hip aBMD (baseline visit)) were included in the model. The results of the logistic regression with backwards rejection are displayed in Table 6.14 – 6.17.

Table 6-14. Results from a logistic regression analysis assessing the combined capability of miR-214-3p and miR-31-5p in predicting incident vertebral fracture risk in the chosen OPUS population (complete model).

Variable	|Z|	p
intercept	1.74	0.08
miR-31-5p	1.92	0.05
Age	2.22	0.03
BMI	0.34	0.73
PINP	2.43	0.02
CTX	1.91	0.06
Baseline visit TH aBMD	3.20	0.001
miR-214-3p	1.97	0.05

Table 6-15. Results from a logistic regression analysis assessing the combined capability of miR-214-3p and miR-31-5p in predicting incident vertebral fracture risk in the chosen OPUS population (BMI rejected from the model).

Variable	|Z|	p
intercept	1.71	0.09
miR-31-5p	1.92	0.06
Age	2.29	0.02
PINP	2.41	0.02
CTX	1.89	0.06
Baseline visit TH aBMD	3.32	0.0009
miR-214-3p	1.95	0.05

Table 6-16 Results from a logistic regression analysis assessing the combined capability of miR-214-3p and miR-31-5p in predicting incident vertebral fracture risk in the chosen OPUS population (CTX rejected from the model).

Variable	|Z|	p
intercept	1.89	0.06
miR-31-5p	1.88	0.06
Age	2.43	0.01
PINP	1.70	0.09
Baseline visit TH aBMD	3.36	<0.001
miR-214-3p	1.89	0.06

Table 6-17 Results from a logistic regression analysis assessing the combined capability of miR-214-3p and miR-31-5p in predicting incident vertebral fracture risk in the chosen OPUS population (PINP rejected from the model).

Variable	|Z|	P value
intercept	2.51	0.01
miR-31-5p	2.11	0.035
Age	2.52	0.01
Baseline visit TH aBMD	3.19	0.001
miR-214-3p	1.98	0.048

Results from this section show that when expression of miR-214-3p and miR-31-5p are combined together, their capability of predicting incident vertebral fracture risk in the OPUS population improved. However, age and total hip aBMD remain stronger determinants of incident vertebral fractures (Table 6.17). The low expression of miR-214-3p and miR-31-5p may be responsible for fewer incident vertebral fractures in the OPUS population.

Correlation between miR-214-3p, miR-31-5p, PINP, CTX and TH aBMD

I performed a Spearman's rank correlation between the 2 OsteomiRs (miR-214-3p and miR-31-5p), PINP, CTX and TH aBMD to assess if these 2 OsteomiRs correlate with each other and if they correlate with bone turnover and bone density. The Spearman's rank correlation coefficient (r) and p value of the association are listed in Table 6.18 and 6.19. miR-214-3p and miR-31-5p did not correlate with each other and neither did they correlate with bone turnover or bone density.

Table 6-18 Spearman's rank correlation and p value of association between miR-214-3p, and PINP, CTX, TH aBMD and miR-31-5p in the chosen OPUS population.

	PINP		CTX		TH aBMD		miR-31-5p	
	r	p	r	p	r	p	r	p
miR-214-3p	0.04	0.12	0.03	0.29	0.02	0.40	-0.02	0.51

Table 6-19 Spearman's rank correlation and p value of association between miR-31-5p, and PINP, CTX and TH aBMD in the chosen OPUS population.

	PINP		CTX		TH aBMD	
	r	p	r	p	r	p
miR-31-5p	0.04	0.20	0.02	0.51	0.001	0.96

6.3.7 Ability of OsteomiRs in predicting bone loss from the hip in the OPUS population

The multiple linear regression model was used to assess the ability of OsteomiRs to predict bone loss from the hip in the OPUS population. Table 6.20 lists the predictive capability of the OsteomiRs as a beta-estimate (95% CI). None of the OsteomiRs were capable of commenting on the rate of bone loss from the hip in the OPUS population.

Table 6-20 Ability of OsteomiRs in predicting bone loss from the hip in the chosen OPUS population, represented as a beta-estimate (95% CI).

OsteomiR	Linear model β1 (95% CI)	p
let-7b-5p	-0.26 to 0.40	0.67
miR-127-3p	-0.23 to 0.14	0.61
miR-133b	-0.21 to 0.27	0.78
miR-141-3p	-0.10 to 0.29	0.34
miR-143-3p	-0.37 to 0.39	0.95
miR-144-5p	-0.32 to 0.38	0.87
miR-17-5p	-0.72 to 0.24	0.33
miR-188-5p	-0.22 to 0.12	0.55
miR-19b-3p	-0.52 to 0.29	0.59
miR-203a-3p	-0.14 to 0.05	0.35
miR-214-3p	-0.06 to 0.48	0.13
miR-152-3p	-0.34 to 0.75	0.45
miR-29b-3p	-0.49 to 0.39	0.83
miR-31-5p	-0.11 to 0.14	0.82
miR-335-5p	-0.21 to 0.312	0.68
miR-375	-0.29 to 0.23	0.81
miR-582-5p	-0.17 to 0.12	0.67
miR-23a-3p	-0.59 to 0.52	0.89
miR-320a	-0.23 to 0.30	0.79
miR-550a-3p	-0.08 to 0.27	0.26

6.3.8 Capability of OsteomiRs in predicting incident non-vertebral fracture risk in the OPUS population

The Cox regression model was used to assess the ability of OsteomiRs to predict incident non-vertebral fractures in the OPUS population. Table 6.21 lists the predictive capability of the

OsteomiRs as a hazard ratio. None of the osteomiRs were capable of predicting incident non-vertebral fractures in the OPUS population.

miR-29b-3p returned a hazard ratio of 0.99 to 1.41 and was borderline significant with p = 0.06 while miR-19b-3p returned a hazard ratio of 0.78 to 1.02 and was also borderline significant with p = 0.09. Although only borderline significant, the model suggests that a 1 Cq decrease in the expression of miR-29b-3p increases the risk of an incident non-vertebral fracture by up to 40%; and a 1 Cq increase in the expression of miR-19b-3p decreases the risk of an incident non-vertebral fracture by up to 22%.

Table 6-21 Ability of OsteomiRs in predicting incident non-vertebral fractures in the chosen OPUS population, represented as a hazard ratio.

OsteomiR	Cox model β1 (95% CI)	Hazard ratio	p
let-7b-5p	-0.15 to 0.07	0.86 to 1.08	0.46
miR-127-3p	-0.07 to 0.056	0.93 to 1.06	0.81
miR-133b	-0.13 to 0.03	0.88 to 1.03	0.16
miR-141-3p	-0.06 to 0.08	0.94 to 1.08	0.81
miR-143-3p	-0.20 to 0.04	0.81 to 1.04	0.16
miR-144-5p	-0.16 to 0.08	0.85 to 1.09	0.55
miR-17-5p	-0.27 to 0.04	0.76 to 1.04	0.12
miR-188-5p	-0.07 to 0.05	0.93 to 1.05	0.73
miR-19b-3p	-0.25 to 0.02	0.78 to 1.02	0.09
miR-203a-3p	-0.03 to 0.03	0.97 to 1.04	0.83
miR-214-3p	-0.09 to 0.10	0.92 to 1.11	0.91
miR-152-3p	-0.07 to 0.30	0.93 to 1.35	0.22
miR-29b-3p	-0.001 to 0.34	0.99 to 1.41	0.06
miR-31-5p	-0.02 to 0.06	0.98 to 1.07	0.25
miR-335-5p	-0.03 to 0.14	0.97 to 1.15	0.19
miR-375	-0.14 to 0.04	0.87 to 1.04	0.28
miR-582-5p	-0.05 to 0.05	0.95 to 1.05	0.96
miR-23a-3p	-0.23 to 0.15	0.79 to 1.16	0.66
miR-320a	-0.07 to 0.11	0.93 to 1.12	0.71
miR-550a-3p	-0.03 to 0.09	0.97 to 1.09	0.29

6.3.9 Combined capability of miR-19b-3p and miR-29b-3p in predicting incident non-vertebral fracture risk in the OPUS population

Since none of the osteomiRs were capable of individually predicting the occurrence of an incident non-vertebral fracture in the OPUS population, we took an exploratory approach and

built a Cox regression model using the 2 osteomiRs that were borderline significant (p value closest to 0.05 - miR-19b-3p, p value = 0.09 and miR-29b-3p, p value = 0.06) (Table 6.22). I next build a Cox regression model with backwards rejection using these 2 OsteomiRs and other variables which we believe to be determinants of incident non-vertebral fractures (age, BMI, PINP, CTX, total hip aBMD (baseline visit)). The results of the Cox regression are listed in Table 6.23 – 6.27.

