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Skeletal Morbidity and Its Determinants in Type 1 Diabetes

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MBChB, MRCPCH

Submitted in fulfilment of the requirements for the degree of Doctor of Medicine

University of Glasgow
College of Medical, Veterinary and Life Sciences
March 2019
Abstract

Adults, and more recently, children with T1D have been found to have a significantly increased risk of fractures compared to the general population. The increased fracture risk is disproportioned to the marginal reduction in bone mineral density (BMD) observed in T1D suggesting that factors other than bone mineral status contribute to skeletal fragility. The National Institute of Health (NIH) Consensus Development Panel on Osteoporosis highlighted that bone strength is in fact dependent on two main factors: bone density and bone quality. Recognising the importance of bone quality as a factor determining bone health, this work set out to examine bone microarchitecture using novel imaging techniques of high-resolution magnetic resonance imaging (HR-MRI). Furthermore, HR-MRI enables the use of magnetic resonance spectroscopy (MRS) to quantify the amount of bone marrow fat, providing invaluable insight into the relationship between bone marrow adiposity and skeletal fragility. The background to this work, the current body of evidence and the rationale for the studies are therefore laid out in Chapter 1. All the methodology used in the thesis is summarised in Chapter 2, including the laboratory techniques carried out.

The clinical study was conducted in children with T1D as not only have these children been shown to have increased fracture risk, but also given that childhood and adolescence are important physiological periods for optimal bone development, it is therefore possible that they may be especially predisposed to abnormalities of bone health. The use of MRI as a research tool in children is relatively new, so I started this work exploring the practicality of this technique in children and using data from a previous HR-MRI study to determine the feasibility of partial set analysis of the images, the latter detailed in Chapter 3. This straightforward exercise confirmed that partial MRI data sets can reliably represent a larger complete set of images when assessing trabecular bone microarchitecture parameters.

The overall objective of this thesis is to assess the bone health of children with Type 1 diabetes, by using HR-MRI to study the trabecular bone microarchitecture, in addition to bone mineral density and bone turnover status as detailed in Chapter 4. A cross-sectional case control study was conducted in 32 children with T1D and compared to 26 healthy age- and gender-matched controls. The primary hypothesis of the study was proven, in that children with T1D were found to have poorer bone microarchitecture with lower trabecular bone volume compared to the controls. Children with T1D also had lower number of trabeculae and the trabeculae were spaced further apart from one another. Although this study demonstrated that children with T1D fracture more than children without the
condition, it did not however show any relationship between the compromised bone microarchitecture to fracture. In fact, the children with T1D who fractured were found to have significantly lower bone mineral density and poorer glycaemic control. There was also no significant disparity in the bone marrow adiposity between the two groups.

In parallel, I performed one year of laboratory-based experiments to study the differentiation of mesenchymal stem cells (MSCs), which are the precursors to bone (osteoblasts) and fat cells (adipocytes), as detailed in Chapter 5. My main aim was to study the effects of metformin, a drug increasingly used in patients with T1D, on the MSC differentiation in order to determine if metformin may have a therapeutic bone protective role. My studies successfully demonstrated in vitro that metformin exerted reciprocal control over the osteogenic transcription factor Runx2, and the adipogenic transcription factor, PPARγ; promoting osteogenesis through an increase in Runx2 transcriptional activities, independently of Runx2 protein expression, and suppressing adipogenesis, through suppression of PPARγ protein expression and activity. I proceeded to study the underlying molecular mechanisms of the metformin action, starting with AMP-activated protein kinase (AMPK) given that metformin is a known AMPK activator. To our fascination, the study found that the suppression of adipogenesis by metformin appeared to be independent of AMPK activation but rather through the suppression of the mTOR/p70S6K signalling pathway.

Chapter 6 summarised all my work for this thesis, highlighting its strengths and limitations as well as providing my perspectives into the future directions of this work.
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Scientific Outputs Arisen from this Thesis

Publications (Attached in Appendices)


Abstracts


Chen SC, Brooks R, Ahmed SF, Yarwood SJ. Metformin regulates the differentiation of murine mesenchymal stem cells via AMPK-independent suppression of p70s6-kinase. *1st European Bone Marrow Adiposity Meeting, Lille, France. Aug 2015. Oral*


Chen SC, Brooks R, Ahmed SF, Yarwood SJ. Metformin regulates the differentiation of murine mesenchymal stem cells via AMPK-independent suppression of p70s6-kinase. *43rd Meeting of the British Society for Paediatric Endocrinology and Diabetes (BSPED), Sheffield, Nov 2014. Endocrine Abstracts 39 Poster EP16*

Chen SC, Brooks R, Ahmed SF, Yarwood SJ. The Role of AMPK Pathway in Mediating the Effects of Metformin on Mesenchymal Stem Cell Differentiation. *42nd Meeting of the British Society for Paediatric Endocrinology and Diabetes (BSPED), Winchester, Nov 2014. Endocrine Abstracts 36 Oral OC6.4*

Achievement and Awards for this Work

**Shortlisted for Nick Hales Young Investigator Award** – Metformin regulates the differentiation of murine mesenchymal stem cells via AMPK-independent suppression of p70s6-kinase. *Diabetes UK Professional Conference, 2016*

**Best Abstract Award** - The Role of AMPK Pathway in Mediating the Effects of Metformin on Mesenchymal Stem Cell Differentiation. *42nd Meeting of the British Society for Paediatric Endocrinology and Diabetes, 2014*

**Best Oral Presentation** – The Role of AMPK in Mediating the Effects of Metformin on Mesenchymal Stem Cell Differentiation. *Yorkhill Research Day, 2014*

**Clinical Research Fellowship Award (£65,000) for the research proposal** – *Glasgow Children’s Hospital Charity, 2013*
Dedicated to my husband, Douglas, and my daughter, Annabel, for their love, understanding and support; as well as my parents, who have always believed in me.
Acknowledgement

This thesis would not have come to fruition without the support and encouragement from my principal supervisor Professor Faisal Ahmed, whose foresight made this work possible and attention to detail provided my drive to excel.

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Finally, I could not thank enough my husband, family and friends, for spurring me on and their moral support to the very end. A special thanks to my cousin, Hooi Ching, for providing in-house help and endless hours of entertainment for my little one to facilitate the writing of this thesis.
Author’s Declaration

I declare, except where reference is made to the contribution of others that all work presented in this thesis was performed entirely by myself and has not been submitted for any other degree at the University of Glasgow, or any other institution.

Dr Suet Ching Chen

I certify that the work reported in this thesis has been performed by Dr Suet Ching Chen and that during the period of study, she has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Medicine, University of Glasgow.

Prof S Faisal Ahmed
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aBMD</td>
<td>areal bone mineral density</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl co-A carboxylase</td>
</tr>
<tr>
<td>AGD</td>
<td>ascorbic acid, β-glycerolphosphate, dexamethasone</td>
</tr>
<tr>
<td>AGEs</td>
<td>advanced glycation end-products</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide riboside</td>
</tr>
<tr>
<td>ALS</td>
<td>acid labile subunits</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APN</td>
<td>adiponectin</td>
</tr>
<tr>
<td>appBV/TV</td>
<td>apparent bone volume to total volume ratio</td>
</tr>
<tr>
<td>appTbN</td>
<td>apparent trabecular number</td>
</tr>
<tr>
<td>appTbSp</td>
<td>apparent trabecular spacing</td>
</tr>
<tr>
<td>appThTh</td>
<td>apparent trabecular thickness</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BA</td>
<td>bone area</td>
</tr>
<tr>
<td>BAP</td>
<td>bone alkaline phosphatase</td>
</tr>
<tr>
<td>BMA</td>
<td>bone marrow adiposity</td>
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<tr>
<td>BMAD</td>
<td>bone mineral apparent density</td>
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<tr>
<td>BMC</td>
<td>bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
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<tr>
<td>BMF</td>
<td>bone marrow fat</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BMMSCCc</td>
<td>bone-marrow-derived mesenchymal stem cells</td>
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<tr>
<td>BMPs</td>
<td>bone morphogenic proteins</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer binding protein</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CSA</td>
<td>cross sectional area</td>
</tr>
<tr>
<td>CSII</td>
<td>continuous subcutaneous insulin infusion</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CTX</td>
<td>cross-linked C-terminal telopeptide of Type 1 collagen</td>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DKA</td>
<td>diabetic ketoacidosis</td>
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<tr>
<td>DKK-1</td>
<td>dipkoff-1</td>
</tr>
<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPP-4</td>
<td>dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Dpyr</td>
<td>deoxypyridoline</td>
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<td>DXA</td>
<td>dual energy X-ray absorptiometry</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>FEA</td>
<td>finite element analysis</td>
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<tr>
<td>FF</td>
<td>fat fraction</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FN</td>
<td>femoral neck</td>
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<td>FOV</td>
<td>field of view</td>
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<td>FRAX</td>
<td>fracture risk assessment tool</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>GLP-1</td>
<td>glycogen-like peptide 1</td>
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<tr>
<td>HbA1c</td>
<td>haemoglobin A1c</td>
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<tr>
<td>hMSC</td>
<td>human mesenchymal stem cell</td>
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<tr>
<td>HPro</td>
<td>hydroxypyroline</td>
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<tr>
<td>HR-MRI</td>
<td>high resolution magnetic resonance imaging</td>
</tr>
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<td>HR-pQCT</td>
<td>high resolution peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IGFBP</td>
<td>insulin-like growth factor-binding protein</td>
</tr>
<tr>
<td>IID</td>
<td>insulin, IBMX, dexamethasone</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>IM</td>
<td>images</td>
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<td>IR</td>
<td>insulin receptor</td>
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<tr>
<td>KO</td>
<td>knock out</td>
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<tr>
<td>LBM</td>
<td>lean body mass</td>
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<tr>
<td>LS</td>
<td>lumbar spine</td>
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<td>LWR</td>
<td>lipid to water ratio</td>
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<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
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<tr>
<td>MDI</td>
<td>multiple daily (insulin) injections</td>
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<tr>
<td>MIL</td>
<td>mean intercept length</td>
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<tr>
<td>miRNA</td>
<td>micro-ribonucleic acid</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>MRS</td>
<td>magnetic resonance spectroscopy</td>
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<tr>
<td>MSCs</td>
<td>mesenchymal stem cells</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>MVD</td>
<td>microvascular diseases</td>
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<tr>
<td>NMV</td>
<td>net magnetisation vector</td>
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<tr>
<td>OC</td>
<td>osteocalcin</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------------------------------------------</td>
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<tr>
<td>OI</td>
<td>osteogenesis imperfecta</td>
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<td>OPG</td>
<td>osteoprotegerin</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<tr>
<td>ORO</td>
<td>oil red O</td>
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<tr>
<td>PHV</td>
<td>peak height velocity</td>
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<td>PIO</td>
<td>pioglitazone</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>pQCT</td>
<td>peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>PREF-1</td>
<td>pre-adipocyte factor-1</td>
</tr>
<tr>
<td>PRESS</td>
<td>point-resolved spectroscopy sequence</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>Pyr</td>
<td>pyridoline</td>
</tr>
<tr>
<td>QCT</td>
<td>quantitative computed tomography</td>
</tr>
<tr>
<td>QUS</td>
<td>quantitative ultrasound</td>
</tr>
<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor κβ</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor κβ ligand</td>
</tr>
<tr>
<td>RCT</td>
<td>randomised controlled trial</td>
</tr>
<tr>
<td>RF</td>
<td>radiofrequency</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RR</td>
<td>relative risk</td>
</tr>
<tr>
<td>Runx</td>
<td>Runt-related proteins</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SDS</td>
<td>standard deviation score</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SGLT-2</td>
<td>sodium/glucose con-transporter 2</td>
</tr>
<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozocin</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TB</td>
<td>total body</td>
</tr>
<tr>
<td>TBLH</td>
<td>total body less head</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with Tween20</td>
</tr>
<tr>
<td>TE</td>
<td>echo time</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factors-β</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factors-α</td>
</tr>
<tr>
<td>TR</td>
<td>repetition time</td>
</tr>
<tr>
<td>TrueFISP</td>
<td>true fast imaging with steady state precession</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>TZDs</td>
<td>thiazolidinediones</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>vBMD</td>
<td>volumetric BMD</td>
</tr>
<tr>
<td>VF</td>
<td>vertebral fracture</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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1 Introduction

1.1 Background to the thesis

T1D affects 300,000 people in the UK, of which 35,000 are children. The incidence of T1D is 1:4000, with peak age at diagnosis between 9-14 years old. In addition to the many target organs that are typically associated with dysfunction in diabetes mellitus, the skeleton has now been recognised as another organ adversely affected in diabetes mellitus. The effects on bone health manifest clinically as early osteoporosis and fragility fractures, with increased incidence of hip fractures particularly in patients with T1D compared to age-matched non-diabetics (Vestergaard 2007, Hothersall et al. 2014). In addition, this increased risk is also evident in young adults (Hothersall et al. 2014). Given that childhood and adolescence are important physiological periods for optimal bone development, it is possible that children and adolescents with T1D may be especially predisposed to abnormalities of bone health.

In adults, patients with T1D have a modest 10% lower BMD than the general population (Vestergaard 2007) which generates an estimated relative hip fracture risk of 1.4-fold (Table 1.1) but instead, a meta-analysis of several large cohort studies demonstrated a disproportionately higher risk of hip fractures by 7-folds (Figure 1.1) (Janghorbani et al. 2007). Such disparities between dual x-ray absorptiometry (DXA)-derived BMD and actual fracture risk in T1D suggest that factors other than BMD need to be considered more comprehensively. This is acknowledged by the National Institute of Health (NIH) Consensus Development Panel on Osteoporosis which recognises the importance of bone microarchitecture as a factor determining bone health (NIH Consensus Development Conference Panel 1993) High resolution MRI provides the necessary information of cortical and trabecular microarchitecture, analogous to high resolution computed-tomography (CT) but without the radiation (Seeman and Delmas 2006, Wehrli 2007). In addition, spectroscopic imaging in MRI also enables evaluation of bone marrow adiposity (BMA) (Wehrli et al. 2000), an issue which is gaining increasing attention in the field of osteoporosis because of the connection between adipogenesis and osteogenesis (Fazeli et al. 2013).

<table>
<thead>
<tr>
<th>Type of diabetes</th>
<th>Spine Z-score</th>
<th>Hip z-score</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1D</td>
<td>-0.22 ± 0.01</td>
<td>-0.37± 0.16</td>
</tr>
<tr>
<td>T2D</td>
<td>0.41± 0.01</td>
<td>0.27± 0.01</td>
</tr>
</tbody>
</table>

Table 1.1 Observed changes in BMD and expected change in fracture risk
Figure 1.1 Meta-analysis of the association between T1D and risk of hip fracture. Results from 6 studies showed that adults with T1D have an almost 7-fold increase in relative risk of hip fracture compared to that of the general population. This is disproportionately high for the expected risk of fracture predicted from the marginal reduction in bone mineral density (see Table 1.1). [Adapted from (Janghorbani et al. 2007)]

NHS Greater Glasgow and Clyde Paediatric Diabetes Service is one of the largest paediatric diabetes centre in the UK looking after over 750 children with Type 1 diabetes. In 2015 the Royal Hospital for Children in Glasgow moved to one of the largest biomedical campuses in Europe with a dedicated £10million clinical research facility and state-of-the-art MRI imaging facilities. This provided me an ideal opportunity to carry out a comprehensive multi-modality assessment looking at the bone health of children with Type 1 diabetes, without radiation exposure.

Evidence suggests that diabetic bone loss involves a complex interplay of chronic hyperglycaemia, insulin deficiency, increased marrow adiposity, inflammation and vasculopathy, although the exact underlying pathogenesis remains unclear. In vitro and in vivo studies in T1D point towards a defect in bone formation (Rico et al. 1989, Verhaeghe et al. 1990, Pater et al. 2010), in contrast to bone resorption which is the underlying mechanism of age-related osteoporosis. Importantly, this highlights therapeutic implications for treating diabetic bone loss with bisphosphonates, which is current practice for age-related osteoporosis and fragility fracture, as it will not be mechanistically sound. The anti-diabetic drug metformin, however, may have a bone protective effect as reduction in fracture risks has been noted in Type 2 diabetes mellitus (T2D) patients on this medication (Vestergaard et al. 2005, Zinman et al. 2010). Metformin is also increasingly used in T1D to improve insulin sensitivity and glycaemic control (Vella et al. 2010), so it would be advantageous to
ascertain the underlying mechanisms by which metformin protects bones. This led me to a one-year laboratory research at the Institute of Molecular, Cell and Systems Biology, University of Glasgow, studying the effects of metformin on mesenchymal stem cell differentiation into bone (osteoblasts) and fat cells (adipocytes).

I hope that this work will contribute to a better understanding of the extent of skeletal morbidity in children with Type 1 diabetes and its relationship to the disease. I further hope that it will not only elucidate the causes, but also provide evidence for measures to protect bone health in this cohort of children from an early age. Based on my laboratory research, I hope to establish the bone protective mechanisms of metformin as a therapeutic potential for use in diabetic osteopathy.
1.2 Bone development and its components

Bone is a mineralised connective tissue with an impressive hierarchical organisation from a macroscopic to microscopic level. This confers it a combination of stiffness and toughness allowing it to perform its multifunctional role from locomotion, support and protection of vital organs such as heart, lungs and brain, to mineral homeostasis and haematopoiesis. More recent discoveries revealed that bone also has an endocrine function, in particular through osteocalcin and its role in glucose metabolism. Circulating undercarboxylated osteocalcin, itself regulated by insulin acts on pancreatic β-cells to further increase β-cell mass and subsequent insulin secretion in a feed-forward loop (Ducy et al. 1996, Lee et al. 2007, Ferron et al. 2010).

1.2.1 Bone architecture

On a macroscopic level, bone is made up of two main entities: cortical and trabecular bone, giving rise to four distinct surfaces called skeletal envelopes which are morphologically distinct with different roles: periosteal, endocortical, trabecular and intracortical envelopes (Figure 1.2).

![Figure 1.2 Bone structure.](image)

(A) Macroscopically, bone appears as either lace-like trabecular bone or denser cortical bone. These create four different kinds of surfaces, called envelopes, upon which bone cells act. (B) Trabeculae in the cancellous bone compartment consist mostly of primary lamellae. However remodelled areas (areas in which bone has been resorbed and reformed) can also form hemiosteons, similar to half osteons. (C) The intracortical envelope in humans is packed with secondary osteons, or Haversian systems. [Adapted from (Burr and Akkus 2014)].
The adult skeleton is composed of 80% cortical bone and 20% trabecular bone (Eriksen et al. 1994). The ratio of cortical to trabecular bone varies in different bones and skeletal sites. Whilst the radial diaphysis is 95:5 cortical to trabecular bone, the vertebra is only 25:75 with trabecular predominance. Within the long bone, the hollow shaft, or diaphysis, is composed primarily of cortical bone whereas the metaphysis and epiphyses are composed of mainly trabecular bone with only a thin shell of cortical bone. Both cortical and trabecular bones are composed of osteons.

### 1.2.1.1 Cortical bone

Cortical bone is the dense and solid thick outer shell of bones, also known as the “compact” bone. Although it constitutes 80% of the total bone mass, cortical bone has a substantially smaller surface area than trabecular bone (Weatherholt et al. 2012). Osteons are the basic units in the cortical bone orientated along the long axis of the bone with a central Haversian canal, together forming the Haversian system. They are cylindrical in shape with circumferential bands of bone where collagen and mineral exist in discrete sheets (known as concentric lamellae) in the walls of the Haversian system, as shown in Figure 1.3. The collagen fibres of the concentric lamellae are orientated in alternating directions to give strength to the tissue.

**Figure 1.3 The hierarchical organisation of bone on a microscopic level.** (A) Schematic diagram of a bone microstructure. (B) Cortical bone osteon or Haversian system with a central Haversian canal, with concentric lamellae and uniformly spaced lacunae. (C) Cross section of a cortical bone osteoid under light microscopy. (D) Trabeculae shown in cross section with lamellae of bone, lacunae and outer covering of endosteum. [Adapted from (Weatherholt et al. 2012)]
There are many interconnected canals (lacunae and canaliculi) through which bone cells communicate and receive vascular supply. These canals give rise to the low 3-5% of porosity in cortical bone (Burr and Akkus 2014). Increased cortical remodeling causes an increase in cortical porosity and decrease in cortical bone mass (reduction in cortical volumetric bone mineral density (vBMD)) (Clarke 2008).

Cortical bone has an outer periosteal surface and an inner endosteal surface. On the periosteal surface, bone formation exceeds resorption, hence bone increases in diameter (appositional growth) with aging. On the endosteal surface, the opposite is true with bone resorption typically exceeding formation, so the marrow space expands with aging. Higher remodelling activity takes place on the endosteal surface, with greater biomechanical strain and greater inflammatory exposure from the adjacent marrow (Clarke 2008). Trabecular bone loss begins first, in early adulthood for both sexes, before cortical bone which begins during middle age for women, though much later in men (after age 75 years) (Riggs et al. 2008). By age 50 years, approximately 40% of total lifetime trabecular loss has occurred, in contrast to only 15% of cortical bone lost (Riggs et al. 2008).

1.2.1.2 Trabecular bone

Trabecular bone, also known as the “spongy” bone, or cancellous bone, is the honeycomb-like network of plate and rod-like structures found predominantly in the metaphyses of long bones, vertebrae and ribs (Figure 1.4A). Trabecular bone constitutes 20% of the total bone mass in the skeleton. Unlike the cortical osteons, the trabecular osteons are hemiosteons without central Harversian canal due to bone remodelling on the longer surface of the trabeculae. The lamellae are arranged parallel to this longer trabecular surface (Burr and Akkus 2014). Trabecular bone has 50-90% higher porosity relative to the cortical bone (Weatherholt et al. 2012).

The porosity helps trabecular bone to be light yet add strength to the skeleton by providing a honeycomb scaffolding to reinforce the ends of long bones, close to the joints, which are often subjected to mechanical stresses from the sides. The trabecular bones help to channel the mechanical stresses imposed on it to the stronger, and more solid cortical bones. In the vertebrae, it helps to cushion axial loading forces on the skeleton.
In healthy humans, the trabeculae tend to be shaped as plates rather than circular or elliptical rods. The architecture of the trabecular bone can be characterised by the number of trabeculae (TbN), how thick they are (TbTh), and how far apart they are (TbSp) all contributing to the overall trabecular bone volume (BV). TbTh ranges between 50-400µm, averaging about 200µm in adults (Eriksen et al. 1994) and 150µm in children up to 16 years (Glorieux et al. 2000), with an estimated total trabecular area of 7m² (Clarke 2008).

The ideal relationship between TbN and TbTh is not only dependent on the site, but also the primary direction of the loading forces (Burr and Akkus 2014). Most importantly, TbN have a bigger impact on the strength of the bone than TbTh, with the loss of a complete trabeculae (reduced TbN) weakening the bone by 2-3 folds more compared to losing the same amount through thinning of the trabeculae (reduced TbTh) (Figure 1.4B). This highlights the importance of connectivity within the trabecular architecture. The loss of connectivity can also occur when trabecular bone plates become more rod-like, as with the case in osteoporosis. Trabecular bone is also preferentially lost; earlier and at a greater rate than cortical bone during aging resulting in skeletal fragility at trabecular rich sites such as distal radius (Riggs et al. 2008) and femoral head (Li and Aspden 1997, Ciarelli et al. 2000).
1.2.2 Bone cells and matrix

Bone is a living tissue made up of minerals, organic matrix, water and lipids, as seen in Figure 1.5. The organic matrix is 90% Type I collagen with a trace amount of Type III and V collagen, providing bone its elasticity and flexibility (Clarke 2008). The collagen fibres are made up of triple helices of three polypeptide chains, connected by different kinds of cross-links, which can alter the material properties of the tissue, and ultimately the mechanical behaviour of the whole bone. These cross links, such as pyridinoline and deoxypyridinoline, can be formed through enzymatic processes, or through non-enzymatic glycation, creating advanced glycation end products (AGEs) such as pentosidine.

![Bone Composition Diagram]

**Figure 1.5 Bone composition.** Bone is comprised predominantly of calcium hydroxyapatite mineral, with Type I collagen making up most of the organic matrix.

The inorganic matrix is predominantly calcium hydroxyapatite which is interspersed with the collagen fibres conferring bone the mechanical rigidity and load-bearing strength to bone. Human bones are 60% mineralised (Seeman and Delmas 2006). The balance of distribution between collagen and mineral is crucial to ensure bone is stiff enough to provide mechanical support yet has the flexibility to absorb and redistribute loading stresses. Alteration in this balance with aging and with diseases can therefore compromise the structural integrity of bone resulting in increased susceptibility to fracture. In diabetes, especially when free sugar is in abundance due to poor glycaemic control, AGEs accumulate in the extracellular matrix (Yamamoto *et al.* 2001). The increased cross linking increases the stiffness of the bone ultimately making it too brittle resulting in fragility fracture (Vashisht *et al.* 2001).
There are three distinct types of cells found within bone: i) the **Osteoblasts** (3-4% of the cells in bone) which synthesise and regulate the deposition and mineralisation of the extracellular matrix of bone. These cells are derived from pluripotent mesenchymal stem cells, as described in detail in section 1.3, and have a lifespan of up to 8 weeks in humans, during which time they lay down osteoid (including proteoglycans, glycoproteins and collagen fibres) before becoming embedded in their own calcified matrix and developing morphologically into an osteocyte. ii) **Osteocytes**, are the most abundant bone cells (90-95%) and the longest lived with a life span of up to 25 years (Capulli et al. 2014). Although derived from osteoblasts, they are distinctively different in function. These spider-shaped cells have long and branched cellular processes that extend throughout bone aiding cell to cell communication and intracellular transport of small signalling molecules. iii) **Osteoclasts** are derived from haematopoetic stem cells, although their formation (osteoclastogenesis) remains dependent on osteoblasts and osteoblast precursors. The osteoblasts secrete receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (CSF) to recruit and activate the differentiation of osteoclast precursors into osteoclasts (Figure 1.6). Osteoprotegerin (OPG), also produced by osteoblasts, is a competitive inhibitor of RANKL binding to RANK receptor so the ratio of RANKL:OPG determines osteoclastogenesis. Functionally, osteoclasts are the only cells capable of resorbing mineralised bone.

**Figure 1.6 Regulation of Osteoclastogenesis by RANKL and OPG.** Osteoclasts are derived from haematopoetic stem cells although their formation remains dependent on osteoblasts. Osteoblasts and osteoblast precursors produce two forms of RANKL which stimulate osteoclast recruitment and activation. The membrane-bound form directly interacts with membrane-bound RANK molecules on adjacent osteoclast precursors. The soluble form diffuses through the intercellular space and interacts with the membrane-bound RANK molecules on nearby osteoclast precursors. Osteoprotegerin (OPG) acts as a decoy receptor (inhibitor) to prevent RANKL or sRANKL from interacting with RANK. The ratio between RANKL and OPG produced determines osteoclastogenesis. [Adapted from (Clarke 2008)]
1.2.3 Bone growth, modelling and remodelling

Bone is a highly dynamic and metabolically active tissue, which undergoes constant growth, modelling and remodelling. Longitudinal growth occurs at the growth plates during childhood and adolescence to allow for growth and attainment of peak bone mass. Cartilages proliferate in the epiphyseal and metaphyseal areas of long bones, before undergoing mineralisation to form primary new bone. Modelling is the process by which bones change their overall shape to accommodate for skeletal loading and radial growth, which continues throughout life although less frequent in adulthood. During bone modelling, bone formation and resorption are not tightly coupled, in that osteoblasts and osteoclasts act independently in response to biomechanical forces. Bones normally widen with age when bone formation at the periosteal envelope exceeds bone resorption at the endosteal surface (Clarke 2008).

Bone remodelling is the process whereby old bone is replaced by newly formed bone, which allows self-repair in both childhood and adulthood. More acutely, remodelling also helps to maintain calcium homeostasis. The process of remodelling occurs at all four skeletal envelopes: endocortical, intracortical and trabecular, with a lesser extent on the periosteal envelope. It involves four sequential phases: activation, resorption, reversal and formation, as shown in Figure 1.7. Osteocytes are thought to sense bone deformation or microdamage (Han et al. 2004), initiating the need for remodelling during which bone resorption by osteoclasts precedes bone formation by osteoblasts. The osteocytes, through their long cytoplasmic processes, relay the information to the osteoblast lining-cells and osteoblast precursors, which in turn activates osteoclastogenesis (as described in Section 1.2.2) and is believed to orchestrate the bone remodelling process (Verborgt et al. 2000, Parfitt 2002). The mononuclear monocyte-macrophage osteoclast precursors recruited from the circulation lift off the bone-lining cells from the endosteum, fuse to form multinucleated osteoclasts underneath, then bind to the matrix resorbing old bone before undergoing apoptosis at the end. Osteoclast-mediated bone resorption takes only approximately 2-4 weeks during each remodelling cycle. Bone resorption transitions to bone formation in the reversal phase, where monocytes, osteocytes released from bone matrix and the osteoblast precursors from the same multicellular remodelling unit migrate to the resorption cavities, under the influence of several growth factors such as TGFβ, IGF-1 and BMPs (Hock et al. 1988, Bonewald and Mundy 1990). The osteoblast precursors then mature to become osteoblasts promoting bone formation, through synthesis of new collagenous organic matrix and regulation of the matrix mineralisation by releasing small membrane-bound matrix vesicles
that concentrate calcium and phosphate. Bone formation takes longer, up to 4-6 months to complete (Clarke 2008).

![Sequential Bone Remodelling Processes on a Trabecula](image)

**Figure 1.7 Sequential Bone Remodelling Processes on a Trabecula.** When microcracks sever canaliculi, the osteocytes sense the damage and signals the osteoblast lining cells to activate osteoclastogenesis (activation phase). The activated osteoclast binds to the matrix and resorbs old bone and the microcrack (resorption phase), taking 2-4 weeks to complete, following which osteoclasts undergo apoptosis and osteoblast precursors migrate to the area under the influence of growth factors (reversal phase). These osteoblast precursors mature into osteoblasts and begin synthesizing new collagenous organic matrix bone formation, laying down osteoid which becomes mineralised over the next 4-6 months (formation phase). [Adapted from (Seeman and Delmas 2006)]

The remodeling process begins before birth and continues until death. The rate, however, decreases as longitudinal growth ceases with epiphyseal closure (Parfitt et al. 2000). Bone remodelling, in particular bone resorption, increases with age in both men and women, although more marked in the latter especially in peri-menopausal and early postmenopausal women, accounting for the higher rate of osteoporosis and fragility fracture in this group.

Bone turnover is the process of bone remodelling, where bone formation and bone resorption are tightly coupled in space and time (Delmas 1991). Biochemical markers of bone formation and resorption enable the assessment of bone turnover as described above. This can allow insight into the effects of diseases on bone health in childhood. Interpretation of the results, however, can be challenging as they depend on age, gender, pubertal stage, nutritional status and other hormonal regulation. Skeletal growth and puberty can lead to substantial changes in raw levels of bone formation and resorption markers with age, as seen with their correlation with growth velocity (van Coeverden et al. 2002). In children, there is
added variability and reduced specificity as these markers are released into the circulation during the process of growth in length, when the actions of osteoclasts and osteoblasts are not coupled. Some markers can also be affected by circadian variation and day-to-day fluctuations. This can be further compounded by methodologic issues with assay sensitivity and specificity.

1.2.3.1 Bone formation markers

The commonly used serum markers for bone formation are bone-specific alkaline phosphatase (BAP) and osteocalcin (OC), which are released at different stages of osteoblast proliferation and differentiation (Calvo et al. 1996).

1.2.3.1.1 Bone Alkaline Phosphatase (BAP)

Serum total alkaline phosphatase (AP) is a combination of four isoenzymes; liver/bone, placental, intestinal and germ cell. The first two, liver and bone isoenzymes, differ only by post-translational glycosylation. Current immunoassays for BAP therefore possess a low cross-reactivity (16%) with the circulating liver isoenzyme (Delmas 1995). It has a relatively long half-life of 1 to 2 days and no circadian variation (Yang and Grey 2006). BAP is produced by osteoblasts and is involved in the initialisation process of osteoid mineralisation.