Table 6-22 Results from a Cox regression analysis assessing the combined capability of miR-19b-3p and miR-29b-3p in predicting incident non-vertebral fracture risk in the chosen OPUS population.

Variable	95% CI	|Z|	p
miR-19b-3p	-0.37 to -0.06	2.72	0.01
miR-29b-3p	0.08 to 0.44	2.82	0.005

Table 6-23 Results from a Cox regression analysis assessing the combined capability of miR-19b-3p and miR-29b-3p in predicting incident non-vertebral fracture risk in the chosen OPUS population (complete model).

Variable	|Z|	p
miR-19b-3p	2.74	0.006
miR-29b-3p	2.68	0.007
Age	1.31	0.19
BMI	1.15	0.25
PINP	0.64	0.52
CTX	0.53	0.60
Baseline visit TH aBMD	3.80	0.0001

Table 6-24 Results from a Cox regression analysis assessing the combined capability of miR-19b-3p and miR-29b-3p in predicting incident non-vertebral fracture risk in the chosen OPUS population.

(CTX rejected from the model).

Variable	|Z|	p
miR-19b-3p	2.76	0.006
miR-29b-3p	2.69	0.007
Age	1.26	0.21
BMI	1.16	0.25
PINP	1.31	0.19
Baseline visit TH aBMD	3.78	0.0002

Table 6-25 Results from a Cox regression results analysis assessing the combined capability of miR-19b-3p and miR-29b-3p in predicting incident non-vertebral fracture risk in the chosen OPUS population (BMI rejected from the model).

Variable	|Z|	p
miR-19b-3p	2.84	0.005
miR-29b-3p	2.67	0.008
Age	1.42	0.16
PINP	1.28	0.20
Baseline visit TH aBMD	3.62	0.0003

Table 6-26 Results from a Cox regression analysis assessing the combined capability of miR-19b-3p and miR-29b-3p in predicting incident non-vertebral fracture risk in the chosen OPUS population (PINP rejected from the model).

Variable	|Z|	p
miR-19b-3p	2.60	0.01
miR-29b-3p	2.72	0.007
Age	1.44	0.15
Baseline visit TH aBMD	3.62	0.0003

Table 6-27 Results from a Cox regression analysis assessing the combined capability of miR-19b-3p and miR-29b-3p in predicting incident non-vertebral fracture risk in the chosen OPUS population (Age rejected from the model).

Variable	|Z|	p
miR-19b-3p	2.66	0.007
miR-29b-3p	2.70	0.007
Baseline visit TH aBMD	4.30	<0.0001

Results from this section summarize that the combined expression of miR-19b-3p and miR-29b-3p increases the capability of these OsteomiRs in predicting incident non-vertebral fractures in the OPUS population. However, total hip BMD was found to be a strong determinant of incident non-vertebral fractures (Table 2.27). The increase in expression of miR-19b-3p and decrease in expression of miR-29b-3p may be responsible for the prediction of incident non-vertebral fractures in the OPUS population.

I next performed a Cox regression between the 2 OsteomiRs (miR-19b-3p and miR-29b-3p) and PINP and CTX to assess if after adjusting for bone turnover, do these 2 osteomiRs together drive the prediction of incident non-vertebral fractures. Indeed, they do (Table 6.28. After adjusting for bone turnover, the results indicate that these two osteomiRs (miR-19b-3p and miR-29b-3p) together can drive the prediction of incident non-vertebral fractures in the OPUS population. The results suggest that an increase in the expression of miR-19b-3p can possibly lead to the occurrence of fewer non-vertebral fractures, while a decrease in the

expression of miR-29b-3p can possibly lead to the occurrence of more non-vertebral fractures. These results also indicate a negative association between miR-19b-3p and miR-29b-3p.

Table 6-28 Results from a Cox regression analysis assessing the combined capability of miR-19b-3p and miR-29b-3p in predicting incident non-vertebral fracture risk in the chosen OPUS population (after adjusting for bone turnover).

Hazard ratio presented as 95% CI.

Variable	95% CI (HR)	|Z|	p
miR-19b-3p	0.68 to 0.93	2.90	0.004
miR-29b-3p	1.09 to 1.56	2.81	0.005
PINP	0.99 to 1.01	0.3	0.76
CTX	0.30 to 3.43	0.02	0.98

Correlation between miR-19b-3p, miR-29b-3p, PINP, CTX and TH aBMD

I performed a Spearman's rank correlation between the 2 OsteomiRs (miR-19b-3p and miR-29b-3p), PINP, CTX and TH aBMD to assess if these 2 OsteomiRs correlate with each other and if they correlate with bone turnover and bone density. The Spearman's rank correlation coefficient (r) and p value of the association are listed in Table 6.29 and 6.30. I found miR-19b-3p and miR-29b-3p to be positively associated with each other (r = 0.30, p <0.001). miR-19b-3p negatively correlated with PINP. However, miR-19b-3p did not correlate with CTX or total hip aBMD. Additionally, miR-29b-3p did not correlate with bone turnover or bone density at the hip.

Table 6-29 Spearman's rank correlation and p value of association between miR-19b-3p, and PINP, CTX, TH aBMD and miR-29b-3p in the chosen OPUS population.

	PINP		CTX		TH aBMD		miR-29b-3p	
	r	p	r	p	r	p	r	p
miR-19b-3p	-0.1	<0.001	-0.03	0.24	0.03	0.30	0.30	<0.001

Table 6-30 Spearman's rank correlation and p value of association between miR-29b-3p, and PINP, CTX and TH aBMD

	PINP		CTX		TH aBMD	
	r	p	r	p	r	p
miR-29b-3p	-0.02	0.51	-0.001	0.97	-0.03	0.24

6.4 Discussion

6.4.1 Summary of main findings

I assessed the capability of the 'OsteomiR kit' to predict fracture risk in the OPUS population. Our analysis found a combined model of adjusted-expression of miR-31-5p and miR-214-3p to be positively associated with the occurrence of incident vertebral fractures and a combined model of adjusted-expression of miR-19b-3p and miR-29b-3p to be negatively associated with the occurrence of incident non-vertebral fractures. In addition to these osteomiRs, I found BMD at the hip to be a strong and robust determinant of incident vertebral and non-vertebral fractures. The 'OsteomiR kit' could not predict bone loss from the hip in the OPUS population.

6.4.2 miR-31-5p, miR-214-3p and incident vertebral fractures

miR-31-5p is considered to be the master regulator of osteogenesis [346]. In the OPUS population, I found the adjusted-expression of miR-31-5p to be positively associated with the occurrence of an incident vertebral fracture. The expression of miR-31-5p was adjusted for bone turnover, age, gender, BMI and total hip BMD. Our finding was in agreement with evidence found in literature. In the literature, the upregulation of miR-31-5p has been shown to play a role in cellular senescence [347] and osteoclastogenesis [348]. The expression of miR-31-5p is induced by the RANKL, and it helps to control osteoclast formation and maturation, as well as bone resorption by regulating the organisation of the cytoskeleton induced by RhoA [348]. Weilneir et al. have shown that the increase in miR-31-5p in plasma of elderly and of osteoporotic patients leads to impaired bone formation and accumulation of senescent endothelial cells [346]. Apart from this, miR-31-5p has recently been shown to be important in Wnt signalling. The increase in miR-31 expression lead to a downregulation in the Wnt/β-catenin signalling pathway by targeting the expression of Satb2 gene, thereby inhibiting osteogenic differentiation [349].

miR-214-3p is considered to be a critical regulator of musculoskeletal metabolism. In this study, I also found miR-214-3p to be positively associated with vertebral fractures. However, after adjusting for bone turnover, age, BMI and total hip BMD, the association between miR-214-3p and incident vertebral fractures was no longer significant.