BAP is a very sensitive diagnostic tool in adults where a small increase is easily detectable and indicating possible pathology (Delmas 1995). In infants, BAP increases after birth due mainly to skeletal growth and is less predictive of bone mineral accrual. In rapidly growing children and adolescents, BAP increases until mid-puberty and decreases in late puberty (after menarche in girls) (Magnusson et al. 1995, Tobiume et al. 1997) (Figure 1.8), correlating positively and significantly with height velocity in both genders. BAP is about 10 times higher than adult values in pubertal girls in contrast to liver AP which remains within normal adult values (Blumsohn et al. 1994). BAP increase has been shown to correlate with the increase in bone mineral density in growth hormone-treated children (Tobiume et al. 1997).

1.2.3.1.2 Osteocalcin

Osteocalcin (OC) is a sensitive and specific marker for bone formation. It is produced almost exclusively by the osteoblasts during the matrix mineralisation phase and incorporated directly into the bone matrix (Delmas 1995). Circulating OC has a short half-life and is
rapidly (5mins) cleared by the kidneys. In adults, one third of the circulating OC is an intact molecule, one third is a large N-terminal mid-region fragment and the rest is N-terminal midregion and C-terminal fragments (Garnero et al. 1994). The distribution of these immunoreactive forms of OC is unknown in children. Serum OC results can vary considerably depending on which immunoreactive forms (intact molecules or OC fragments) the immunoassay measures. Assays that measure the intact molecule and large N-terminal fragment are more robust as well as sensitive.

Figure 1.8 Serum bone alkaline phosphatase (BAP) in children and adolescents. Females had their peak serum BAP levels during puberty about two years earlier than males [Adapted from (Tobiume et al. 1997)].

Figure 1.9 Serum osteocalcin levels in children and adolescents. Values are presented as mean and standard error of mean. [Adapted from (Johansen et al. 1988)]
Similar to BAP, circulating OC varies with age and pubertal stage in children as shown in Figure 1.9 (Johansen et al. 1988). The concentration is 10-20 times higher in growing children compared to pre-menopausal women (Matikainen et al. 1999). Additionally, there is also a circadian rhythm to OC production with the highest levels in the morning (Heuck et al. 1998). Circulating OC detectable in urine also demonstrates a similar diurnal variation (Taylor et al. 1990, Matikainen et al. 1999).

1.2.3.2 Bone resorption markers

The commonly used bone resorption markers are urinary hydroxyproline (HPro), pyridinoline (Pyr) and deoxypyridinoline (Dpyr) released during matrix resorption into the blood and excreted in the urine. They reflect bone resorption as they are not reutilised in collagen synthesis. Urinary bone markers have several limitations including the practical difficulties associated with serial urine collection, units of expression which may need size correction and expression in concentration relative to creatinine which itself is subjected to age-related changes due to muscle mass variation (Forbes and Bruining 1976, Rauch et al. 1994). Hence, the measurement of serum bone resorption marker, C-terminal telopeptide of type I collagen (CTX), in particular, is preferred.

1.2.3.2.1 Cross-linked Telopeptides (CTX)

During bone resorption, osteoclasts cleave off short fragments of the N- and C-terminal domains of collagen molecules into the circulation. The level of circulating CTX is highest in neonates and then markedly decreased in children after 1 year of age. The second peak was observed in girls 11-13 years old and in boys who were 14-17 years old (Crofton et al. 2002, Rauchenzauner et al. 2007), again demonstrating the effects of puberty, as seen in Figure 1.10. Urinary crosslinks show diurnal variation; highest in the morning and lowest late evening although this has not been shown in serum CTX.
1.2.3.3 Other markers

1.2.3.3.1 Sclerostin

Sclerostin is the product of SOST gene, secreted by osteocytes, and expressed almost exclusively in bone (Bellido T 2014). It is an inhibitor of the Wnt signalling pathway and thus inhibits bone formation. Sclerostin competes with Wnt ligands to bind to LRP5/6 co-receptors resulting in degradation of the intracellular β-catenin and hence impair osteoblast differentiation and function (Li et al. 2005, Bellido T 2014). Mechanical loading in rats and mice suppresses sclerostin level resulting in greater bone formation (Robling et al. 2008) whilst immobilisation in humans increases sclerostin levels leading to bone loss (Gaudio et al. 2010). These strongly suggest sclerostin as the crucial link between osteocytic mechanosensing and osteoblastic bone formation. Sclerostin level rises from early childhood and peaks at mid puberty (Tanner stage 3), and diminishes thereafter (Tsentidis et al. 2016). Boys have higher sclerostin levels than girls (Tsentidis et al. 2016). Most importantly, sclerostin has been shown to be an independent predictor of fracture risks (Ardawi et al. 2012, Starup-Linde et al. 2016).
1.2.4 Bone marrow and its composition

Bone marrow occupies approximately 85% of the medullary cavity, surrounded by a network of trabecular bone. In addition to the mesenchymal stem cells (described in section 1.3), it contains haematopoietic elements (red marrow) and a variable amount of fat (yellow marrow). At birth, the bone cavities are filled mainly with haematopoietic marrow, which is gradually replaced by fatty yellow marrow during childhood. By early adulthood, approximately 50% of the bone marrow volume is yellow marrow confined primarily in the appendicular skeleton, whilst the red marrow is concentrated to the axial skeleton (including proximal humerus and femur).

This physiological conversion occurs in a centripetal fashion from the distal to the more proximal appendicular skeleton (Vande Berg et al. 1998). Within the long bones, the fatty marrow conversion begins in the epiphyses at the onset of epiphyseal ossification and is completed within 6-8 months, followed by the diaphysis before extending to the metaphyses (Vogler and Murphy 1988, Jaramillo et al. 1991). The conversion also occurs concurrently from the central medullary canal to the endosteum. In the tibia specifically, the conversion begins in the diaphysis between age 1-5, and is completed by age 10-15 years (Vande Berg et al. 1998). The marrow conversion continues throughout adult life, although at a slower pace than during childhood (Vogler and Murphy 1988, Vande Berg et al. 1998) resulting in a positive correlation between bone marrow adiposity (BMA) and age (Meunier et al. 1971, Wehrli et al. 2000).

There is no gender differences in the rate of marrow conversion during childhood (Dooms et al. 1985, Waitches et al. 1994), although males have 6-10% more fat than females between the ages 20-60 years. This gender difference in BMA is reversed in adults >60 years. Vertebral bone fat increases sharply in females in the post-menopausal years between ages 55-65 years and stays approximately 10% higher in males thereafter (Kugel et al. 2001, Griffith et al. 2012).
1.3 Mesenchymal stem cells and bone biology

1.3.1 Origins

Bone marrow was thought to be the home of the haematopoetic stem cells, until the German pathologist Cohnheim elucidated the presence of non-haematopoetic stem cells as the source of fibroblasts within the same compartment in the late 1800s (Prockop 1997). It was not until the 1970s when Friedenstein discovered that the whole bone marrow he cultured in petri dishes, after discarding the haematopoetic cells, could differentiate into colonies of spindly-shaped fibroblasts and small deposits of bone or cartilage, suggesting that these marrow cells were multi-potential (Friedenstein et al. 1976). Since then, it has been established that the non-haematopoetic stem cells, now referred to as mesenchymal stem cells (MSCs) or marrow stromal cells, are capable of: i) self-renewing, i.e. proliferating in the undifferentiated form, and ii) pluripotency, i.e. differentiating into ‘committed’ progenitor cells such as osteoblasts, chondrocytes, adipocytes and even myoblasts, to give rise to the mesenchymal tissues bone, cartilage, adipose tissue and muscle respectively (Figure 1.11).

Figure 1.11 The differentiation of mesenchymal stem cells (MSCs). MSCs are pluripotent cells capable of differentiating into bone, muscle, fat and other connective tissues. [Adapted from image source: frontiersin.org]
Apart from the bone marrow, MSCs can also be isolated from periosteum, adipose tissue, amniotic fluid, and fetal tissues (Nakahara et al. 1991, Zuk et al. 2002, Anker et al. 2003). These are sometimes referred to as mesenchymal precursor cells which can be phenotypically similar to MSCs but exhibit heterogeneity in differentiation potential compared to the more traditional bone marrow-derived MSCs (Anker et al. 2003). Indeed, cultured human MSCs can have differentiation capacity not present in primary fibroblasts, which are mature mesenchymal cells (Pittenger et al. 1999).

Importantly, bone-marrow-derived MSCs (BMMSCs) express signature cell markers such as CD29, CD44, CD73, CD105 and CD166 and are negative for haematopoietic markers, specifically CD45, CD34, CD14 or CD11 (Chamberlain et al. 2007). The gene expression of MSCs continues to shift once induced to commit to a specific lineage, during differentiation and until the phenotype is characteristic of the target cell, for instance the expression of CD24, CD29 and CD36 among others in lipid-laden mature adipocytes (Berry and Rodeheffer 2013, Vroegrijk et al. 2013).
1.3.2 MSC Differentiation and signalling cascades involved

Pluripotent MSCs undergo “commitment” to a more lineage-specific cell line before differentiating into its respective mesenchymal tissue (Figure 1.12). This process of “commitment” happens under the influence of factors/cytokines that activate the expression of certain genes, or proteins which direct the MSCs down a designated cell line (Davis et al. 1987, Tang et al. 2004b). It is less well understood compared to the differentiation process of the more committed progenitor cells.

Figure 1.12 Osteogenic and adipogenic commitment and differentiation pathways in mesenchymal stem cells (MSCs). MSCs are pluripotent progenitor cells capable of differentiating into adipocytes and osteocytes, depending upon the stimulation cues. The lineage-specific differentiation is a multiple stage and well coordinated process regulated by master regulators such as PPARγ and C/EBPβ for adipogenesis and Runx2 and Osterix for osteogenesis. Osteogenic differentiation can be staged by measuring alkaline phosphatase (early marker) and osteocalcin and osteopontin (late markers). Expression of FABPs and production of lipids are indicators of terminal adipogenic differentiation. [Adapted from (Wagner et al. 2010)]

The differentiation of MSCs to adipocytes is not completely understood as yet. It can be broadly categorised into two phases: the determination phase and the terminal differentiation phase (Muruganandan et al. 2009). The determination phase is when multipotent MSCs commit to the adipocyte lineage, becoming pre-adipocytes which have a fibroblastic phenotype morphologically but are not readily distinguishable from their MSC precursors. This phase is believed to be ligand-dependent, with endogenous molecules such as fatty acids (Schopfer et al. 2005) and exogenous ligands such as thiazolidinediones which are
potent peroxisome proliferator-activated receptor γ (PPARγ) agonists (Lehmann et al. 1995), although some studies have shown otherwise (Walkey and Spiegelman 2008). In the terminal differentiation phase, pre-adipocytes become mature adipocytes with distinctive gene expression markers and new functions including lipid synthesis, storage and adipocyte-specific protein production. This is a sequentially and temporally ordered process involving multiple signalling cascades that converges at PPARγ transcriptional activity.

Similarly in MSC differentiation to osteoblasts, the process starts with commitment of osteoprogenitor cells and differentiation into pre-osteoblasts, which eventually develop into mature osteoblasts (Neve et al. 2011). The development of an immature osteoblast into a mature one involves proliferation, maturation, matrix synthesis and mineralisation before becoming entombed in osteoid to turn into an osteocyte. Osteoblast differentiation requires the key transcription factors runt-related transcription factor 2 (Runx2) as described above, but also others such as BMPs.

The differentiation of MSC towards an adipogenic or osteogenic cell fate depends on a variety of transcription factors and signalling cascades. The two main lineage-specific transcription factors are Runx2 for osteogenesis and PPARγ for adipogenesis. Due to its common precursor from the pluripotent MSC, in vitro evidence exist to suggest a mutually exclusive and reciprocal lineage selection of one or the other, whereby adipogenesis occur at the expense of osteogenesis and vice versa (Figure 1.13) (Beresford et al. 1992, Yang et al. 2008). Typically, increased expression of one is associated with downregulation of the other (Li et al. 2003, Zhang et al. 2006), further affirming the bone-fat balance hypothesis.

**Figure 1.13 Bone-fat balance.** Osteogenesis and adipogenesis are proposed to be mutually exclusive given that osteoblasts and adipocytes originate from the common progenitor mesenchymal stem cells. PPARγ is the key regulator of adipogenesis whilst Runx2 activation determines osteogenesis. [Adapted from (Yang et al. 2008)]
1.3.2.1 PPARγ: The master regulator of adipogenesis

Peroxisome proliferator-activated receptors (PPAR) are members of steroid/thyroid hormone receptor gene superfamily with three structural analogs: PPARα, PPARδ and PPARγ. All three are expressed during adipogenesis, but PPARγ expression is exclusive to adipocytes and increases more rapidly during early adipogenesis (Wagner et al. 2010). PPARγ is expressed in two isoforms, PPARγ1 and PPARγ2, generated by alternative splicing, during adipogenesis. In response to PPARγ activators, such as thiazolidinediones (TZDs) and free fatty acid molecules, PPARγ heterodimerises with the nuclear receptor, retinoid X receptor (RXR) to form a transcription complex that can bind to DNA and regulate transcription of the adipogenic genes (Figure 1.14).

PPARγ plays a very diverse role in different tissues including its function in adipogenesis, atherosclerosis, inflammation and insulin sensitisation (Kliewer et al. 1995, Lehmann et al. 1995, Takeda et al. 2000). It is considered the master regulator of adipogenesis as no other factor can rescue adipocyte formation when PPARγ is knocked out. Selective deletion of PPARγ in murine adipose tissue leads to a loss of both brown and white adipocytes (Rosen and MacDougald 2006). PPARγ-deficient (-/-) embryonic stem cells from genetically manipulated mice failed to differentiate into adipocytes, and instead differentiated into osteoblasts (Tzameli et al. 2004). In addition, cells derived from PPARγ+/− mice demonstrated a reduced ability to differentiate into adipocytes with increased osteoblastogenesis instead.

![Figure 1.14 Mode of action for PPARγ](image)

Figure 1.14 Mode of action for PPARγ. PPARγ and RXR form a heterodimer which is activated by the respective ligands. The activated PPARγ/RXR heterodimer will be translocated into the nucleus and regulates downstream target genes in concert with nuclear receptor co-activators. [PGJ2 prostaglandin J2; Adapted from (Wagner et al. 2010)]
1.3.2.2 Runx2: The master regulator of osteogenesis

The Runx family consists of three distinct proteins: Runx1-3 which share a common β subunit, but a different α subunit (Ogawa et al. 1993). Runx proteins have a DNA-binding domain, known as Runt, homologous to that identified in the *Drosophila melanogaster*. Similar to PPARγ, Runx proteins form a heterodimer with core binding factor β (Cbfβ), a co-transcriptional factor, which displaces the inhibitory domain and allows binding to DNA to effect downstream targets. Runx have distinct tissue-specific expression and their functions are dependent on cellular context as summarised in Table 1.2 (Ito et al. 2015).

Phosphorylated Runx proteins are very susceptible to degradation (Biggs et al. 2006, Shen et al. 2006). Runx genes are intimately linked to carcinogenesis so its protein levels need to be tightly regulated in order to switch between tumour suppressive and oncogenic functions. Runx1 aberrations have been associated with leukaemia, Runx2 with osteosarcoma and Runx3 with stomach, colon and bladder cancers to name a few (Nomoto et al. 2008, Mangan and Speck 2011, Martin et al. 2011). They are expressed interdependently and often, more than one Runx family member is expressed in the same cell. Levels of Runx1 and Runx2 mRNAs are inversely correlated in skeletal development (van der Deen et al. 2012). Importantly, Runx protein levels oscillate during cell differentiation with Runx2 expression in osteoblasts highest at G0-G1 phases (early differentiation) of the cell cycle and downregulated during G1-S transition (mid differentiation) (Galindo et al. 2005). Runx have the ability to exert opposite effects on transcription due to its complex post-translational modifications, which modulate its interactions with DNA.

**Table 1.2 Function of Runx proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runx1</td>
<td>Haematopoeisis</td>
<td>Haematopoietic stem cell maintenance</td>
</tr>
<tr>
<td></td>
<td>Immune response</td>
<td>T-cell and B-cell development</td>
</tr>
<tr>
<td>Runx2</td>
<td>Skeletal development</td>
<td>Differentiation of osteoblasts and chondrocytes</td>
</tr>
<tr>
<td></td>
<td>Alveolar cell maturation in mammary glands</td>
<td>Directs MSCs to the osteoblast lineage</td>
</tr>
<tr>
<td></td>
<td>Regulates mammary epithelial progenitors during pregnancy</td>
<td>Necessary for the specification of luminal progenitor cells</td>
</tr>
<tr>
<td>Runx3</td>
<td>Stomach</td>
<td>Required for proper differentiation</td>
</tr>
<tr>
<td></td>
<td>Nervous system</td>
<td>Involved in neuronal cell fate</td>
</tr>
<tr>
<td></td>
<td>Immune response</td>
<td>Required for macrophage and T-cell differentiation, and dendritic cell maturation</td>
</tr>
</tbody>
</table>
In the mesenchymal stem cells, Runx participates in the decision to proliferate or differentiate. Specifically, Runx2 directs MSCs to the osteoblast lineage for bone development. Runx2 level, however, must be tightly regulated in osteogenesis. Its expression increases at the onset of quiescence in preparation for differentiation, but must reduce in time for osteoblast maturation as overexpression results in the accumulation of a larger immature osteoblast population (Pratap et al. 2003, Komori 2010). Heterozygous mutation of Runx2 (Runx2+/−) causes hypoplastic clavicles and open fontanelles in a condition called cleidocranial hypoplasia (Mundlos et al. 1997) whilst Runx2−/− mice die soon after birth from respiratory failure due to lack of bone ossification in the thoracic cage (Komori et al. 1997). Due to defective osteoblast differentiation, the skeleton of Runx2−/− mice are cartilaginous with the complete absence of mineralisation, with the exception of very mild calcification at tibia, fibula, radius and ulna detectable on Alizarin staining. It has been noted that Runx2−/− mesenchymal stem cells are capable of differentiating into adipocytes and chondrocytes, but completely lack the potential to differentiate into osteoblasts indicating its key role in skeletal development (Kobayashi et al. 2000).

1.3.2.3 Signalling pathways in MSC differentiation

In addition to the transcription factors, many complex signalling pathways are involved in the commitment and differentiation of MSC towards adipocytes or osteocytes. There are several pro-osteogenic/anti-adipogenic signalling pathways including (i) β-catenin dependent Wnt signalling (as well as the non-canonical β-catenin Wnt signalling) (Taipaleenmaki et al. 2011, D’Alimonte et al. 2013), (ii) Hedgehog signalling (Fontaine et al. 2008, James et al. 2010) and (iii) NEL-like protein 1 (NELL-1) signalling (James et al. 2011, James et al. 2012). The β-catenin-dependent Wnt signalling pathway is the most well-established one involved in both bone formation. β-catenin plays a direct role in the regulation of osteoblasts with deficiency causing arrest in pre-osteoblast development at an early stage and impaired maturation and mineralisation in more committed osteoblasts, and consequent embryonic skeletal defects (Day et al. 2005, Holmen et al. 2005, Chen and Long 2013). There is also an antagonistic effect on osteoclastogenesis with suppression of bone resorption through the canonical β-catenin/Wnt signalling pathway (through upregulation of OPG expression and down-regulation of RANKL expression) and increased bone resorption through activation of the non-canonical pathway (Takahashi et al. 2011). Accordingly, trials involving antibody against sclerostin, which is a Wnt inhibitor, have shown promising results in phase II clinical trials demonstrating increased bone mineral density (Padhi et al. 2011). Its reciprocal anti-adipogenic effect is exerted through several members of the Wnt
family, such as Wnt10b and Wnt3a, which inhibits adipogenesis mainly through suppression of PPARγ and/or C/EBP-α expressions (Ross et al. 2000, Kawai et al. 2007).

Contradicting the bone-fat balance, there are signalling cascades which demonstrate dual pro-osteogenic and pro-adipogenic effects, namely (i) BMP signalling and (ii) IGF signalling. The precise determinant that governs the cell fate of BMP signalling is not well understood. Studies in mouse MSCs have shown a lower concentration of BMP-2 favouring adipogenesis and a higher concentration directing MSCs towards osteogenic differentiation (Wang et al. 1993). The effects of different BMP receptor expression on the lineage differentiation of MSCs have also been shown (Chen et al. 1998).

The identification of the genes/proteins involved in the signalling pathway for these commitment and differentiation steps in MSC differentiation is crucial as it may lead to potential targets for therapeutic interventions, as seen with the anti-sclerostin described above. Of note, MSC differentiation can also be influenced by many micro-environmental factors such as mechanical forces (Kelly and Jacobs 2010), electrical currents (Hronik-Tupaj et al. 2011) and magnetic fields (Yan et al. 2010).
1.3.3 C3H10T1/2 Mesenchymal stem cell model

Human bone-marrow-derived MSCs (BMMSCs) are ideal for *in vitro* studies investigating their own multi-lineage differentiation potential (Pittenger *et al.* 1999) and the various signalling pathways involved. This attractive cell source, however, can be difficult to obtain, usually dependent on invasive marrow aspirates of volunteer donors or samples obtained during orthopaedic surgery for an unrelated reason. In addition, BMMSCs can show variable heterogeneity in colony sizes, colony-forming rates and cell morphology during cell isolation/expansion (Bianco *et al.* 2001) which may have a false positive or false negative impact on study findings.

In contrast, the mouse mesenchymal stem cells C3H10T1/2, which are commercially available, have a homogenous population and do not undergo spontaneous differentiation under normal culture conditions making it an ideal alternative. The C3H10T1/2 cell line was established in 1973 from 14-17 day old C3H mouse embryos (Reznikoff *et al.* 1973). These cells display a fibroblastic morphology under normal culture conditions and are functionally similar to mesenchymal stem cells in their ability to develop into osteoblasts, chondrocytes and adipocytes with specific inductions (Katagiri *et al.* 1990, Tang *et al.* 2004a). It is worth noting that the C3H10T1/2 cell line has been reported to have a lower adipogenic differentiation potential of only 10% compared to 70% in primary BMMSCs (Zhao *et al.* 2009).
1.4 Bone fragility

1.4.1 Normal bone mineral accrual

The skeleton grows, in length, in breadth and in mass, as the body grows. Skeletal health is largely regulated by genetic factors, which determine up to 70% of peak bone mass (Gortz and Fassbender 2001) with other key environmental factors such as body composition (Baxter-Jones et al. 2003, Farr et al. 2014a), physical activity (Bailey et al. 1999, Baxter-Jones et al. 2008) and nutrition (Whiting et al. 2004, Vatanparast et al. 2005) affecting its accrual. Bone mass increases substantially in the first two decades of life, reaching a plateau in the late-teens or young adulthood before declining after the age of about 50 years (Figure 1.15). The actions of sex steroids, growth hormone (GH) and insulin-like growth factors released and active during the adolescent growth periods (Saggese et al. 2002) also contribute significantly to bone mineral acquisition and skeletal changes, underpinning the effects of puberty and the accompanying growth spurt on bone health.

![Figure 1.15 Bone mass accrual throughout life. Diagramatic representation of the bone mass accrual in individuals who achieve their full genetic potential (solid line) and in those who did not (dashed line), with the latter having lower peak bone mass predisposing to skeletal fragility in later years. [Adapted from (Heaney et al. 2000)]](image)

As captured in a comprehensive longitudinal study, bone first plateaus in bone area, and then roughly 1 to 2 years later, plateaus in mineralisation (Baxter-Jones et al. 2011). Peak bone mass is reached approximately 7 years after peak height velocity (PHV), in both genders, at an approximate chronological age of 18.8 in females and 20.5 years in males (Baxter-Jones et al. 2011). The attainment of peak bone mass varied with skeletal site - femoral neck peak bone mass is attained first, 3 years after PHV, before that of lumbar spine at 5 years after PHV (Baxter-Jones et al. 2011). Similarly in another study in males, peak bone mass occurred by 18-20 years at the femoral neck, lumbar spine and total body but had not yet
been reached at the distal radius and tibia (Lorentzon et al. 2005). Depending on site, 33-46% of the total adult bone mineral content is accrued in the adolescent growth period within the 5 years surrounding PHV (Baxter-Jones et al. 2011). In females, this represents double the amount of bone mineral that will be subsequently lost during the post-menopausal years from 50-80 years of age (Arlot et al. 1997). As bone mass tracks throughout life (Slemenda et al. 1990, Ferrari et al. 1998, Loro et al. 2000), achieving a higher peak bone mass is therefore crucial in the protection against later-life fragility fracture. Pre-pubertal children at the high end of the bone mass distribution gained the most bone across puberty and remained at the high end of the distribution 2 years later (Loro et al. 2000).

In the same way that adolescence is a critical window for laying down bone, it is also a time of crucial vulnerability. Any interruption to the normal physiology by either illness (period of immobilisation, chronic inflammatory diseases, interruption to normal reproductive hormone production, eating disorders) and/or lifestyle factors (smoking, inadequate dietary intake, particularly of calcium or vitamin D, oral contraceptive use) may lead to a significantly lower peak bone mass constituted for by the genetic potential. There is a controversy that combined oral contraceptive, especially very low dose ethinyl oestradiol, during adolescent age can be detrimental to the acquisition of peak bone mass, although subsequent prospective studies and RCT have shown otherwise (Cromer et al. 2004, Cibula et al. 2012, Gargano et al. 2008). Additionally, medication such as glucocorticoids and anti-epileptics, necessary for the treatment in many childhood diseases, are detrimental to skeletal health. Young people affected by any of these factors will enter adulthood with low bone mass, and will have a higher long-term risk of fracture even if they lose only a modest amount of bone thereafter. Hence, optimal skeletal development during growth is protective against osteoporosis and fragility fracture in adulthood.
1.4.2 Determinants of bone strength and its clinical implications

The mechanical competence, or strength, of any structure is dependent on a combination of the mass, the physical properties of the component materials, and their geometric arrangement in space (Figure 1.16). This is no different when it comes to bone. Bone strength is determined by the whole bone – bone mass, architecture and geometry.

Bone mass is an important determinant of bone strength, accounting for approximately 60-70% of the variation in bone strength (Cheng et al. 1997). The relationship between bone mass and structural strength holds at all ages, with a clear positive association between bone mass and fracture throughout life (Heaney et al. 2000). For the last several decades, as bone mass became measurable in vivo, quantified as bone mineral density by DXA, BMD has been used as the main surrogate for bone strength. However, when: i) useful anti-osteoporosis medications produced a reduction in fracture risk with only marginal change in BMD (SR et al. 1996), and ii) BMD values of subjects with and without osteoporotic fracture imbricated (Siris et al. 2001, Schuit et al. 2004), there is an increasing realisation that bone fragility is not only dependent on bone mineral mass (bone quantity) but also alterations in bone quality including bone geometry and microarchitecture.

Some of the important parameters of bone quality include bone size and shape, trabecular bone microarchitecture and cortical bone thickness and porosity. Trabecular and cortical bone parameters were found to be more discriminatory of men and women with and without fractures (Chang et al. 2015) compared to BMD. It also became clear from studying the microarchitecture of osteoporotic subjects and bone specimens that there was a predominance of cortical over trabecular bone loss in peripheral sites (Zebaze et al. 2010),

---

**Figure 1.16 Determinants of mechanical competence or strength, of a structure.** The strength of any structure is determined by the triad of the mass, physical properties of its components and the geometric arrangements of the structure.
which helped explain why most fractures in old age are non-vertebral (Riggs et al. 1981). The study found a significant increase in cortical porosity which was poorly captured by densitometry (Zebrze et al. 2010). Increased porosity reduces bone strength as a 4% rise in porosity increases crack propagation through bone by 84% (Diab and Vashishth 2005), with a 10% rise in porosity halving the maximum load before fracture occur (Martin and Burr 1989). As well as disruption to the lace-like trabecular scaffolding, another important finding of the paper from studying the post-mortem bone specimens was the presence of cortical remnants, or trabecularisation of the cortex, which can mislead cortical and trabecular quantification by non-invasive imaging methods. In addition, the apparent trabecular bone volume (BV/TV) was found to be the best morphometric predictor of bone strength amongst the trabecular parameters (Thomsen et al. 1998). The intrinsic material properties of bone including the matrix mineralisation and collagen cross-linking can also affect bone stiffness compromising bone strength.

As there is currently no accurate measure of overall bone strength, fracture prediction may be more accurate when measures of bone mass and microarchitecture parameters can all be incorporated together. Advances in non-invasive imaging techniques such as HR-pQCT and micro-MRI can now provide information on bone quality that was not previously available to enhance the assessment of fracture risk and skeletal health in a variety of diseases related to bone abnormalities, which cannot be fully studied by DXA alone.
1.4.3 Osteoporosis in Adults

Osteoporosis is a skeletal disorder characterised by the loss of bone mass (bone quantity), and deterioration of bone microarchitecture (bone quality) resulting in compromised bone strength and increased risk of fractures (NIH Consensus Development Panel on Osteoporosis Prevention and Therapy 2001, Camacho et al. 2016). A comparison of the bone microarchitecture of a normal vertebra and one with osteoporotic deficit is shown in Figure 1.17.

![Figure 1.17 Bone microarchitecture of normal and osteoporotic specimen. (A) Healthy vertebra (B) Compressed osteoporotic vertebra with reduced trabecular number, connectivity and density with more trabecular spaces. [Adapted from (Lorentzon and Cummings 2015)]](image)

1.4.3.1 Epidemiology of osteoporosis

Approximately 30% of all postmenopausal women are affected in the UK and 40% of them will go on to develop a fracture (Safaei et al. 2007). 1 in 2 women and 1 in 5 men over the age of 50 will suffer a fragility fracture (van Staa et al. 2001). Fragility fractures are fractures that result from low-level (or low energy) mechanical forces that would not ordinarily result in fracture (Kanis et al. 2001). The World Health Organisation has quantified this as forces equivalent to a fall from standing height or less. It is usually asymptomatic until fragility fracture occurs resulting in substantial pain and severe disability. Fractures of the hip (proximal femur), wrist (distal radius) and spine (vertebra) are most common.

Osteoporosis is a significant public health problem affecting over 9 million people worldwide (Johnell and Kanis 2006) and 3 million people in the UK (National Osteoporosis Society 2015). Every year 500,000 people suffer a fragility fracture (British Orthopaedic Association 2007), including 90,000 hip fractures with a projected rise in the incidence to 101,000 by 2020 (Department of Health (England) 2006). Hip fracture nearly always
requires hospitalisation, is fatal in 20% of cases and leaves 50% permanently disabled (Sernbo and Johnell 1993). Only 30% of patients fully recover. Fragility fractures have a massive impact on healthcare budget, costing the NHS an estimated annual cost of £2 billion (over £5 million per day) in combined hospital and social care for hip fracture alone (Burge et al. 2001).

1.4.3.2 Risk factors of osteoporosis

Although osteoporosis can occur at any age, the risk increases significantly with aging and after menopause in women. Osteoporosis can arise from secondary causes (Table 1.5) including glucocorticoid use and chronic diseases such as diabetes mellitus which is of particular relevance to this thesis. Other risk factors for osteoporosis includes premature menopause, amenorrhoea in women or hypogonadism in men, cigarette smoking, dietary factors such as low calcium intake and vitamin D deficiency, low body weight, glucocorticoid therapy, family history of osteoporosis, Asian or Caucasian ethnic origin, and long term immobilisation (Kanis 2002).