Another important observation was that, the combined adjusted-expression of miR-31-5p and miR-214-3p was associated with an even lower risk of an incident vertebral fracture. To date, we have no data on the combined expression of miR-31-5p and miR-214-3p on osteogenesis, bone strength and other cellular processes important in bone metabolism. Our data suggests that these two miRNAs do not work in synergy as they do not correlate with each other. Hence, more work is required on miRNA-miRNA interactions at a cellular level and at a circulating

level to understand if these two miRNAs work in synergy or independent of each other in reducing the risk of incident vertebral fractures. Our results also suggest that these two miRNAs do not correlate with PINP or CTX, suggesting that they may be predicting vertebral fractures independent of changes in bone turnover.

6.4.3 miR-19b-3p, miR-29b-3p and incident non-vertebral fractures

In the OPUS population, I found none of the osteomiRs to be independently associated with incident non-vertebral fractures. However, I found the combined expression of miR-19b-3p and miR-29b-3p to be associated with incident non-vertebral fractures. In particular, the increase in expression of miR-19b-3p was significantly associated with a lower occurrence of incident non-vertebral fractures and the decrease in expression of miR-29b-3p was significantly associated with a higher occurrence of incident non-vertebral fractures. The adjusted-expression of these two miRNAs combined, slightly reduced their capability of predicting incident non-vertebral fractures, but the interaction still remained significant. The miRNA expression was adjusted for bone turnover, age, BMI and total hip BMD.

The literature provides evidence for miR-19b-3p to be a regulator of osteogenesis by promoting the differentiation of human bone marrow mesenchymal stromal cells into osteoblasts by targeting WWP1 and Smurf2 of the Wnt/β-catenin signalling pathway and in turn enhancing fracture healing [350]. This supports our finding of a positive correlation between miR-19b-3p and PINP. miR-29b-3p is another important regulator of osteoblast formation. It functions by directly modifying the expression of bone matrix structural genes, collagen and matrix proteins, osteonectin during osteoblast formation. Further, Feichtinger et al. have demonstrated a positive correlation between miR-29b and mineral apposition rate and bone formation rate [351]. To date, we have no data on the combined expression of miR-19b-3p and miR-29b-3p on osteogenesis, bone strength and other cellular processes important in bone metabolism and it is unclear if they function in synergy or independent of each other.

In the Spearman correlation analysis, I observed miR-19b-3p and miR-29b-3p to be positively associated with each other (Table 6.29). However, from the Cox regression modelling (Table 6.28), I found these 2 OsteomiRs to have a negative relationship between each other. If we believe there to be a mechanism by which the increase in miR-19b-3p would cause a decrease in miR-29b-3p, then we should have observed a negative relationship between these 2 OsteomiRs. The predictive r value of correlation between them was only 0.3, which doesn't rule out the possibility of these 2 OsteomiRs contributing independently to the risk of non-vertebral fractures. At present, since we have no evidence for a biological mechanism of action that may be driving these 2 OsteomiRs, we don't suspect there to be any mediation or synergy

between them. I speculate the finding of a positive correlation between miR-19b-3p and miR-29b-3p to be a chance finding and a false positive result.

In this study, I assessed the capability of the miRNAs in predicting incident vertebral and non-vertebral fractures individually. Only one other study has assessed the capability of miRNAs in predicting fracture risk [253] In the OFELY cohort, Feurer et al. assessed the capability of 32 miRNAs is predicting incident fragility fractures (vertebral and non-vertebral fractures combined). These miRNAs were chosen from a literature search. The assessment of 5 miRNAs overlapped between our study and Feurer et al.'s study, namely, miR-127-3p, miR-133b, miR-17-5p, miR-214-3p and miR-29b-3p; and Feurer and collegues did not observe any associations between their selection of 32 miRNAs and incident fragility fractures [253]. In our study, I observed miR-214-3p to be capable in predicting incident vertebral fractures, however, this observation was not significant after adjusting for covariates we believe to be important in altering miRNA expression. The difference in results between our study and in the OFELY cohort could be due to the fact that the OFELY cohort included a broader population of women in the age group of 44 – 94 years and their analysis was performed by combining vertebral and non-vertebral fractures together. Our study was limited to a women in the age group of 55 – 79 years; and we stratified our analysis individually for vertebral and non-vertebral fractures.

miR-550a is responsible for osteogenesis Research done by Heilmeier et al. showed that patients who recently sustained a low-traumatic fracture had a significantly higher expression of miR-550a-3p when compared to those without a fracture [30]. Feichtinger et al. have reported a positive correlation between miR-550a-3p and mineral apposition rate and bone surface in a population of Caucasians with idiopathic osteoporosis or postmenopausal osteoporosis and at least one fragility fracture [351]. miR-550a-3p is a miRNA included in the OsteomiR panel. In our analysis I found no relationship between miR-550a-5p and incident vertebral or non-vertebral fractures. This may be due to the difference in the background of the study population and the analysis strategy. Our study included people from Britain and Europe, and our analysis was stratified individually for incident vertebral and non-vertebral fractures. Feichtinger et al. included Caucasians and their analysis was done by pooling all incident fractures (prevalent fractures) together.

Of note, apart from miR-19b-3p, the remaining miRNAs analysed in this study showed low abundance in serum (Cq > 30). The reason for this could be that these low abundant miRNAs are transcribed in peripheral tissues or in cell types that secrete very small amounts of the miRNA and hence, there is a lesser amount in circulation in blood.

6.4.4 Strengths and limitations of the study

To our knowledge, this is the first study that has assessed the capability of the commercially available ‘OsteomiR kit’ in predicting fracture risk. In this study, the serum used for miRNA measurements was collected in a non-fasted state and at mid-day. This may be one of the reasons why I was unable to find positive outcomes for the entire ‘OsteomiR kit’. We do not know the conditions at which the samples used to validate the miRNAs in the ‘OsteomiR kit’.

In general, when making serial measurements of an analyte, it is important that it exhibits low day-to-day or week-to-week within subject variability with the expected change, and a high signal-to-noise ratio. At present, there is limited data on variability of miRNAs in serum, and these tended to show large variability [342], [343]. Moreover, there is no data available on the variability and signal-to-noise ratio of miRNAs as well as if feeding influences miRNA variability. This information is important before we choose to use miRNAs as biomarkers for osteoporosis and fractures.

6.4.5 Summary

I was able to validate the fracture risk prediction capability of miRNAs in the ‘OsteomiR kit’. In addition to these miRNAs, I provided evidence for aBMD at the hip to be a strong determinant of incident fractures.

Chapter 7 – Overall discussion and conclusion

The research in this thesis aimed to identify miRNAs as novel biomarkers for assessment of bone health in type 1 and type 2 diabetes. The key findings below suggest that although miRNAs are promising biomarkers for diabetes and osteoporosis, additional research is needed to understand the variability of miRNAs before they can be used as diagnostic markers and therapeutic agents for diabetes and osteoporosis.

7.1 Key findings

- The systematic review on miRNAs in diabetic bone disease identified 64 dysregulated miRNAs in diabetes and abnormal bone phenotype (fractures, bone turnover, aBMD). Of these, miR-21, miR-335, miR-17, miR-375, miR-320a, miR-155, miR-124, miR-25, miR-133a and miR-223 were identified as the top 10 promising miRNA biomarkers for the assessment of diabetes and bone health.
- Bone turnover and aBMD in patients with T1D and neuropathy does not reduce further after a follow-up period of 4 years and 4 months.
- Patients with T1D and neuropathy do not experience excessive bone loss after a follow-up period of 4 years and 4 months.
- Cortical porosity at the tibia does not increase further in patients with T1D and neuropathy after a follow-up period of 4 years and 4 months.
- miR-133a-3p is a biomarker of T1D and could be related to bone health
- In T2D, age, gender and BMI are important determinants of aBMD. Age, gender and BMI-adjusted total hip aBMD was lower in T2D.
- T2D is characteristic of low bone turnover and positive bone balance using the T-score bone marker plot.
- miR-24-3p and miR-574-3p are promising biomarkers of T2D and bone health.
- aBMD is a strong determinant of incident vertebral and non-vertebral fractures.
- The combined-adjusted expression miR-124-3p and miR-31-5p can predict incident vertebral fracture risk in postmenopausal women.
- The combined-adjusted expression of miR-19b-3p and miR-29b-3p can predict incident non-vertebral fracture risk in postmenopausal women.