1.4.3.3 Diagnosis of osteoporosis

The current clinical diagnosis of osteoporosis is based on densitometry cut-offs proposed by the World Health Organisation (WHO) and modified by the International Osteoporosis Foundation (WHO Study Group 1994, Kanis and Gluer 2000) as shown in Table 1.3. Because bone density values follow a normal Gaussian distribution, BMD values can be expressed in relation to a reference population in standard deviation (SD) units. When the reference population is that of a young healthy adult, the BMD values are expressed as T-scores.

<table>
<thead>
<tr>
<th>Table 1.3 WHO/IOF Definition of osteoporosis in adults</th>
</tr>
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<tbody>
<tr>
<td><strong>Definition of Osteoporosis in Adults</strong></td>
</tr>
<tr>
<td>WHO and International Osteoporosis Foundation Diagnostic Criteria</td>
</tr>
<tr>
<td><strong>Category</strong></td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Osteopenia</td>
</tr>
<tr>
<td>Osteoporosis</td>
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<tr>
<td>Severe osteoporosis</td>
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</tbody>
</table>
Although there is set diagnostic criteria for the diagnosis of osteoporosis, the assessment of fracture risk which is of greater clinical interest is much less straight forward. DXA-derived measures of BMD have been an important determinant of fracture risk with an increase in the relative risk of fracture by approximately 2-fold for every 1 SD decrease in bone mineral density, depending on skeletal sites (Marshall et al. 1996). However, BMD alone has a very low sensitivity in predicting the likelihood of fractures as considerable overlap in BMD values was seen in patients with and without fragility fractures (Siris et al. 2001, Schuit et al. 2004). Furthermore, therapeutic interventions for osteoporosis demonstrated reduction in the risk of fractures with minimal change in BMD (Cummings et al. 1996). Kanis described the use of BMD alone to predict fracture risk is akin to the reckless use of blood pressure alone in predicting the risk of stroke, where there is not a clear discriminatory cut off. With this in mind, Kanis and McCloskey founded the Fracture Risk Assessment Tool (FRAX) to evaluate the 10-year probability of hip and other major osteoporotic fractures (Kanis et al. 2008). This tool integrates clinical risk factors such as age, gender, previous fracture, glucocorticoid use, and BMD at femoral neck to give the fracture risk estimates. In FRAX, T1D and T2D has been recognised as a secondary cause of osteoporosis which increases the fracture probability independent of BMD (Giangregorio et al. 2012).
1.4.4 Osteoporosis in Children

1.4.4.1 Definition of osteoporosis

The 2013 International Society for Clinical Densitometry provided a position statement for the definition of osteoporosis in children which is clinically useful and enabled scientifically valid comparison between studies, as summarised in Table 1.4 (Bishop et al. 2014).

Table 1.4 ISCD Definition of osteoporosis in children

<table>
<thead>
<tr>
<th>Definition of Osteoporosis in Children:</th>
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<tbody>
<tr>
<td>ISCD 2013 Position Statement</td>
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</table>

(1) The presence of one or more vertebral compression (crush) fracture,

or,

(2) The presence of a clinically significant fracture and a BMD Z-score \( \leq -2.0 \)

A clinically significant fracture is:

(a) two or more long bone fractures by age 10 years

(b) three or more long bone fractures by age 19 years

BMC/BMD Z-score > -2.0 does not preclude the possibility of skeletal fragility and increased fracture risk. The term osteopenia should be avoided.

This position statement emphasises that the diagnosis of osteoporosis in children is not based on densitometry alone and highlights the importance of establishing a detailed fracture history. In addition, vertebral fractures in children are themselves diagnostic of osteoporosis. It also recognises that children with bone fragility can have recurrent fractures despite normal bone mineral density values.

1.4.4.2 Causes of Secondary Osteoporosis

The aetiology of primary osteoporosis, where primary bone disorders result in recurrent fractures is out with the context on this thesis. Secondary osteoporosis is increasingly recognised in children with chronic diseases and is caused by the underlying disease or its treatment (Table 1.5) (Korula et al. 2015).
### Table 1.5 Causes of secondary osteoporosis

<table>
<thead>
<tr>
<th>Category</th>
<th>Aetiological causes</th>
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<tbody>
<tr>
<td>Reduced mobility</td>
<td>Prolonged immobilisation, cerebral palsy, Duchenne muscular dystrophy (DMD), spinal cord injury and Rett syndrome</td>
</tr>
<tr>
<td>Pubertal delay</td>
<td>Chronic illness, primary hypogonadism and induction by drugs</td>
</tr>
</tbody>
</table>
| Chronic illnesses      | **Haematological**: leukaemia, childhood cancers, thalassaemia and post- bone marrow transplant  
                         | **Renal**: chronic renal failure and post-renal transplant  
                         | **Gastrointestinal**: inflammatory bowel disease, coeliac disease, and chronic liver disease  
                         | **Rheumatological**: systemic lupus erythematosus (SLE) and juvenile idiopathic arthritis (JIA)  
                         | **Others**: anorexia nervosa, cystic fibrosis, severe burns and HIV                                                                                  |
| Endocrine causes       | Hypogonadism, diabetes mellitus, hyperthyroidism, hyperprolactinaemia, and metabolic bone disease of prematurity                                           |
| Drug-induced           | Glucocorticoids, methotrexate, GnRH analogues, cyclosporin, anticonvulsants, heparin and radiotherapy                                                |
| Inborn errors of metabolism | Glycogen storage disorder, lysinuric protein intolerance, galactosaemia, Gaucher disease, homocystinuria and Menke’s disease                        |

#### 1.4.4.2.1 Reduced mobility

Normal bone development is dependent on mechanical loading and bone-muscle interaction which increase bone formation and bone mineral accrual (Hind and Burrows 2007). Reduced ambulation in children with cerebral palsy and DMD predisposes them to osteoporosis and lower limb fractures. (McDonald et al. 2002, Mergler et al. 2009). Vertebral fractures have also been found in up to 40% of children with DMD (McDonald et al. 2002). Studies have also found greater trabecular vBMD, tibial cortical thickness and tibial muscle cross-sectional area Z-scores in children with partial spinal cord injury who can stand compared to those who are bed bound (Biggin et al. 2013).
1.4.4.2 Pubertal delay
As described in section 1.4.1, sex steroids play a significant role in bone mineral accrual and longitudinal bone growth, hence the resultant growth spurt in puberty. Pubertal delay negatively affects skeletal development and increases long term fracture risk. The Bone Mineral Density in Childhood Study cohort demonstrated that age of onset of puberty was a strong negative predictor of DXA bone mineral density at skeletal maturity in both genders, independent of the BMD at the beginning of puberty and the length of puberty (Gilsanz et al. 2011). Hogler et al showed that pubertal induction halted the reduction in BMD which was seen in pre-pubertal children (Hogler et al. 2004).

1.4.4.2.3 Drug-induced
Many medications used to treat chronic illnesses in children predispose them to iatrogenic secondary osteoporosis. Glucocorticoid-induced osteoporosis is very well recognised with an increased risk of fracture in children with a history of frequent oral corticosteroid use [adjusted OR of 1.32 (95% CI, 1.03-1.69)] and a doubling of humeral fracture risk in those who receive four or more courses of oral corticosteroids in a year (van Staa et al. 2003). Impairment of bone health is through a direct effect on bone turnover with increase in bone resorption followed by a decrease in bone formation, and indirect influence on muscle and calcium homeostasis (Buckley and Humphrey 2018). In adults, studies have shown the greatest negative effect on trabecular bone in the spine with concomitant vertebral fractures being most common (De Vries et al. 2007). This is reversible with fracture rate reported to normalise one year after glucocorticoid discontinuation (Korula et al. 2015).

1.4.4.2.4 Chronic inflammatory conditions
A wide variety of chronic inflammatory diseases can result in secondary osteoporosis summarised beautifully in the review by Makitie (Makitie 2013). This occurs not only through the increased inflammatory milieu which is toxic to the bones but also often multifactorial from reduced mobility, pubertal delay, poor nutrition and drug-induced bone loss. Diabetes mellitus, in particular T1D, is one of the disorders where systemic and local inflammatory responses have been demonstrated from the onset of the disease with detrimental effects on bone turnover and resultant skeletal fragility as discussed in more details in section 1.5.3.5.
1.4.5 Fractures in Healthy Children

Fractures in healthy children can be common. In the UK, approximately one third of children are reported to suffer a fracture before the age of 17 years, with a fracture incidence of 3% in boys and 1.5% in girls at their peak ages (Cooper et al. 2004) (Figure 1.18A), although other studies have reported differently (Jones et al. 2002, Mills and Simpson 2013). These differences could reflect the variation with the geographical area studied.

The fracture incidence varied with age and gender; increasing through childhood, with a steady rise in each 5-year age group, starting at approximately 1% risk in the under 5s, 2% in the 5-9 year olds, 3% in the 10-14 year olds and 4% in the 15-19 years old. Boys consistently had higher fracture incidence than girls, with a widening gap in the teenage years, peaking at 5% in the older teenage boys. (Figure 1.18B) (Mills and Simpson 2013). Across the literature, there is generally an agreement in the peak age of fracture incidence around 14 years old for boys and 11 years old for girls (Jones et al. 2002, Cooper et al. 2004), with slightly later ages for the Scottish cohort (Mills and Simpson 2013).

Seasonal differences with peak in fractures during the summer and a much lower rate during the winter have been noted (Cooper et al. 2004). Evidence on socioeconomic status on the incidence of childhood fractures was conflicting. The study in Glasgow using neighbourhood-type analysis demonstrated higher fracture incidence in those living in deprived areas compared to those in more affluent areas (175.3/10,000 vs 140.1/10,000) (Stark et al. 2002) whilst the study in South Wales using Townsend scores found no association (Lyons et al. 2000). In a large prospective cohort study of over 1400 children...
aged 6-17 from the US, children of white Caucasian origin have been found to have substantially higher fracture risk compared to children of black African origin (Wren et al. 2012).

A population-based study in Finland has shown a significantly decreased incidence of fractures in the last two decades, following a rise between the 1960s and 1980s (Mayranpaa et al. 2010), possibly due to the implementation of injury prevention measures over the years. Studies in Finland and Europe have found a changing pattern in the skeletal sites of childhood fractures with increase in forearm fractures (Oskam et al. 1998, Mayranpaa et al. 2010) and decrease in femoral (Bridgman and Wilson 2004) and tibial (Emami et al. 1996) shaft fractures. Upper limb fractures are more common in healthy children, accounting for 65% of all the fractures compared to 7-28% of lower limb fractures (Lyons et al. 2000, Wren et al. 2012). The most common skeletal site is distal radius (25-43%), followed by fingers and carpal bones (Landin 1983, Moustaki et al. 2001). Vertebral fractures are uncommon, with one study reporting the incidence of traumatic fractures of 66 per million (Puisto et al. 2010) although asymptomatic vertebral fractures have been identified in fracture-prone children (Mayranpaa et al. 2010), which should prompt further investigations into underlying skeletal fragility. Pelvic fracture is the least common in children (Cooper et al. 2004).

The risk of further fractures is doubled in children who have had a fracture, especially those in early childhood (Landin 1983, Wren et al. 2012). Up to 3% of children experience further fractures in their childhood (Cooper et al. 2004).
1.5 Type 1 Diabetes Mellitus (T1D)

1.5.1 Background

Type 1 diabetes (T1D) is a disorder characterised by severe insulin deficiency due to an autoimmune destruction of the pancreatic β cells in genetically predisposed individuals. The disorder affects 300,000 people in the UK, of which 35,000 are children. The incidence of T1D is 1:4000, with peak age at diagnosis between 9-14 years old. It is the second most common chronic disorder in childhood (Gale 2002), although it can occur at any age.

1.5.2 Complications of T1D

T1D is associated with well-known long term microvascular and macrovascular complications. The microvascular complications include retinopathy, nephropathy and neuropathy which lead to visual impairment, renal failure and loss of sensation in the feet with muscle weakness respectively. Macrovascular diseases, similar to that seen in Type 2 diabetes, such as coronary heart disease, peripheral vascular disease and stroke also affect adults with Type 1 diabetes. Although clinically evident diabetes-related vascular complications are rare in childhood and adolescence, early functional and structural abnormalities may be present a few years after diagnosis.

Landmark papers from the Diabetes Control and Complications Trial (DCCT), Epidemiology of Diabetes Interventions and Complications (EDIC) and large randomised controlled trials categorically demonstrated that glycaemic control is the single most important predictor of complications (Nathan et al. 1993, Fullerton et al. 2014, Lagani et al. 2015, Nordwall et al. 2015). DCCT was a multicentre, randomised controlled trial involving 1441 patients with Type 1 Diabetes conducted in North America from 1983-1993 whereby patients were randomised to two treatment arms of intensive and conventional treatment (Nathan et al. 1993). The intensive therapy group had average HbA1c of 7% whilst the conventional group had HbA1c of 9%. The trial results showed conclusively that intensive therapy reduced the risk of retinopathy and other microvascular complications by 35-76% in the primary prevention (no retinopathy at baseline) and secondary intervention cohorts.

Results from the EDIC follow-up study showed that early intensive therapy reduced albuminuria and long term risk of developing clinically significant kidney dysfunction by 50% (de Boer et al. 2011) Compared to the age-matched general population, people with T1D have 10-fold increased risk of cardiovascular death (Dorman et al. 1984, Krolewski et al. 1987) with 57% reduction in the risk of macrovascular complications including non-fatal
heart attack, stroke or death from cardiovascular diseases with intensive glycaemic control (The Diabetes Control and Complications Trial (DCCT) Research Group 1995).

Apart from glycaemic control, the other main driver for the development of microvascular complications is the duration of diabetes. Longer duration of diabetes has been associated with increased risk of renal complications, including microalbuminuria, proteinuria and end-stage renal disease (ESRD) (Elley et al. 2013, Lagani et al. 2015) and retinopathy (Lachin et al. 2008). Nordwall et al showed increasing cumulative incidence of retinopathy with longer diabetes duration, although the role of glycaemic control was difficult to be apportioned (Nordwall et al. 2015). Despite this, total glycaemic exposure (HbA1c and duration of diabetes) only explained 11% of the variation in retinopathy risk (Lachin et al. 2008) with other factors including environmental and genetic factors, explaining the remaining 89% of the variation independent of HbA1c.

Diabetic end-organ damage are rare before puberty, and the onset of puberty accelerates the microvascular complications, in particular nephropathy (Andersen et al. 1983, Krolewski et al. 1985). Lawson et al postulated that the physiological changes of increased renal volume and the accompanying macroalbuminuria during puberty may contribute to this (Lawson et al. 1996). Children with diabetes appear to go through puberty at the same time as their peers (Thorn et al. 2005, Ostman et al. 2008) although some studies have shown evidence of delayed menstruation (Kjaer et al. 1992, Lombardo et al. 2009, Deltsidou 2010). Indeed, the mechanism by which puberty contributes to the development of microvascular complication remains largely unclear. Women with diabetic retinopathy and nephropathy were found to undergo later menarche (Harjutsalo et al. 2016) and earlier menopause (Sjoberg et al. 2011), further providing evidence to support the effect of sex hormones on the risk of microvascular complications.

Other specific risk factors such as hypertension and adverse cholesterol profile have been associated with retinopathy and diabetic kidney disease (Hovind et al. 2004, Gheith et al. 2016) as well as macrovascular complications (Diabetes Control and Complications Trial/Epidemiology of Diabetes and Complications Research Group 2016). A recent large cohort study (27,195 T1D patients) demonstrated that younger age at onset of T1D was not only associated with increased macrovascular complications but also all-cause mortality as shown in Figure 1.19 (Rawshani et al. 2018).
Figure 1.19 Life years lost in relation to age at onset T1D. Loss of life years was estimated by use of separate Cox regression analyses fitted to individuals with T1D and their matched controls within each age group. Conditional medial survival was estimated from the upper limit of each age interval. Life-years lost because of diabetes were calculated as the difference in the expected median survival between people with T1D and controls. [Adapted from (Rawshani et al. 2018)]

Interestingly, complications are less likely to occur individually. Recent evidence has highlighted that micro- and macrovascular complications tend to co-occur (Bjerg et al. 2018, Sauder et al. 2019) with co-occurrence of T1D complications in 6% of adolescents and children, more than would be expected by chance alone (Sauder et al. 2019). Specifically, retinopathy with diabetic kidney disease, retinopathy with arterial stiffness, and arterial stiffness with cardiovascular autonomic neuropathy, co-exist most frequently in the ascending order (Sauder et al. 2019). Longer duration of diabetes, poor glycaemic control, non-Hispanic white and lower socioeconomic status were identified as predisposing risk factors. The presence of two complications increases the risk of developing another complication. (Bjerg et al. 2018).

In the last decade or so, diabetes mellitus has also been implicated with rarer known complications such as hearing impairment (Bainbridge et al. 2008, Malucelli et al. 2012, Schade et al. 2018) and skeletal fragility. The latter may be manifested as reduced bone mass, abnormal bone microarchitecture, increased bone marrow adiposity or fragility fractures, as detailed in the rest of this thesis.

1.5.3 Pathogenesis of altered bone metabolism

1.5.3.1 Hyperglycaemia

Hyperglycaemia contributes to diabetic osteopathy through a variety of effects on the bone cells. Acute hyperglycaemia causes direct glucose toxicity to osteoblast (Cunha et al. 2014)
affecting osteoblast numbers and function; suppresses genes involved in osteoblast maturation through osmotic and non-osmotic pathways (Zayzafoon et al. 2000, Botolin and McCabe 2006); and affects matrix mineralisation (Balint et al. 2001, Cunha et al. 2014). Chronic hyperglycaemia impairs osteoblast maturation from 1,25(OH)2D3-induced osteocalcin secretion by down regulation of vitamin D receptor (Inaba et al. 1995); and promotes osteoblast apoptosis through glucose autoxidation hydrogen peroxide-induced oxidative stress (Wolff et al. 1991, Fatokun et al. 2006). Hyperglycaemia-induced acidosis might also enhance bone resorption (Frassetto and Sebastian 2012). Emerging evidence suggests that hyperglycaemia may also alter the differentiation of the MSCs promoting adipogenesis instead of osteogenesis (Keats and Khan 2012). Hyperglycaemia diverts the MSC differentiation signalling pathways toward adipogenesis at the expense of osteogenesis. This effect is achieved via ERK-1/2-activated P13K/Akt-regulated pathway in mouse MSCs (Chuang et al. 2007), and via suppression of HO-1 expression (Barbagallo et al. 2010) and Wnt/protein kinase C non-canonical pathway in human MSCs (Keats and Khan 2012), evidenced by increased PPARγ expression in high glucose stimuli and reduction of osteocalcin levels. Indeed, mice with spontaneously and pharmacologically induced diabetes with bone loss have shown increased marrow adiposity, determined by histology and by mRNA markers of adipogenesis (PPARγ and aP2) (Botolin and McCabe 2007). Wang et al. demonstrated that hyperglycemia can also promote adipogenesis through synthesis of hyaluronan matrix which promotes an inflammatory response culminating in demineralisation of trabecular bone (Wang et al. 2014).

In addition, hyperglycemia leads to increased non-enzymatic protein glycation and excessive formation of these modified proteins called advanced glycosylation end-products (AGEs). AGEs appear to be the common biochemical entity accumulating in individuals with long term diabetic complications. They are seen in various sites such as vascular tissues, kidneys and bones (Bucala and Vlassara 1995, Yamamoto et al. 2001, Bohlender et al. 2005). AGEs exert their biological effects by engaging the receptor for AGEs (RAGEs), a multiligand receptor involved in immune and inflammatory responses (Schmidt et al. 1999, Chavakis et al. 2003). Consequently, AGEs, largely via RAGE, cause oxidative stress contributing to cellular dysfunction and ultimately damage target organs leading to complications such as atherosclerosis (Cipollone et al. 2003, Wendt et al. 2006) and nephropathy (Tanji et al. 2000, Wendt et al. 2003).

The accumulation of AGEs increases formation of collagen cross-links which results in increased stiffness of the collagen network in the cortical bone. This results in more brittle
bones that are less able to deform before fracturing. Accumulation of pentosidine, a well-characterised AGE, is negatively correlated to trabecular bone volume and structural strength (Schwartz et al. 2009, Silva et al. 2009). Bones of diabetic rats with higher content of pentosidine correlated negatively with BMD and bone strength (Tomasek et al. 1994, Saito et al. 2006). The pentosidine content in cortical and trabecular bones of people with femoral neck fracture is higher compared to post-mortem controls (Saito et al. 2006). Urinary and serum pentosidine is also increased in diabetic patients with vertebral fractures (Yamamoto et al. 2001, Schwartz et al. 2009). Iliac crest biopsies showed a significantly higher level of pentosidine in the trabecular bone of fracturing T1D patients compared to non-fracturing T1D and healthy controls (Farlay et al. 2016). In addition to affecting bone physical properties, AGEs also have direct effects on bone cells. Elevated AGEs can inhibit osteoblast proliferation and differentiation (Kume et al. 2005), function (Sanguineti et al. 2008), synthesis of type 1 collagen (Katayama et al. 1996) and osteoblast mineralisation (Ogawa et al. 2007). Okazaki et al. demonstrated that the inhibition of osteoblastic differentiation by AGEs can occur independently of hyperglycaemia (Okazaki et al. 2012). It is also associated with reduced osteocalcin mRNA expression as seen in rodents with diabetes (Tomasek et al. 1994). Furthermore, AGEs can also cause osteoblast death by provoking inflammation and generation of reactive oxygen species (ROS) which in turn lead to cell apoptosis as a result of oxidative stress (Coe et al. 2011b, Weinberg et al. 2014). The influence of AGEs on osteoclasts showed inconsistent effects with some evidence of increased (Miyata et al. 1997), as well as decreased (Valcourt et al. 2007) bone resorption.

Not only does hyperglycaemia and AGEs suppress osteoblastic differentiation and mineralisation, the expression of RAGE is also enhanced (Ogawa et al. 2007). More importantly, the AGE-RAGE interaction has been shown to affect bone quality with increased risk of vertebral fractures independent of BMD in T2D patients (Yamamoto et al. 2009).

1.5.3.2 Insulin, IGF-1 and other growth factors

Insulin has been shown to have an anabolic effect on bones that is distinct from the effect of its analogue IGF-1. This effect is mediated either indirectly through insulin regulation of renal 1alpha-hydroxylase activity and serum vitamin D levels in response to PTH (Hough et al. 1982, Ikeda et al. 1987), or directly on bone cells through specific insulin receptors (IRs) in osteoblasts. IRs are present on osteoblasts and its expression varies with differentiation status of the osteoblast (Levy et al. 1986, Thomas et al. 1996b). In vitro studies on primary
osteoblasts and clonal osteoblast cell lines show insulin promotes glucose uptake (Hahn et al. 1988), alkaline phosphatase activity, collagen synthesis (Canalis 1983, Pun 1989) and osteoblast proliferation (Wergedal and Baylink 1984) of these cells. Maor et al. demonstrated reduced IRs in the skeletal growth centres of mice with streptozotocin (STZ)-induced diabetes and these were reversed with insulin therapy (Maor and Karnieli 1999). Evidence for a direct link between insulin action and bone formation in vivo can be observed in IR knock out mice which show altered bone formation (Ferron et al. 2010), abnormal trabecular microarchitecture (Fulzele et al. 2010) and reduced bone strength (Thrailkill et al. 2014). Insulin positively regulates bone formation by stimulating osteocalcin production. Observational studies in people with T1D and T2D, with insulin deficiency and resistance respectively, demonstrate reduced osteocalcin levels compared with controls (Diaz-Lopez et al. 2013, Gower et al. 2013). Furthermore, there is a positive correlation between insulin dose and BMD (Weinstock et al. 1989, Fukunaga et al. 1997). The dichotomy of lower BMD in T1D with insulinopenia and higher BMD in T2D with clinical hyperinsulinemia further implicates a causal anabolic effect of insulin on bone.

The direct effect on bone may also be mediated through the IGF-1 pathway. IGF-binding protein (IGFBP) serves as a carrier protein for IGF-1. Insulin inhibits IGFBP-1 and IGFBP-4 expression in osteoblasts. Lack of insulin in T1D leads to an increase in these IGF-binding proteins hence reducing the availability of unbound IGF-1 for anabolic effects on bone (Conover et al. 1996). In addition to insulin deficiency, T1D individuals and animal models demonstrate dysregulation of a variety of endocrine factors including reduced amylin and IGF-1 (Rosen 2004, Hofbauer et al. 2007). Amylin is another osteotropic factor that is co-secreted by pancreatic beta-cells and absent in T1D (Horcajada-Molteni et al. 2001). Amylin-deficient mice displayed low bone mass with increased number of osteoclasts (Dacquin et al. 2004) and that treatment with amylin in diabetic rat result in increased BMD and bone strength (Horcajada-Molteni et al. 2001). Similarly, diabetic animals with low blood IGF-1 and knock-out mice for its receptor displayed diminished bone formation (Bouxsein et al. 2002, Zhang et al. 2002). Furthermore, serum IGF-1 level was shown to be an independent predictor of vertebral fractures in postmenopausal women with T2D (Kanazawa et al. 2011).

1.5.3.3 Calcium, Vitamin D and calcitropic hormones

It is well known that calcium homeostasis plays a major role in regulating bone metabolism, Therefore, imbalance in systemic factors which are capable of regulating calcium balance
have been found to influence diabetic bone loss (Zhang et al. 2011). Studies in diabetic animal models suggested reduced levels of calcium, phosphate and PTH (McCabe et al. 2011), with lower 1,25-dihydroxyvitamin D3 (Verhaeghe et al. 1990, McCabe et al. 2011) and vitamin-D binding proteins (Verhaeghe et al. 1990). Many studies have also shown vitamin D deficiency in T1D and T2D individuals albeit normal calcium, phosphate and low normal PTH (Frazer et al. 1981, Hampson et al. 1998). The effect of diabetes mellitus on calcium metabolism is complex, but essentially it is associated with a negative calcium balance hallmark by both bone and renal loss (Schwartz 2003). Children with T1D have been shown to have markedly reduced 1,25-dihydroxyvitamin-D but raised 24,25-dihydroxyvitamin-D, and this was associated with a decrease in cortical bone mass (Frazer et al. 1981). This could be explained by the altered vitamin D metabolic enzyme expressions and decreased expression of renal calcium transporter found in STZ-induced diabetic mice although these mice have reduced pQCT measured trabecular BMD and histologically poorer trabecular, instead of cortical, bone microstructure (Zhang et al. 2011). In support of this, treatment with calcitriol in STZ-induced rats demonstrated recovery of BMD (Del Pino-Montes et al. 2004). In terms of PTH, several in vivo and in vitro studies indicate that an imbalance in PTH was associated with bone loss and increased fracture risk (Wang et al. 2005, Motyl et al. 2012). PTH encourages bone formation and turnover by increasing the expression of osteocalcin, IGF-1, IGFBP-3, β-fibroblast growth factors (FGF), matrix metallopeptidases (MMP)-1 and MMP-13 in rat osteoblasts in vitro (Schmid et al. 1994), as well as MMP-13 in mice in vivo (Porte et al. 1999). Moreover, treatment with PTH to T1D and T2D mice demonstrated reversal of trabecular bone parameters through its anabolic effects on osteoblast differentiation and maturation (Motyl et al. 2012, Hamann et al. 2014). PTH-treatment was also associated with reduced TUNEL-staining of osteoblast suggesting its antiapoptotic effect on osteoblasts. It has been suggested that PTH promotes repair of DNA damage by increasing proliferating cell nuclear antigen (PCNA) and forkhead box protein O3a (FoxO3a) (Schnoke et al. 2009), hence prolonging osteoblast survival. Intermittent PTH treatment reversed bone loss to baseline in mice compared to only partial reversal in continuous PTH-treatment, although this effect may be due to underlying differences between the T1D and T2D mouse models (Motyl et al. 2012, Hamann et al. 2014). Similarly, treatment with PTH-related protein, which is produced at high levels by differentiating osteoblasts, also reverses trabecular bone loss in STZ-induced diabetic mice suggesting its modulatory effect on osteoblast function and role in diabetic osteopenia (Lozano et al. 2009).
1.5.3.4 Marrow adiposity and adipokines

There is a growing body of evidence that suggests that the bone-fat connection plays an important role in the pathophysiology of bone loss. Osteoblasts and adipocytes share a common precursor, both derived from the pluripotent MSCs located in the bone marrow suggesting a mutually exclusive and reciprocal lineage selection of one or the other. The main lineage-specific transcription factor which controls adipogenesis is PPARγ (Rosen and MacDougald 2006). In mice, there is evidence of increased PPARγ expression and an increase in visible adipocytes in tibia of T1D mouse models (Botolin et al. 2005) and increase in bone marrow adiposity in both spontaneously and STZ-induced T1D mice compared to controls (Botolin and McCabe 2007, Lozano et al. 2009, Motyl et al. 2011). Rzonca et al. demonstrated in vivo that rosiglitazone (PPARγ agonist) administration results in significant bone loss (Rzonca et al. 2004) whilst Cock et al. demonstrated that congenitally PPARγ deficient mice had increased BMD and bone trabecular microarchitecture parameter including BV/TV and trabecular thickness (Cock et al. 2004). However, Botolin and McCabe found that inhibition of PPARγ by administration of an antagonist to insulin deficient T1D mice prevented bone marrow adiposity but not bone loss (Botolin and McCabe 2007). This inability in preventing bone loss in T1D mice suggested that bone marrow adiposity may only be partially responsible for diabetic bone loss. Bone marrow adiposity can also have direct effects on osteoblasts as demonstrated by several co-culture studies in mouse and human cells (Maurin et al. 2000, Elbaz et al. 2010, Coe et al. 2011a). The presence of adipocytes inhibits osteoblast proliferation (Maurin et al. 2000) through the lipotoxic effect of free fatty acid in the bone marrow microenvironment [81].

There have been limited studies looking at marrow fat content in diabetes individuals to date with paucity in those with T1D. Most clinical studies of bone marrow adiposity in T2D women have shown no difference in marrow fat content between cases and controls, although T2D women consistently had lower unsaturated lipids (Baum et al. 2012, Patsch et al. 2013b, Kim et al. 2017). Sheu et al demonstrated higher BMF content in men with T2D, although this became insignificant after excluding two individuals on thiazolidinediones (PPARγ agonists) (Sheu et al. 2017, Yu et al. 2017). However, a new study published has now demonstrated that the bone marrow in adults with T2D have increased fat accumulation compared to controls (Ferland-McCollough et al. 2018), with diabetes itself causing the preferential differentiation of the bone marrow stem cells into adipocytes. Importantly, a positive correlation between HbA1c and marrow fat content has been shown suggesting that BMA may influence or be influenced by glucose metabolism and glycaemic control (Baum et al. 2012).
In addition to releasing large amounts of free fatty acid, adipocytes in the bone marrow also secrete cytokines including leptin, adiponectin, monocyte chemoattractant protein-1 (MCP-1) and TNFα, the last of which will be discussed in the next section. Leptin is an adipokine which has a complex regulatory role on bone metabolism, with an indirect inhibitory effect on osteoclastogenesis and a direct stimulatory effect on osteoblastogenesis through a central (hypothalamic) and peripheral pathway (Steppan et al. 2000, Reseland et al. 2001, Cornish et al. 2002). Despite being an adipokine, in vitro studies indicate that leptin promotes an osteoblast rather than adipocyte lineage (Reseland et al. 2001, Cornish et al. 2002). In contrast to T1D patients who demonstrate increased (Luna et al. 1999) or slightly decreased leptin level (Karaguzel et al. 2006), leptin levels were found to be significantly suppressed in T1D mice, its absence results in reducing bone mass with increasing marrow adiposity (Steppan et al. 2000). T2D is typically associated with obesity, which has been associated with higher leptin level and higher BMD. Vasilkova et al., however, demonstrated that leptin has an independent positive correlation to BMD, irrespective of BMI (Vasilkova et al. 2011). In addition, interventional studies in mice have demonstrated that leptin administration reduces bone marrow adiposity and increases bone mass (Hamrick et al. 2005, Martin et al. 2005). However, similar to their work on PPARγ antagonist, Motyl and McCabe concluded that leptin administration to T1D mice modify and prevent marrow adiposity but did not prevent diabetic bone loss (Motyl and McCabe 2009).