7.2 Ten promising miRNA biomarkers for the assessment of bone fragility in diabetes

This is the first systematic review of literature to report dysregulated miRNAs in diabetes and abnormal bone phenotype (fractures, bone turnover and aBMD). This systematic review

identified 64 miRNAs to be dysregulated in diabetes and abnormal bone phenotype (Chapter 3). Further, a sensitivity analysis identified miR-21, miR-335, miR-17, miR-375, miR-320a, miR-155, miR-124, miR-25, miR-133a and miR-223 to be the top 10 promising biomarkers for assessment of bone fragility in diabetes. The direction of expression of these top 10 miRNAs are listed in Table 7.1.

Table 7-1. List of the top 10 dysregulated miRNAs in diabetes and abnormal bone phenotype.

miRNA	Direction of expression (compared to a control / healthy population)
miR-21	upregulated
miR-335	upregulated
miR-17	downregulated
miR-375	upregulated
miR-320a	upregulated
miR-155	downregulated
miR-124	upregulated
miR-25	downregulated
miR-133a	upregulated / downregulated ?
miR-223	downregulated

7.3 After a follow-up of 4 years and 4 months, there is no excessive bone loss and impairment in bone microarchitecture in type 1 diabetes and neuropathy

The findings in Chapter 4 are the first to describe longitudinal changes in bone turnover, aBMD and bone microarchitecture in type 1 diabetes and neuropathy. The time difference between the baseline and follow-up visit was 4 years and 4 months. After 4 years and 4 months, bone turnover did not reduce further in T1D and neuropathy and these patients experienced a steady state of low bone turnover. Additionally, these patients did not experience further bone loss from the lumbar spine, total hip or femoral neck. Cortical porosity at the radius and tibia did not increase further, suggesting that the prolonged duration of type 1 diabetes and neuropathy did not weaken the bone further.

7.4 T-score bone marker method is a method applied to T2D here for the first time

The T-score bone marker method is used to identify the overall state of bone remodeling qualitatively and quantitatively by combining bone turnover and bone balance in relation to healthy premenopausal women. Using this method, T2D was characterised by low bone turnover and positive bone balance (Chapter 5). However, the relationship between the

observed low aBMD, high bone balance and observed increased fracture risk in T2D is yet to be understood.

7.5 miRNAs as biomarkers for bone health in diabetes

The utility of miRNAs as suitable biomarkers for the assessment of bone fragility and bone health in diabetes in the thesis has not been fully established. In T1D, I have shown that the expression of miR-133a-3p only showed a pattern of downregulation in T1DN- and T1DN+ but not significantly. On combining the two diabetic groups together (T1DN- and T1DN+), I observed a significant downregulation in expression of miR-133a-3p in T1D (Chapter 4). However, miR-133a-3p did not correlate with BTMs (PINP and CTX) or HbA1c. In T2D, I show evidence for miR-24-3p and miR-574-3p to be positively associated with PINP, suggestive of their use as promising miRNA biomarkers for T2D and bone health (Chapter 5). Our results on miRNAs in T1D, T2D and bone health are preliminary and require further validation.

7.6 Capability of the 'OsteomiR kit' in predicting incident fracture risk

Using an independent population of postmenopausal women, we believe to the first ones to test the capability of a commercially available bone biomarker kit called the 'OsteomiR kit' in predicting incident vertebral and non-vertebral fractures.

Our results suggested miR-124-3p to be capable of predicting incident vertebral fractures in postmenopausal women, but its predictive power was not significant and robust enough after adjusting for covariates of miRNA expression we believe to be important, such as age, gender, BMI and bone turnover. Further, our results suggested that the combined-adjusted expression of miR-124-3p and miR-31-5p is capable in predicting incident vertebral fractures in postmenopausal women. None of the miRNAs in the 'OsteomiR kit' could predict bone loss from the hip in postmenopausal women. Further, our results suggested that no single miRNA in the 'OsteomiR kit' could predict incident non-vertebral fractures in postmenopausal women. However, the combined-adjusted expression of miR-19b-3p and miR-29b-3p could predict incident non-vertebral fractures in postmenopausal women. Moreover, our results confirmed BMD as a strong predictor of incident vertebral and non-vertebral fractures.

7.7 miRNA variability

During miRNA measurements in this study, I observed unexpected findings: (i) the expression of a given miRNA within a given study group had a heterogeneous distribution of expression irrespective of the sample type (serum and plasma) it was measured in (Chapter 4) and (ii) The Cq values (> 25) of a synthetic miRNA used for normalization (UniSp4) was above the threshold accepted in our laboratory (Chapter 5). The reasons for these observations remain

unexplained. I was unable to find positive outcomes for the entire 'OsteomiR kit'. We do not know the sample type the 'Osteomir kit' was validated in neither do we know the protocol that was used for sample collection. In this thesis, I measured the OsteomiRs in serum and sample was collected at a random time of day in a non-fasted state. By referring back to literature to understand why I was unable to identify positive outcomes for the entire 'OsteomiR kit' I gathered that there is very little known in literature on miRNA variability.

7.8 Overall strengths of this thesis

Throughout the thesis several strengths contributed to the findings.

The dysregulated miRNAs in diabetes and altered bone phenotype were identified in a systematic manner following PRISMA guidelines. This allowed me to capture a large amount of information. The quality of the data collected was assessed based on the NOS score and the inconsistency in the miRNA expression pattern identified in the different studies due to the different sample types the miRNAs were measured in or the synthetic miRNA used to normalize the expression of the endogenous miRNA was addressed by performing a sensitivity analysis.

Consistency in the measurement of BTMs throughout the thesis was maintained by using an automated immunoassay in a centralised laboratory facility and thus minimising human error. Consistency in the measurement of miRNAs was throughout the thesis was maintained by using an automated system to prepare the 384-well plate for miRNA measurement, thus minimising human error, and the RT-PCR was performed using high standards at a centralised commercial laboratory facility. Consistency in the measurement of aBMD using DXA and outcomes of bone microarchitecture using HR-pQCT at both study visits was maintained by having the same experienced technician perform the measurements. Moreover, longitudinal changes in bone microarchitecture were measured by using the baseline scan site as a template while performing the scan at the follow-up visit and applying the method of 3D scan registration.

Subjects from a large population of postmenopausal women (N = 1329) with well-characterized clinical data were used to assess the capability of the 'OsteomiR kit' in predicting incident vertebral and non-vertebral fractures. With regards to fracture risk assessment, there were a large number of non-vertebral fracture cases (N = 157) in this cohort. Having a large study size and well-characterised clinical data to validate a diagnostic kit or assay ensures the accuracy and reliability of the kit.

7.9 Overall weakness of the thesis

There were also some limitations that affected the findings presented in this thesis.

The first limitation was identified during the systematic review (Chapter 3). The set-up of the systematic review's search strategy was extensive, allowing me to capture dysregulated miRNAs measured in many different sample types, including saliva and aqueous humour. Moreover, the search was not limited to just free-circulating miRNAs but also captured dysregulated miRNA expression in exosomes and extracellular vesicles. This led to a collection of heterogeneous information on the direction of expression of the identified dysregulated miRNAs. Although I balanced for this while performing the sensitivity analysis, this heterogeneity may contribute to the findings of the review.

The second limitation was identified in Chapter 4 which investigated longitudinal changes in bone health in type 1 diabetes and neuropathy. Almost half of the study population was lost during the follow up visit 4 years and 4 months later, which reduced the sample size to 29 and the study was underpowered. However, the population who came for the follow up visit were representative of the population who attended the baseline visit of the study. I speculate that the small sample size contributed to the results of no significant differences in bone turnover, aBMD and cortical porosity in the T1DN- and T1DN+ population with prolonged duration of T1D and neuropathy.

The third limitation was identified in Chapter 5 which assessed bone fragility in T2D. In this chapter, the population of T2D and healthy controls compared were from different geographic locations and could have possibly had other demographic characteristics. The T2D population was recruited from Odense, Denmark, while the healthy controls were recruited from Sheffield, UK. Nevertheless, best efforts were taken to match the T2D population to the healthy controls based on age, gender, height and BMI.

The fourth limitation was identified during the measurement of miRNAs in Chapter 4 and Chapter 5. In chapter 4, I observed that during the measurement of miRNAs at baseline, within a given study group, a given miRNA displayed a heterogeneous split in miRNA expression. This was observed irrespective of the sample type (serum and plasma) the miRNA was measured in. This made it impossible to measure miRNA expression at baseline and further study longitudinal changes in miRNA expression in T1D and neuropathy. In Chapter 5, I observed high Cq values (> 25), particularly in the healthy control group, for a synthetic miRNA UniSp4, which is used for data normalisation. These high Cq values were beyond the acceptable limits of our laboratory. Due to this, the healthy control samples needed to be removed from the analysis. This made it impossible to validate the findings from the systematic

review (Chapter 3) and identify dysregulated miRNAs as novel biomarkers in T2D and bone health.

7.10 Future work

The miRNAs measured in the population of T1D and T2D as part of this thesis were chosen from a systematic review of previous literature. An alternative methodology would be to use deep sequencing and perform next-generation sequencing in a population of T1D and T2D (with and without fractures) and matched healthy controls to identify dysregulated miRNAs in the context of diabetes and bone health. Next-generation sequencing allows for the detection and quantification of novel miRNAs. It is a highly sensitive and dynamic method gained by the high sequencing depth [352]. The use of next-generation sequencing may change the findings of this thesis by allowing the identification of dysregulated miRNA expression patterns in diabetes and bone health.

Longitudinal changes in bone turnover, aBMD and bone microarchitecture in T1D and neuropathy were assessed after a follow-up period of 4 years and 4 months. We suggest that this study population of T1D and neuropathy needs to be followed for a longer duration of time (~11 years) to investigate if the prolonged duration of T1D and neuropathy lead to further bone loss and a negative impact on bone or if the negative impact on bone occurs early on during disease onset and stabilises with time. The reason for suggesting a follow-up time of 11 years is because Mathiassen et al. have previously reported a 0.5% decrease in bone mineral content in the trabecular bone in insulin-dependent diabetic patients after a follow-up time of ~11 years [89]. To our knowledge, this is the longest longitudinal study in assessing bone health in diabetes known to date.

The reasons for the observed low aBMD, positive bone balance and increased fracture risk in T2D still remains a question. In order to address the above, I believe that assessment of bone microarchitecture using HR-pQCT in T2D may be informative.

All throughout the measurement of miRNAs in this thesis, in particular during the assessment of the capability of the ‘OsteomiR kit’ in predicting fractures, I realised there is very little known in the literature on the variability of miRNAs. Additional research is required to understand the day-to-day and week-to-week inter-variability of miRNAs, sample types miRNA measurements are most stable in, standardisation of miRNA normalisation methods and if feeding and fasting affect miRNA expression. This information is required before miRNAs can be developed as biomarkers for the diagnostics of diabetes and osteoporosis.

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Appendix 1

Table 1 Summary of the direction of expression of the overlapping miRNAs from the eligible studies in both searches.

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
miRNA	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
let-7b	Unique molecular signatures of microRNAs in ocular fluids and plasma in diabetic retinopathy [138]	downregulated	upregulated/ downregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	downregulated
	miRNA signatures of insulin resistance in obesity [183]	upregulated				
	Decreased expression of microRNAs targeting type-2 diabetes susceptibility genes in peripheral blood of patients and predisposed individuals [186]	downregulated				
	Identification of Neuroendocrine Stress Response-Related Circulating MicroRNAs as Biomarkers for Type 2 Diabetes Mellitus and Insulin Resistance [219]	upregulated				
let-7g	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	Noncoding RNA miR-205-5p mediates osteoporosis pathogenesis and osteoblast differentiation by regulating RUNX2 [145]	downregulated	downregulated
	miRNA signatures of insulin resistance in obesity [183]	upregulated				
miR-7	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	downregulated
	Increased serum miR-7 is a promising biomarker for type 2 diabetes mellitus and its microvascular complications [234]	upregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
miRNA	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-16	Plasma-derived exosome characterization reveals a distinct microRNA signature in long duration Type 1 diabetes [140]	downregulated	upregulated/ downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated		MiR-16-5p regulates postmenopausal osteoporosis by directly targeting VEGFA [156]	upregulated	
				Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	
miR-17	Circulating miRNA Profiles in Patients with Metabolic Syndrome [167]	downregulated	downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Serum miR-17 levels are downregulated in obese, African American women with elevated HbA1c [136]	downregulated				
	Diagnostic and Prognostic Role of Serum miR-20b, miR-17-3p, HOTAIR, and MALAT1 in Diabetic Retinopathy [246]	downregulated				
miR-19a	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	MiRNA-19a-3p alleviates the progression of osteoporosis by targeting HDAC4 to promote the osteogenic differentiation of hMSCs [155]	downregulated	downregulated
	miRNA signatures of insulin resistance in obesity [183]	downregulated		Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	
	Four-microRNA signature for detection of type 2 diabetes [216]	upregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
miRNA	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-19b	miRNA signatures of insulin resistance in obesity [183]	downregulated	downregulated	MicroRNA-19b-3p promotes cell proliferation and osteogenic differentiation of BMSCs by interacting with lncRNA H19 [152]	upregulated	upregulated
				Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	
				Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	
miR-20a	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated/ downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Evaluation of Serum microRNAs in Patients with Diabetic Kidney Disease: A Nested Case Controlled Study and Bioinformatics Analysis [236]	downregulated				
miR-21	Molecular and functional characterization of circulating extracellular vesicles from diabetic patients with and without retinopathy and healthy subjects [141]	upregulated	upregulated	Serum Circulating MicroRNAs as Biomarkers of Osteoporotic Fracture [252]	upregulated	upregulated
	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated		Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	
	Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes [239]	upregulated		The Role of Serum Expression Levels of Microrna-21 on Bone Mineral Density in Hypostrogenic Postmenopausal Women with Osteoporosis: Study on Level of RANKL, OPG, TGFβ-1, Sclerostin, RANKL/OPG Ratio, and Physical Activity [256]	upregulated	