Adiponectin (APN), the most abundant adipokine secreted exclusively by adipocytes, regulates energy homeostasis and exerts well-characterized insulin sensitizing properties. The insulin-sensitising effect of OC is known be due to the upregulation of APN gene in adipocytes (Lee et al. 2007) but there is some suggestion that in humans the link between OC and APN may be gender specific (Buday et al. 2013). APN seems to have an anabolic effect on osteoblasts and an inhibitory effect on osteoclasts in vitro (Williams et al. 2009). The peripheral and central effects of APN on bone metabolism are beginning to be explored but are still not clearly understood. APN-knockout (APN-KO) mice fed a normal diet exhibit decreased trabecular structure and mineralisation and increased bone marrow adiposity whilst central administration of APN into the brain ventricles decreased osteoclast numbers and increased osteoblastogenic marker expression and trabecular bone mass, in both APN-KO and WT mice (Wu et al. 2014). Clinically, serum APN levels have been shown to be positively associated with BMD at the distal radius in Japanese individuals with T2D, although the evidence linking adiponectin to bone mass is conflicting with some studies showing an inverse relationship (Lenchik et al. 2003, Tamura et al. 2007, Napoli et al. 2010).
1.5.3.5 Chronic inflammation

The pathogenesis of both T1D and T2D are associated with activation of the immune system, especially so in T1D which involves an autoimmune destruction of pancreatic β-cells whilst T2D involves a more chronic low-grade inflammatory process (Bending et al. 2012, Esser et al. 2014). Several experimental studies using T1D mice model indicate that systemic and local inflammatory cytokines are increased at the onset of diabetes with rapid suppression of osteoblast markers and increase in adipocyte markers, indicating that bone inflammation may be another contributing factor to the diabetic bone pathology (Motyl et al. 2009, Coe et al. 2011a, Roszer 2011). Serum cytokine levels (TNFα, IFNγ, IL-1Rα and lymphotoxin-β) and corresponding bone cytokine mRNA expression were increased from as early as 5 days after induction of diabetes in mouse models (Botolin and McCabe 2007, Motyl et al. 2009) with decrease in osteocalcin mRNA expression in bone RNA extracts, and it remains suppressed at 40 days postinduction of diabetes in the mouse models. These inflammatory cytokines can either directly suppress osteoblast differentiation (Gilbert et al. 2000), promote osteoblast death directly (Coe et al. 2011a) or indirectly by contributing to elevation of ROS causing osteoblast apoptosis from oxidative stress (Hamada et al. 2009, Coe et al. 2011b), and activate osteoclastogenesis (Glantschnig et al. 2003). Coe et al. also found that TNFα in the bone marrow microenvironment directly mediates osteoblast death with increase in expression of proapoptotic factors and osteoblast TUNEL staining, further contributing to T1D bone loss (Coe et al. 2011a). Inhibition of the cytokines with TNFα neutralizing antibodies prevented osteoblast apoptosis (Coe et al. 2011a) but transgenic mice with IFNγ KO proceeded to have diabetic bone pathology (Motyl et al. 2009), supporting the idea that diabetic inflammatory bone loss involves an interplay of more than one cytokine and/or a combination of other factors. Apart from proinflammatory cytokines, abnormal hyaluronan production in bones of diabetic rodents also induced monocyte and macrophage infiltration into the bone collagen matrix, promoting adipogenesis at the expense of osteogenesis (Wang et al. 2014). The concept of T2D as an inflammatory disease is relatively new with increased fat depots in T2D implicated as the source for more proinflammatory cytokines and adipokines (Esser et al. 2014). The cytokine MCP-1, secreted by mature adipocytes in the bone marrow, acts in a paracrine manner, further promoting MSC differentiation into adipocytes (Ferland-McCollough et al. 2018). However, a large prospective population study (EPIC) in Germany found that systemic inflammatory markers are independent predictors for the development of T2D, independent of degree of insulin resistance and obesity (Spranger et al. 2003).
### 1.5.3.6 Vasculopathy

Similar to other diabetic microvascular complications of retinopathy, nephropathy and neuropathy, bone microangiopathy has been insinuated as another possible mechanism for diabetic bone loss. In hypoxic condition (2% oxygen), the bone marrow shifts toward adipogenic lineage by enhancing expression of genes associated with adipogenic/lipogenic phenotype (C/EBPβ, PPARγ2, and aP2) and by suppressing expression of genes associated with osteoblast differentiation (AP) (Irwin et al. 2007). Oikawa et al. found that the cumulative vascular density was reduced by threefold in bone marrow of STZ-induced diabetic mice compared to control, along with reduction in blood flow (Oikawa et al. 2010). In addition, there is also evidence of reduction in bone marrow volume and bone marrow remodeling with cell depletion mainly affecting the osteoblastic niche secondary to hypoperfusion and oxidative stress (Oikawa et al. 2010). Correspondingly, boosting the antioxidative pentose phosphate pathway with benfotiamine supplementation prevented microangiopathy and hypoperfusion in the bone marrow with reduction in cell apoptosis, providing further compelling evidence for vasculopathy in diabetic bone pathology. Regenerating mouse tibia has reduction in blood vessels with lower expression of vascular endothelial growth factor (VEGF), a signalling protein which regulates angiogenesis, and its receptor (Lozano et al. 2009). Clinically, histomorphometric evaluation of iliac crest bone biopsy in 118 diabetic patients revealed evidence of diabetic bone microangiopathy in 82% with significant osteopenia and reduction in the sinusoidal capillaries (Burkhardt et al. 1981).

### 1.5.3.7 Anti-diabetic medications

Different classes of anti-diabetic medication, such as thiazolidinediones (TZDs), metformin and glycogen-like peptide 1 (GLP-1) inhibitors have been reported to have varying effects on bone.

#### 1.5.3.7.1 Insulin

Although insulin is considered to have an anabolic effect on bones, individuals with T1D on higher insulin dose have been shown to have lower BMD (Leger et al. 2006, Eller-Vainicher et al. 2011) whilst individuals with T2D on higher insulin dose have been shown to have increased risk of falls from hypoglycaemic attacks resulting in fractures (Monami et al. 2008, Johnston et al. 2012). The positive correlation between daily insulin dose and HbA1c (Hofbauer et al. 2007) may help explain the negative correlation between daily insulin dose with BMD, reflecting possibly an issue with compliance or possibly the presence of a more
severe disease (more inflammatory processes) in T1D (Leger et al. 2006). Findings from the HABC study showed that T2D insulin-treated patients with HbA1c ≤ 6% had an increased risk of falls (Schwartz et al. 2008). This finding should be interpreted with caution as T2D patients on insulin usually had longer duration of diabetes and other concurrent microvascular complications such as retinopathy and neuropathy which predispose to falls. Nephropathy with impaired renal function also has a linear correlation to falls (Schwartz et al. 2008), possibly as impaired renal function may interfere with vitamin D metabolism resulting in reduced muscle strength.
Figure 1.20 Pathophysiological mechanisms of diabetic bone disease. Type 1 (T1D) and Type 2 Diabetes (T2D) mellitus affect bone turnover, bone mineral density and bone quality through a complex interplay of hyperglycaemia, insulinopenia, GH/IGF-1 deficit, chronic inflammation and vasculopathy to name a few, ultimately leading to osteoporosis and increased fracture risk. IGF-1 insulin growth factor-1, PTH parathyroid hormone, MVCs microvascular complications, AGEs advanced glycation end products, ROS reactive oxygen species, TNFa tumour necrosis factor-alpha, IFNy interferon-gamma, IL(1,6,8) interleukin-(1,6,8), MCP-1 monocyte chemoattractant protein-1, MSCs mesenchymal stem cells, BMD bone mineral density. Solid lines represent pathways affecting both T1D and T2D, with broken lines specific to T2D only.
1.5.3.7.2 Thiazolidinediones

TZDs such as pioglitazone and rosiglitazone have been shown to increase adipocyte differentiation and decreased osteoblast differentiation through activation of PPARγ activity (Rzonca et al. 2004, Ali et al. 2005, Schwartz 2008). Consistent with these preclinical observations, a comprehensive meta-analyses of 10 randomised controlled-trials (total 13,715 participants) and 2 observational (total 31,679 participants) studies, clearly indicated an increased risk of fractures (OR 2.23, 95% CI 1.65-3.01) with TZD (both pioglitazone and rosiglitazone) use, particularly in women (Loke et al. 2009). A more recent meta-analysis, including 22 randomised controlled-trials (total 24,544 participants) further confirmed this gender difference in fracture risk (OR 1.94, 95% 1.6-2.35 in women vs OR 1.02, 95% CI 0.83-1.27 in men), which is independent of age and duration of TZD exposure (Zhu et al. 2014). Use of this medication should therefore be avoided in women.

1.5.3.7.3 Metformin

Metformin is the standard first line pharmacologic treatment for T2D. It exerts a direct osteogenic effect in vitro by stimulating proliferation and differentiation of osteoblasts (Cortizo et al. 2006, Gao et al. 2008, Kanazawa et al. 2008, Ma et al. 2010a, Jang et al. 2011) through preventing AGE-induced deleterious effects in osteoblastic cells (Schurman et al. 2008) and various other signalling pathways including the PI3K/Akt pathway (Ma et al. 2010b), ERK-1/2 (Cortizo et al. 2006) and AMPK activation (Kanazawa et al. 2008, Jang et al. 2011). Zhen et al. also demonstrated an indirect effect of metformin on osteoblast survival through reduction of intracellular ROS (Zhen et al. 2010). In addition to its osteoblastogenic potency in vitro, metformin can further prevent bone loss by inhibiting osteoclastic differentiation (Mai et al. 2011). However, ovariectomized rodents treated with metformin demonstrated inconsistent radiological findings of no change in bone microarchitecture (Jeyabalan et al. 2013) to increased BMD (Mai et al. 2011). In clinical studies however, metformin has been shown to have a neutral (Monami et al. 2008, Colhoun et al. 2012, Napoli et al. 2014) or positive (Vestergaard et al. 2005, Zinman et al. 2010) effect on bone metabolism. One of the larger observational studies (total 498,617 participants) reported that metformin was associated with a decreased risk of any fractures (HR 0.81; 95 % CI, 0.70–0.93) (Vestergaard et al. 2005). The only randomised trial, ADOPT (total 4351 participants, 1840 women) designed to compare metformin with rosiglitazone and glyburide found that the cumulative incidence of fracture is 1.2 per 100 patient years for metformin and glyburide but up to 9.3 per 100 in women for rosiglitazone (Kahn et al. 2006). To date, only one study has investigated the role of metformin in young patients. In the
Treatment Options for type 2 Diabetes in Adolescents and Youth (TODAY) clinical trial, 699 patients aged 10–17 years with T2D were divided into three different treatment groups: metformin plus rosiglitazone; metformin alone; metformin plus lifestyle. BMD and BMC increased in all groups, but increased less in metformin plus rosiglitazone compared with the other two groups after 24 months (0.06 g/cm2 in metformin plus rosiglitazone vs 0.076 g/cm2 in metformin plus lifestyle; \( p=0.0041 \)) (Today Study Group 2013).

1.5.3.7.4 Sulphonylureas

Sulphonylureas such as glimepiride and glyburide are anti-hyperglycaemic drugs which cause increased secretion of insulin by binding to the \( K_{ATP} \)-channel on the cell membrane of pancreatic beta cell. Consequently, these agents have been reported to cause increased risk of hypoglycaemia (Monami et al. 2008) and increased fracture risk. Similar to metformin, sulfonylureas have been reported to have either neutral (Melton et al. 2008, Monami et al. 2008) or a reduction in risk of fractures in patients treated with these agents (Vestergaard et al. 2005, Zinman et al. 2010). The ADOPT RCT showed comparable cumulative incidence of fractures to metformin of 1.2 per 100 patient years, with 5 year cumulative incidence of fractures (95 % CI) of 5.7 % (3.9–7.6) (Kahn et al. 2006). A large population-based case–control study in Denmark (total 124,655 cases with 3:1 age-and gender matched controls of 373,962) found that treatment with sulfonylureas was associated with a significant decrease in the risk of hip fracture (HR 0.74, 0.58–0.93) versus patients with T2D without treatment (Vestergaard et al. 2005). Given the high rate of hypoglycaemia with sulphonylureas, this group of antidiabetic drug should be used with caution in those at higher risks of fragility fractures.

1.5.3.7.5 Incretin-based therapies

Incretins are a group of gastrointestinal hormones which act to lower blood glucose levels by inhibiting glucagon release. It also reduces gastric emptying and can help to increase the secretion of insulin from pancreatic beta cells. The two main groups are the glucagon-like peptide-1 (GLP-1) and dipeptidyl peptidase-4 (DPP-4) inhibitor, the latter is an enzyme which inactivates GLP-1 (Drucker and Nauck 2006). Three meta-analyses have looked at the fracture incidence with GLP-1 and DPP-4 inhibitors showing contradicting and favourable effects respectively on bone. GLP-1 liraglutide has been associated with reduced fracture risk (OR 0.38, 95% CI 0.17-0.87) whilst exenatide was correlated with increased fracture risk (OR 2.09, 95% CI 1.03-4.21) (Su et al. 2015). Although a meta analyses of DPP-4 inhibitors have shown a reduction in fracture risk (OR 0.60, 95% CI 0.37-0.99)
Monami et al. (2011), more recent studies have demonstrated varying results with sitagliptin reported to have anabolic effects on the bone (Hegazy 2015) but not with saxagliptin in the SAVOR-TIMI trial (Mosenzon et al. 2015).

1.5.3.7.6 Sodium/glucose co-transporter 2 (SGLT-2)

SGLT-2 such as dapagliflozin and canagliflozin are a new generation of antidiabetic drugs which work to reduce hyperglycaemia by inhibiting renal reabsorption of glucose from the proximal tubules (DeFronzo et al. 2012, Ljunggren et al. 2012, Bolinder et al. 2014). Dapagliflozin and empagliflozin seem to have a neutral effect on bone metabolism, with no significant changes in bone turnover or BMD parameters (Ljunggren et al. 2012). Concerns have been raised for canagliflozin, which might cause bone loss at the hip (Bilezikian et al. 2016, Watts et al. 2016) and increase the risk of hip fractures (Watts et al. 2016).

1.5.4 Clinical evidence of fractures

1.5.4.1 Type 1 Diabetes (T1D)

Adults with T1D have been shown to have a significantly higher risk of hip fractures, of eight- to 18-folds compared to the general population (Miao et al. 2005, Ahmed et al. 2006). Two large meta-analyses published following that confirmed the increased hip fracture risk of 6.9- and 6.3-folds respectively compared to individuals without T1D (Janghorbani et al. 2007, Vestergaard 2007). In a large observational study in Scotland (total of 21,033 individuals with T1D) a consistently increased but lower relative risk of hip fracture, of 3.2 in men and 3.5 in women, was reported (Hothersall et al. 2014). A more recent meta-analysis (total 27,300 individuals with T1D) further validated this lower relative fracture risk of over three-folds in all fractures, and not just hip alone (Shah et al. 2015). Fractures at the spine, although less marked (OR 2.5, 95% CI 1.3-4.6), are also moderately increased in the T1D population (Vestergaard et al. 2005). Zhukouskaya et al reported a significantly higher risk of morphometric vertebral fractures, assessed by VFA, in young adults with T1D (24% vs 6% in healthy controls) with a meta-analysis reporting a pooled relative risk of 2.88 (95% CI 1.71-4.82) in individuals with T1D (Zhukouskaya et al. 2013, Shah et al. 2015).