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
	Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study [139]	upregulated		Plasma miRNA levels correlate with sensitivity to bone mineral density in postmenopausal osteoporosis patients [259]	downregulated	
	Circulating microRNA-21 is an early predictor of ROS-mediated damage in subjects with high risk of developing diabetes and in drug-naïve T2D [248]	upregulated		Expression of microRNAs that regulate bone turnover in the serum of postmenopausal women with low bone mass and vertebral fractures [264]	downregulated	
				Correlation of plasma microRNA-21 expression and bone turnover markers in postmenopausal women [267]	upregulated	
				Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	
miR-23a	Downregulated-regulation of miR-23a inhibits high glucose-induced EMT and renal fibrogenesis by upregulated-regulation of SnoN [172]	upregulated	upregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	miRNA signatures of insulin resistance in obesity [183]	upregulated		Expression of microRNAs that regulate bone turnover in the serum of postmenopausal women with low bone mass and vertebral fractures [264]	downregulated	
	Serum miR-23a, a potential biomarker for diagnosis of pre-diabetes and type 2 diabetes [203]	downregulated		Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	
	Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment [222]	upregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-23b	miRNA signatures of insulin resistance in obesity [183]	upregulated	upregulated/ downregulated	Serum miRNAs miR-140-3p and miR-23b-3p as potential biomarkers for osteoporosis and osteoporotic fracture in postmenopausal Mexican-Mestizo women [255]	upregulated	upregulated
	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-hyperglycemic patients with type 2 diabetes mellitus [194]	downregulated				
miR-24	Determination of 14 Circulating microRNAs in Swedes and Iraqis with and without Diabetes Mellitus Type 2 [184]	upregulated	upregulated/ downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Decreased expression of microRNAs targeting type-2 diabetes susceptibility genes in peripheral blood of patients and predisposed individuals [186]	downregulated				
	MicroRNAs from urinary extracellular vesicles are non-invasive early biomarkers of diabetic nephropathy in type 2 diabetes patients with the 'Asian Indian phenotype' [197]	downregulated				
	Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes [239]	upregulated				
	Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study [139]	upregulated				
	Blood-based analysis of 84 microRNAs identifies molecules deregulated in individuals with type-2 diabetes, risk factors for the disease or metabolic syndrome [244]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-25	MicroRNA-25 inhibits high glucose-induced apoptosis in renal tubular epithelial cells via PTEN/AKT pathway [232]	downregulated	downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
miR-26a	miRNA signatures of insulin resistance in obesity [183]	upregulated	upregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Elevated Circulating hsa-miR-106b, hsa-miR-26a, and hsa-miR-29b in Type 2 Diabetes Mellitus with Diarrhea-Predominant Irritable Bowel Syndrome [217]	upregulated				
	Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment [222]	upregulated				
miR-27a	Circulating miRNA Profiles in Patients with Metabolic Syndrome [167]	upregulated	upregulated	miR-27a-3p negatively regulates osteogenic differentiation of MC3T3-E1 preosteoblasts by targeting osterix [276]	upregulated	upregulated/ downregulated
	Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes [239]	upregulated		MiR-27a is Essential for the Shift from Osteogenic Differentiation to Adipogenic Differentiation of Mesenchymal Stem Cells in Postmenopausal Osteoporosis [279]	downregulated	
miR-27b	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated/ downregulated	Noncoding RNA miR-205-5p mediates osteoporosis pathogenesis and osteoblast differentiation by regulating RUNX2 [145]	upregulated	upregulated
	MicroRNAs from urinary extracellular vesicles are non-invasive early biomarkers of diabetic nephropathy in type 2 diabetes patients with the 'Asian Indian phenotype' [197]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-29a	miRNA signatures of insulin resistance in obesity [183]	upregulated	upregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study [185]	upregulated		Expression of microRNAs that regulate bone turnover in the serum of postmenopausal women with low bone mass and vertebral fractures [264]	downregulated	
	Identification of Neuroendocrine Stress Response-Related Circulating MicroRNAs as Biomarkers for Type 2 Diabetes Mellitus and Insulin Resistance [219]	upregulated				
	MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus [228]	upregulated				
miR-29b	miRNA signatures of insulin resistance in obesity [183]	upregulated	upregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [264]	downregulated	downregulated
	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-hyperglycemic patients with type 2 diabetes mellitus [194]	upregulated				
	Elevated Circulating hsa-miR-106b, hsa-miR-26a, and hsa-miR-29b in Type 2 Diabetes Mellitus with Diarrhea-Predominant Irritable Bowel Syndrome [217]	upregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-30a	MicroRNAs from urinary extracellular vesicles are non-invasive early biomarkers of diabetic nephropathy in type 2 diabetes patients with the 'Asian Indian phenotype' [197]	downregulated	upregulated/ downregulated	Zhang et al 2020 Characterization of Dysregulated lncRNA-Associated ceRNA Network Reveals Novel lncRNAs With ceRNA Activity as Epigenetic Diagnostic Biomarkers for Osteoporosis Risk	upregulated	upregulated
	Clinical verification of a novel urinary microRNA panal: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis [243]	upregulated		LncRNA XIXT promotes osteogenic differentiation of bone mesenchymal stem cells and alleviates osteoporosis progression by targeting miRNA-30a-5p [151]	upregulated	
miR-30b	Circulating ectosomes: Determination of angiogenic microRNAs in type 2 diabetes [162]	upregulated	upregulated	Identification of suitable reference gene and biomarkers of serum miRNAs for osteoporosis [263]	downregulated	downregulated
	The variant at TGFBRAP1 is significantly associated with type 2 diabetes mellitus and affects diabetes-related miRNA expression [173]	upregulated				
	Molecular and functional characterization of circulating extracellular vesicles from diabetic patients with and without retinopathy and healthy subjects [141]	downregulated				
miR-30e	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	upregulated/ downregulated
	miRNA signatures of insulin resistance in obesity [183]	upregulated		Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	
	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-hyperglycemic patients with type 2 diabetes mellitus [194]	upregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-34a	Diabetes induces the activation of pro-ageing miR-34a in the heart, but has differential effects on cardiomyocytes and cardiac progenitor cells [164]	upregulated	upregulated	Characterization of Dysregulated lncRNA-Associated ceRNA Network Reveals Novel lncRNAs With ceRNA Activity as Epigenetic Diagnostic Biomarkers for Osteoporosis Risk [258]	upregulated	upregulated
	miRNA signatures of insulin resistance in obesity [183]	downregulated				
	Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study [185]	upregulated				
	Decreased expression of microRNAs targeting type-2 diabetes susceptibility genes in peripheral blood of patients and predisposed individuals [186]	downregulated				
	Sequence variation in microRNA-34a is associated with diabetes mellitus susceptibility in a southwest Chinese Han population [212]	upregulated				
	Senescence-associated miR-34a and miR-126 in middle-aged Indians with type 2 diabetes [229]	upregulated				
	Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes [239]	upregulated				
	Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study [139]	upregulated				
miR-93	The variant at TGFBRAP1 is significantly associated with type 2 diabetes mellitus and affects diabetes-related miRNA expression [173]	upregulated	upregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Plasma level of miR-93 is associated with higher risk to develop type 2 diabetic retinopathy [193]	upregulated		Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-96	Serum microRNA profiling and bioinformatics analysis of patients with type 2 diabetes mellitus in a Chinese population [177]	upregulated	upregulated/ downregulated	MiR-96 regulates bone metabolism by targeting osterix [272]	upregulated	upregulated
	Serum miR-23a, a potential biomarker for diagnosis of pre-diabetes and type 2 diabetes [203]	downregulated				
miR-98	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	downregulated	circRNA_0016624 could sponge miR-98 to regulate BMP2 expression in postmenopausal osteoporosis [266]	upregulated	upregulated
	Decreased expression of microRNAs targeting type-2 diabetes susceptibility genes in peripheral blood of patients and predisposed individuals [186]	downregulated				
	Circulatory miR-98-5p levels are deregulated during diabetes and it inhibits proliferation and promotes apoptosis by targeting PPP1R15B in keratinocytes [199]	downregulated				
miR-100	Circulating and visceral adipose miR-100 is downregulated-regulated in patients with obesity and Type 2 diabetes [178]	downregulated	downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
miR-101	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [177]	upregulated	upregulated	MiR-373 promotes the osteogenic differentiation of BMSCs from the estrogen deficiency induced osteoporosis [271]	downregulated	downregulated
	miRNA signatures of insulin resistance in obesity [183]	downregulated				
	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-hyperglycemic patients with type 2 diabetes mellitus [194]	upregulated				
	Identification of circulating miR-101, miR-375 and miR-802 as biomarkers for type 2 diabetes [241]	upregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-103a	Circulating miR-103 family as potential biomarkers for type 2 diabetes through targeting CAV-1 and SFRP4 [187]	upregulated	upregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
miR-106b	Elevated Circulating hsa-miR-106b, hsa-miR-26a, and hsa-miR-29b in Type 2 Diabetes Mellitus with Diarrhea-Predominant Irritable Bowel Syndrome [217]	upregulated	upregulated	Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	upregulated
miR-122	miRNA signatures of insulin resistance in obesity [183]	downregulated	upregulated	Serum Circulating MicroRNAs as Biomarkers of Osteoporotic Fracture [252]	upregulated	downregulated
	Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment [222]	upregulated		Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	
	Evaluation of Serum microRNAs in Patients with Diabetic Kidney Disease: A Nested Case Controlled Study and Bioinformatics Analysis [236]	upregulated		Circulating microRNAs as potential diagnostic biomarkers for osteoporosis [261]	downregulated	
	Whole blood microRNA levels associate with glycemic status and correlate with target mRNAs in pathways important to type 2 diabetes [249]	upregulated				
miR-124	Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study [185]	upregulated	upregulated	Expression of microRNAs that regulate bone turnover in the serum of postmenopausal women with low bone mass and vertebral fractures [264]	upregulated	upregulated
	Roles of microRNA-124a and microRNA-30d in breast cancer patients with type 2 diabetes mellitus [190]	upregulated				
	Decreased Plasma Level of TRIB3 is Associated with Circulating miR-124a in Patients with Type 2 Diabetes [226]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-125b	Circulating and visceral adipose miR-100 is downregulated-regulated in patients with obesity and Type 2 diabetes [178]	downregulated	downregulated	Serum Circulating MicroRNAs as Biomarkers of Osteoporotic Fracture [252]	upregulated	upregulated/ downregulated
	Profiling of Circulating MicroRNAs Reveals Common MicroRNAs Linked to Type 2 Diabetes That Change With Insulin Sensitization [201]	downregulated		Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	
miR-128	Altered circulatory levels of miR-128, BDNF, cortisol and shortened telomeres in patients with type 2 diabetes and depression [198]	upregulated	upregulated	MiR-128 inhibits the osteogenic differentiation in osteoporosis by downregulated-regulating SIRT6 expression [251]	upregulated	upregulated
miR-130b	Profiling of Circulating MicroRNAs Reveals Common MicroRNAs Linked to Type 2 Diabetes That ChangeWith Insulin Sensitization [201]	downregulated	downregulated	Characterization of Dysregulated lncRNA-Associated ceRNA Network Reveals Novel lncRNAs With ceRNA Activity as Epigenetic Diagnostic Biomarkers for Osteoporosis Risk [258]	upregulated	upregulated
	Circulating MiRNAs of 'Asian Indian Phenotype' Identified in Subjects with Impaired Glucose Tolerance and Patients with Type 2 Diabetes [230]	upregulated				
	The changes in miR-130b levels in human serum and the correlation with the severity of diabetic nephropathy [242]	downregulated				
	Potential serum biomarkers for early detection of diabetic nephropathy [245]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-133a	Decreased expression of microRNAs targeting type-2 diabetes susceptibility genes in peripheral blood of patients and predisposed individuals [186]	downregulated	upregulated/ downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	upregulated
	Correlation between miR-103 and miR-133a Expression and the Circulating ANGPTL8 in Type 2 Diabetic Patients and Healthy Control Subjects [240]	upregulated		Plasma miRNA levels correlate with sensitivity to bone mineral density in postmenopausal osteoporosis patients [259]	upregulated	
				MiR-133a in Human Circulating Monocytes: A Potential Biomarker Associated with Postmenopausal Osteoporosis [265]	upregulated	
				MiRNA-133a is involved in the regulation of postmenopausal osteoporosis through promoting osteoclast differentiation [277]	upregulated	
miR-133b	Clinical verification of a novel urinary microRNA panel: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis [243]	upregulated	upregulated	Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	upregulated
miR-135b	MicroRNAs from urinary extracellular vesicles are non-invasive early biomarkers of diabetic nephropathy in type 2 diabetes patients with the 'Asian Indian phenotype' [197]	upregulated	upregulated	Abnormal expression of miR-135b-5p in bone tissue of patients with osteoporosis and its role and mechanism in osteoporosis progression [150]	upregulated	upregulated
miR-140	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	Serum miRNAs as potential biomarkers for osteoporosis and osteoporotic fracture in postmenopausal Mexican-Mestizo women [255]	upregulated	upregulated/ downregulated
	Profiling Circulating MicroRNAs Reveals Common MicroRNAs Linked to T2D That Change With Insulin Sensitization [201]	upregulated		Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	
	Hyperglycaemia suppresses microRNA expression in platelets to increase P2RY12 and SELP levels in type 2 diabetes mellitus [214]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-142	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-patients with T2D [194]	upregulated	upregulated	Identification of suitable reference gene and biomarkers of serum miRNAs for osteoporosis [263]	downregulated	downregulated
	Profiling of Circulating MicroRNAs Reveals Common MicroRNAs Linked to T2D That Change With Insulin Sensitization [201]	upregulated		MiR-373 promotes the osteogenic differentiation of BMSCs from the estrogen deficiency induced osteoporosis [271]	downregulated	
	Identification of Neuroendocrine Stress Response-Related Circulating MicroRNAs as Biomarkers for Type 2 Diabetes Mellitus and Insulin Resistance [219]	downregulated				
	Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment [222]	upregulated				
miR-143	MicroRNA-130a expression is decreased in Xinjiang Uygur patients with type 2 diabetes mellitus [220]	downregulated	downregulated	Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	upregulated
miR-144	Serum microRNA profiling and bioinformatics analysis of patients with type 2 diabetes mellitus in a Chinese population [177]	upregulated	upregulated	miR-144 promotes the proliferation and differentiation of bone mesenchymal stem cells by downregulating the expression of SFRP1 [146]	upregulated	upregulated
	miRNA signatures of insulin resistance in obesity [183]	downregulated				
	Identification of Neuroendocrine Stress Response-Related Circulating MicroRNAs as Biomarkers for Type 2 Diabetes Mellitus and Insulin Resistance [219]	upregulated				
	MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus [228]	upregulated				
	Biomarkers Associated with Ischemic Stroke in Diabetes Mellitus Patients [237]	upregulated				
	Whole blood microRNA levels associate with glycemic status and correlate with target mRNAs in pathways important to type 2 diabetes [249]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-146a	Salivary microRNA 155, 146a/b and 203: A pilot study for potentially non-invasive diagnostic biomarkers of periodontitis and DM [134]	downregulated	downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Impaired miR-146a expression links subclinical inflammation and insulin resistance in Type 2 diabetes [182]	downregulated				
	Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study [185]	upregulated				
	Increased MicroRNA-146a Levels in Plasma of Patients with Newly Diagnosed Type 2 DM [192]	upregulated				
	Serum miR-23a, a potential biomarker for diagnosis of pre-diabetes and type 2 diabetes [203]	downregulated				
	Expression and clinical significance of microRNA 146a in peripheral blood mononuclear cells from type 2 diabetic neuropathy patients [215]	downregulated				
	Study on inflammation-related genes and microRNAs, with special emphasis on the vascular repair factor HGF and miR-574-3p, in monocytes and serum of patients with T2D [218]	downregulated				
	Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment [222]	upregulated				
	MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 DM [228]	downregulated				
	Decreased Serum Level of miR-146a as Sign of Chronic Inflammation in Type 2 DM [353]	downregulated				
	Altered immune metabolism at the interface of increased endoplasmic reticulum (ER) stress in patients with type 2 diabetes [235]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
	Detection of platelet microRNA expression in patients with diabetes mellitus with or without ischemic stroke [238]	downregulated				
	Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes [239]	upregulated				
miR-148a	A unique plasma microRNA profile defines type 2 diabetes progression [174]	upregulated	upregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated				
	Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes [239]	upregulated				
	Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study [139]	upregulated				
	Whole blood microRNA levels associate with glycemic status and correlate with target mRNAs in pathways important to type 2 diabetes [249]	downregulated				
miR-152	MicroRNA-152 regulates insulin secretion and pancreatic β cell proliferation by targeting PI3Kα [354]	downregulated	downregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	upregulated	upregulated
				Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-155	Salivary microRNA 155, 146a/b and 203: A pilot study for potentially non-invasive diagnostic biomarkers of periodontitis and DM [134]	downregulated	downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Expression Level of Circulating Cell Free miR-155 Gene in Serum of Patients with Diabetic Nephropathy [179]	downregulated		Evaluation of circulating miRNA-208a-3p, miRNA-155–5p and miRNA-637 as potential non-invasive biomarkers and the possible mechanistic insights into pre- and postmenopausal osteoporotic females [270]	downregulated	
	miRNA signatures of insulin resistance in obesity [183]	upregulated				
	Regulatory T cells in the pathogenesis of type 2 diabetes mellitus retinopathy by miR-155 [204]	upregulated				
	Circulating microRNA-155 is associated with insulin resistance in chronic hepatitis C patients [209]	downregulated				
	Gene polymorphism and plasma levels of miR-155 in diabetic retinopathy [223]	downregulated				
miR-186	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	downregulated
	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-hyperglycemic patients with type 2 diabetes mellitus [194]	upregulated				
	Serum miR-23a, a potential biomarker for diagnosis of pre-diabetes and type 2 diabetes [203]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-194	MicroRNA-194 Modulates Glucose Metabolism and Its Skeletal Muscle Expression Is Reduced in Diabetes [355]	downregulated	downregulated	Medical examination powers miR-194-5p as a biomarker for postmenopausal osteoporosis [250]	upregulated	upregulated
				Characterization of Dysregulated lncRNA-Associated ceRNA Network Reveals Novel lncRNAs With ceRNA Activity as Epigenetic Diagnostic Biomarkers for Osteoporosis Risk [258]	upregulated	
miR-203a	Salivary microRNA 155, 146a/b and 203: A pilot study for potentially non-invasive diagnostic biomarkers of periodontitis and diabetes mellitus [134]	downregulated	upregulated/ downregulated	MiR-203 is essential for the shift from osteogenic differentiation to adipogenic differentiation of mesenchymal stem cells in postmenopausal osteoporosis [268]	downregulated	downregulated
	Extracellular Vesicle Encapsulated MicroRNAs in Patients with Type 2 Diabetes Are Affected by Metformin Treatment [222]	upregulated		MiR-203 is involved in osteoporosis by regulating DKK1 and inhibiting osteogenic differentiation of MSCs [157]	downregulated	
miR-214	Blood-based analysis of 84 microRNAs identifies molecules deregulated in individuals with type-2 diabetes, risk factors for the disease or metabolic syndrome [244]	downregulated	downregulated	Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	upregulated
miR-217	Changes of serum Mir-217 and the correlation with the severity in type 2 diabetes patients with different stages of diabetic kidney disease [191]	upregulated	upregulated	Circ-VANGL1 promotes the progression of osteoporosis by absorbing miRNA-217 to regulate RUNX2 expression [144]	upregulated	upregulated
miR-221	Serum microRNA-221 as a biomarker for diabetic retinopathy in patients associated with type 2 diabetes [221]	upregulated	upregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-222	A unique plasma microRNA profile defines T2D s progression [174]	downregulated	upregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-hyperglycemic patients with type 2 diabetes mellitus [194]	upregulated				
	Profiling of Circulating MicroRNAs Reveals Common MicroRNAs Linked to T2D That Change With Insulin Sensitization [201]	upregulated				
miR-223	Co-expression analysis among microRNAs, long non-coding RNAs, and messenger RNAs to understand the pathogenesis and progression of diabetic kidney disease at the genetic level [137]	downregulated	downregulated	Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort [253]	downregulated	downregulated
	A novel transthyretin/STAT4/miR-223-3p/FBXW7 signaling pathway affects neovascularization in diabetic retinopathy [207]	upregulated				
	Hyperglycaemia suppresses microRNA expression in platelets to increase P2RY12 and SELP levels in type 2 diabetes mellitus [214]	downregulated				
	Four-microRNA signature for detection of type 2 diabetes [216]	upregulated				
	Biomarkers Associated with Ischemic Stroke in Diabetes Mellitus Patients [237]	downregulated				
	Detection of platelet microRNA expression in patients with diabetes mellitus with or without ischemic stroke [238]	downregulated				
	Altered levels of circulating cytokines and microRNAs in lean and obese individuals with prediabetes and type 2 diabetes [239]	upregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-320a	Circulating miRNA Profiles in Patients with Metabolic Syndrome [167]	upregulated	upregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	upregulated	upregulated
	The variant at TGFBRAP1 is significantly associated with type 2 diabetes mellitus and affects diabetes-related miRNA expression [173]	upregulated				
	Determination of 14 Circulating microRNAs in Swedes and Iraqis with and without Diabetes Mellitus Type 2 [184]	upregulated				
	Effect of diabetes on exosomal miRNA profile in patients with obesity [135]	upregulated				
miR-320b	Differential expression of microRNAs in plasma of patients with prediabetes and newly diagnosed type 2 diabetes [170]	downregulated	upregulated/ downregulated	Noncoding RNA miR-205-5p mediates osteoporosis pathogenesis and osteoblast differentiation by regulating RUNX2 [145]	upregulated	upregulated
	Effect of diabetes on exosomal miRNA profile in patients with obesity [135]	upregulated				
miR-324	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	downregulated	downregulated
miR-328	The variant at TGFBRAP1 is significantly associated with type 2 diabetes mellitus and affects diabetes-related miRNA expression [173]	upregulated	upregulated	Identification of suitable reference gene and biomarkers of serum miRNAs for osteoporosis [263]	downregulated	downregulated
miR-335	The variant at TGFBRAP1 is significantly associated with type 2 diabetes mellitus and affects diabetes-related miRNA expression [173]	upregulated	upregulated	Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures [275]	upregulated	upregulated
	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated		Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-338	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	Circulating miR-338 Cluster activities on osteoblast differentiation: Potential Diagnostic and Therapeutic Targets for Postmenopausal Osteoporosis [273]	upregulated	upregulated
	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-patients with type 2 diabetes mellitus [194]	upregulated				
miR-340	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated	upregulated	Characterization of Dysregulated lncRNA-Associated ceRNA Network Reveals Novel lncRNAs With ceRNA Activity as Epigenetic Diagnostic Biomarkers for Osteoporosis Risk [258]	upregulated	upregulated
miR-342	A unique plasma microRNA profile defines T2D progression [174]	downregulated	upregulated	miR-342-3p promotes osteogenic differentiation via targeting ATF3 [356]	downregulated	downregulated
	T2DM inhibition of endothelial miR-342-3p facilitates angiogenic dysfunction via repression of FGF11 signaling [180]	downregulated				
	MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus [142]	upregulated				
	microRNA-342, microRNA-191 and microRNA-510 are differentially expressed in T regulatory cells of type 1 diabetic patients [143]	downregulated				
	Assessment of DNA damage and mRNA/miRNA transcriptional expression profiles in hyperglycemic versus non-hyperglycemic patients with type 2 diabetes mellitus [194]	upregulated				
	MicroRNAs 342 and 450 together with NOX-4 activity and their associ ation with coronary artery disease in diabetes [231]	upregulated				
	Clinical verification of a novel urinary microRNA panal: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis [243]	upregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-375	Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study [185]	upregulated	upregulated	Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	upregulated
	Expression and DNA methylation status of microRNA-375 in patients with type 2 diabetes mellitus [195]	upregulated				
	Apelin-13 serum levels in type 2 diabetic obese women: possible relations with microRNAs-107 and 375 [210]	upregulated				
	DNA methylation of microRNA-375 in impaired glucose tolerance [211]	upregulated				
	Identification of circulating miR-101, miR-375 and miR-802 as biomarkers for type 2 diabetes [241]	upregulated				
	Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study [139]	upregulated				
miR-486	Change in circulating microRNA profile of obese children indicates future risk of adult diabetes [171]	upregulated	upregulated/ downregulated	Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures [278]	upregulated	upregulated
	Serum miR-23a, a potential biomarker for diagnosis of pre-diabetes and type 2 diabetes [203]	downregulated				
	Sex Differences in Plasma MicroRNA Biomarkers of Early and Complicated Diabetes Mellitus in Israeli Arab and Jewish Patients [213]	upregulated				
	Evaluation of Serum microRNAs in Patients with Diabetic Kidney Disease: A Nested Case Controlled Study and Bioinformatics Analysis [236]	downregulated				

	Studies of search part 1 containing the overlapping miRNAs			Studies of search part 2 containing the overlapping miRNAs		
	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)	Title and reference	Direction of expression (compared to the control group)	Direction of expression (majority)
miR-574	Plasma-derived exosome characterization reveals a distinct microRNA signature in long duration Type 1 diabetes [140]	downregulated	downregulated	Characterization of Dysregulated lncRNA-Associated ceRNA Network Reveals Novel lncRNAs With ceRNA Activity as Epigenetic Diagnostic Biomarkers for Osteoporosis Risk [258]	downregulated	downregulated
	The variant at TGFBRAP1 is significantly associated with type 2 diabetes mellitus and affects diabetes-related miRNA expression [173]	upregulated				
	Study on inflammation-related genes and microRNAs, with special emphasis on the vascular repair factor HGF and miR-574-3p, in monocytes and serum of patients with T2D [218]	downregulated				
miR-589	Whole blood microRNA levels associate with glycemic status and correlate with target mRNAs in pathways important to type 2 diabetes [249]	upregulated	upregulated	Characterization of Dysregulated lncRNA-Associated ceRNA Network Reveals Novel lncRNAs With ceRNA Activity as Epigenetic Diagnostic Biomarkers for Osteoporosis Risk [258]	upregulated	upregulated