Another most disconcerting finding is that the increased risk of fracture extends across all ages, affecting even young adults and children with T1D, with widening discrepancy in the risks from age 40 years. (Hothersall et al. 2014, Weber et al. 2015). Studies have shown an almost linear association between duration of disease (T1D) and fracture risk (Nicodemus et al. 2001, Miao et al. 2005), although this was contradicted by others (Ahmed et al. 2006).
There is an intriguing proposal of a bimodal relationship with the highest incidence of fracture occurring in the first 2.5 years of diagnosis and again beyond 5 years of diabetes being diagnosed (Vestergaard et al. 2005). Most studies, but not all (Neumann et al. 2011), have found no association between glycaemic control, as measured by HbA1c, with risk of fracture. Many studies have invariably reported an association between fracture risk and the presence of all types of diabetic complications (Miao et al. 2005), and more specifically retinopathy (Ivers et al. 2001, Weber et al. 2015), nephropathy (Miao et al. 2005), neuropathy (Miao et al. 2005, Weber et al. 2015) and cerebrovascular disease (Strotmeyer et al. 2005).

Lower extremity fractures (hip/femur, lower leg/ankle and foot) were more common than upper extremity/vertebral fractures in those with T1D (31.1% vs 25.1% in males, 39.3% vs 32% in females; p<0.001) (Weber et al. 2015).

1.5.4.2 Type 2 Diabetes (T2D)

T2D has also been associated with increased risk of fractures. The Study of Osteoporotic Fractures (SOF) cohort found that women with T2D have an increased risk of hip and proximal humeral fractures (RR 1.82, 95% CI 1.24-2.69 and RR 1.94, 95% CI 1.24-3.02) compared to the non-diabetic population (Schwartz et al. 2001). A meta-analysis of eight prospective studies for the hips and three studies for the wrists in adults with T2D concluded that the relative risk of hip and wrist fractures were 1.4 and 1.2, respectively with no increase in vertebral fracture risk (RR 0.93, 95% CI 0.63-1.37) (Vestergaard 2007). Another meta-analysis published in the same year of 16 epidemiologic studies, of which 4 investigated vertebral fractures, substantiated the neutral vertebral fracture risk but found a slightly higher relative risk of hip fracture of 1.8. This relative risk rose to 2.7 (95% CI 1.7-4.4) when the analysis was restricted to four cohort studies with >10 years of follow-up (Janghorbani et al. 2007). In fact, a history of T2D was shown to be the single most important and independent predictor of low-energy subtrochanteric and diaphyseal femoral fractures (Napoli et al. 2013). Subsequent to the meta-analysis, more studies have suggested than T2D may also be associated with higher vertebral fracture risks with up to 23% of vertebral fractures reported in post-menopausal women (Ardawi et al. 2013). A Japanese cohort study (total participants 996, 1 case:3 controls) further elucidated a gender preponderance with men (OR 4.7, 95% CI 2.19-10.20) more likely to have vertebral fractures than women (OR 1.9 (95% CI 1.11-3.12) (Yamamoto et al. 2009, Viegas et al. 2011). Fractures of the wrists (de et al. 2005) and
foot (Schwartz et al. 2001, Bonds et al. 2006) also seemed to be more prevalent in individuals with T2D than the general population.

Duration of T2D, glycaemic control, anti-diabetic medications, propensity to fall and T2D-related complications were observed as possible predicting factors for the increased fracture risk in these studies. Cross-sectional studies have indicated that those with increased fractures are older (Nicodemus et al. 2001, Janghorbani et al. 2007, Yamamoto et al. 2008) and have a longer duration of T2D (Yamamoto et al. 2008, Viegas et al. 2011). The incidence was also higher in those treated with insulin (Janghorbani et al. 2007, Melton et al. 2008). In T2D, falls are common (Schwartz et al. 2001, Schwartz 2008) and the risk of falling may be higher in those with poor balance, arthritis, cardiovascular disease, depression, poor vision and use of medications for sleeplessness or anxiety (Schwartz et al. 2001). It is unclear whether this increased risk of fall is also associated with more fractures. The presence of microvascular complications have also been associated with increased risk of fractures, in particular neuropathy (Melton et al. 2008) and retinopathy (Viegas et al. 2011).

1.5.5 Bone turnover studies

1.5.5.1 T1D

T1D is associated with a low bone turnover state with dissociation between bone formation and bone resorption (Starup-Linde 2013). Both pharmacologically-induced and spontaneously diabetic NOD mice with chronic disease have impaired bone formation and resorption, with concomitant histomorphometric data (Hough et al. 1981, Hough et al. 1983). In humans, the marker of bone formation, osteocalcin has been found to be low in both adults and children with T1D (Bouillon et al. 1995). A recent large meta-analysis further substantiated this with significantly lower osteocalcin -1.51 (-2.76, -0.26) ng/ml) in individuals with diabetes compared to non-diabetes controls. Subgroup analysis demonstrated specifically that the osteocalcin level was depressed in T1D, but not in T2D (Starup-Linde et al. 2014). Glycaemic control, as measured by HbA1c was found to be negatively correlated to the osteocalcin levels (Adami 2009, Maggio et al. 2010). The level of another bone formation marker, bone alkaline phosphatase (BAP) has been found to be more variable with studies showing a suppressed level (Lumachi et al. 2009) and a meta-analysis showing an increased BAP compared to controls (Masse et al. 2010, Starup-Linde et al. 2014). This imbalance of bone formation markers (increased BAP, decreased osteocalcin) may reflect an impairment of osteoblast differentiation and maturation as BAP is expressed early in the development of osteoblast whilst osteocalcin is released from the
mature osteoblast. Studies on the markers of bone resorption were conflicting with either normal (Alexopoulou et al. 2006) or reduced (Campos Pastor et al. 2000, Maggio et al. 2010, Abdalrahaman et al. 2015) circulating levels in individuals with T1D. Pooled data from the meta-analysis concluded that bone resorption is depressed with reduced CTX at -0.14 (-0.22,-0.05) ng/ml in individual with diabetes mellitus, but no sub-analyses by diabetes type was available to distinguish whether bone resorption differed between T1D and T2D. The low bone turnover state appears to be present from the onset of T1D in childhood with normalisation after 3 months (Pater et al. 2010), which may be a function of concurrent improvement in the HbA1c with insulin treatment, as adults with T1D continued to have lower osteocalcin, BAP and CTX compared to controls as detailed above. Bone histomorphometry data in T1D is scarce. One study in two patients with T1D showed markedly depressed bone formation compared to non-diabetics (Krakauer et al. 1995) but a larger case-control study of 18 T1D patients showed no difference in bone metabolism between the groups (Armas et al. 2012). Further sub-analyses of the T1D patients demonstrated a significantly depressed bone formation in the small group who had fractures compared to those without fractures (Armas et al. 2012). The incorporation of bone turnover markers into the FRAX model has not explained the increased fracture risk in diabetes patients compared to healthy controls (Bhattoa et al. 2013) so at present, bone turnover markers are of limited use in fracture prediction. Heterogeneity between all the studies such as the differences in patient characteristics (age, ethnicity, disease duration, glucose control) and the discrepancies in timing of serum collection and assay variations may have influenced the results.

Sclerostin, produced almost exclusively by osteocytes, is an inhibitor of the canonical Wnt/β-catenin signalling pathway involved in bone formation, thus playing an essential role in osteoblast differentiation and bone turnover (Baron and Kneissel 2013). In animal models, overexpression of SOST gene causes osteopenia and limb defects (Loots et al. 2005, Baron and Kneissel 2013), while lack of SOST causes 3–4 times more bone mass, consistent with human phenotypes in a condition called sclerosteosis (Collette et al. 2012). Many studies have found that sclerostin levels were higher in patients with T1D (Catalano et al. 2014, Neumann et al. 2014) compared to controls, with gender predilection for higher levels in females than males (Catalano et al. 2014). The duration of diabetes has also been shown to positively correlate with higher sclerostin levels, further impairing bone health and increasing fracture risk. T1D mice subjected to sclerostin-neutralising antibody treatment demonstrated reversal in the osteopenia, reduced bone marrow adipogenesis and improved fracture outcome (Yee et al. 2016). Intriguingly however, sclerostin levels have been shown
to be inversely associated with fracture in T1D patients (Starup-Linde et al. 2016). T1D patients with the highest tertile of sclerostin had an 81% decreased risk of fracture compared with the lowest tertile which is counterintuitive and remains unexplained. The reproducibility of this study or further mechanistic explanation has yet to be seen.

1.5.5.2 T2D

Similar to T1D, individuals with T2D are reported to have suppressed bone turnover. Bone formation is consistently lower in people with T2D, as evidenced by lower serum osteocalcin, BAP and procollagen type 1 N-terminal propeptide (P1NP) levels (Dobnig et al. 2006, Gennari et al. 2012) with suppressed bone resorption as demonstrated by lower CTX levels compared to healthy controls (Dobnig et al. 2006, Gennari et al. 2012, Reyes-Garcia et al. 2013). A large meta-analysis, however, did not show a significant difference ($p=0.06$) in osteocalcin in T2D individuals compared to healthy controls (Starup-Linde et al. 2014) and did not analyse CTX by subtype of diabetes hence it remains unclear whether bone resorption is affected in T2D. In accordance with the former, bone histomorphometry demonstrated reduced histologic measures of mineralising surface and bone formation rate (Manavalan et al. 2012). Serum PTH tends to be lower (Dobnig et al. 2006, Garcia-Martin et al. 2012, Reyes-Garcia et al. 2013) in T2D. The relative (moderate or subclinical) hypoparathyroidism in diabetes patients, with normal calcium and phosphate levels, could contribute to the low bone turnover state as the levels of osteocalcin and CTX have been shown to correlate with PTH levels (Yamamoto et al. 2012, Reyes-Garcia et al. 2013).

Increased sclerostin levels have been found in patients with T2D compared to healthy controls (Garcia-Martin et al. 2012, Gaudio et al. 2012, van Lierop et al. 2012). The elevated sclerostin levels were associated with osteoporotic fractures (Ardawi et al. 2012, Heilmeier et al. 2015) in postmenopausal women and also importantly, an increased risk of vertebral fractures (Ardawi et al. 2012) in T2D patients of both genders independently of BMD.
1.5.6 Bone imaging studies

1.5.6.1 T1D

Imaging studies have provided the wealth of evidence linking diabetes mellitus, both T1D and T2D, with impaired bone health. The effects of T1D on bone health may be manifested as aberrant bone mass (Gunczler et al. 1998, Heap et al. 2004, Saha et al. 2009b) and abnormal bone microarchitecture (Abdalrahaman et al. 2015), predisposing ultimately to the clinically significant increased risk of fractures (Vestergaard 2007, Hothersall et al. 2014) in this population.

1.5.6.1.1 Bone mineral density (BMD)

Almost universally, using the more sensitive modern dual energy X-ray absorptiometry (DXA) scanners, studies have shown reduced BMD in adults (Campos Pastor et al. 2000, Strotmeyer et al. 2005, Rakic et al. 2006, Mastrandrea et al. 2008, Eller-Vainicher et al. 2011) and children (Gunczler et al. 1998, Valerio et al. 2002, Leger et al. 2006, K. Heilman et al. 2009) with T1D at either lumbar spine (LS), femoral neck (FN) or total body (TB), bar a few exceptions (Lunt et al. 1998, Pascual et al. 1998, Ingberg et al. 2004, Bridges et al. 2005). All major studies reporting on bone densitometry in children with T1D have been summarised in Table 1.6. The studies which showed no difference in BMD between diabetic and non-diabetic population were methodically suboptimal, in not adjusting for group differences in BMI (Lunt et al. 1998) as BMI is a very important determinant of BMD or taking into account size correction for DXA interpretation in children (Pascual et al. 1998). The reduction in BMD varied markedly from 8-67%, with a reported pooled estimated LS BMD Z-score of -0.22 ± 0.01 and FN BMD Z-score -0.37 ± 0.16 (both p<0.05) in a meta-analysis comparing individuals with T1D and without (Vestergaard 2007). Lower BMD was associated with the presence of microvascular complications although further meta-regression showed that neither duration of disease nor glycaemic control, as measured by HbA1c, was associated with BMD (Vestergaard 2007). Several studies have demonstrated a reduction in BMD from the onset of T1D diagnosis (Gunczler et al. 2001, Lopez-Ibarra et al. 2001) suggesting a predisposing variant rather than chronic metabolic consequences of the disease on bones.
Table 1.6 Bone densitometry studies in children with T1D, based on dual-energy x-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT).

<table>
<thead>
<tr>
<th>Author, year</th>
<th>n (F/M)</th>
<th>Age (yrs)†</th>
<th>Disease duration (yrs)†</th>
<th>Modality</th>
<th>Site</th>
<th>Bone density findings</th>
<th>Additional findings of bone-related parameters/Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettgen et al. 1995</td>
<td>21</td>
<td>NA</td>
<td>NA</td>
<td>pQCT</td>
<td>NA</td>
<td>↓Trabecular and cortical vBMD ↓TB BMD</td>
<td>Trabecular vBMD inversely associated with disease duration. TB BMD inversely correlated with HbA1c</td>
</tr>
<tr>
<td>Gunczler et al. 1998</td>
<td>26 (11/15)</td>
<td>12.1±3.1</td>
<td>4.3±2.9</td>
<td>DXA</td>
<td>TB,LS,FN</td>
<td>↓LS BMD</td>
<td>DXA interpretation not size-corrected. No association with disease duration or HbA1c.</td>
</tr>
<tr>
<td>Gunczler et al. 2001</td>
<td>23 (16/7)</td>
<td>9.5±2.2</td>
<td>0.5±0.1</td>
<td>DXA</td>
<td>TB,LS,FN</td>
<td>↓LS BMD</td>
<td>Matched to age and height. All prepubertal and newly diagnosed T1D.</td>
</tr>
<tr>
<td>Heap et al. 2004</td>
<td>55 (25/35)</td>
<td>M 14.6±1.7 F 14.7±1.9</td>
<td>5.8±4.3</td>
<td>pQCT</td>
<td>Tibia, Radius</td>
<td>↓Trabecular and cortical vBMD ↓TB and FN BMD</td>
<td>BMD adjusted for LBM. Trabecular vBMD and TB BMD inversely associated within HbA1c.</td>
</tr>
<tr>
<td>(Moyer-Milieuer et al. 2004)</td>
<td>42 (16/26)</td>
<td>M 16.0±1.7 F 15.1±1.8</td>
<td>4.2±3.1</td>
<td>pQCT</td>
<td>Tibia, Radius</td>
<td>↓Cortical vBMD, ↓TB and LS BMD</td>
<td>BMD adjusted for LBM and LBM-for-height. Lower annual gain of tibia cortical bone and TB BMD; inverse association to HbA1c.</td>
</tr>
<tr>
<td>(Leger et al. 2006)</td>
<td>127 (54/73)</td>
<td>12.9 (10.2-15.2)</td>
<td>5.6(4.6-8.8)</td>
<td>DXA</td>
<td>TB,LS</td>
<td>↓ TB vBMD</td>
<td>Adjusted for LBM and LBM-for-height. Girls have lower LS and TB BMC than boys. Lower IGF1 in T1D, but no correlation to HbA1c or disease duration.</td>
</tr>
<tr>
<td>(Bechtold et al. 2006)</td>
<td>88 (42/46)</td>
<td>11.7±3.0</td>
<td>5.6±3.7</td>
<td>pQCT</td>
<td>Radius</td>
<td>↔ Trabecular and cortical vBMD ↓Cortical BMD, ↓TB BMD.</td>
<td>Early age at T1D diagnosis associated with reduced total, cortical and muscle CSA. No difference with HbA1c or disease duration. NB: Higher weight and BMI in T1D cohort.</td>
</tr>
<tr>
<td>(Bechtold et al. 2007)</td>
<td>41 (20/21)</td>
<td>15.4±2.3</td>
<td>9.8±2.8</td>
<td>pQCT</td>
<td>Radius</td>
<td>↔ Cortical vBMD</td>
<td>Normalisation of total, cortical and muscle CSA. Reduced trabecular vBMD but no comment to reference population. NB: Higher weight and BMI in T1D cohort.</td>
</tr>
<tr>
<td>(Brandao et al. 2007)</td>
<td>44 (22/22)</td>
<td>8.8±4.4</td>
<td>6.6±3.9</td>
<td>DXA</td>
<td>LS (L1-L4)</td>
<td>↔ LS BMD</td>
<td>66% prepubertal. Negative association of disease duration and HbA1c with BMD only in girls.</td>
</tr>
<tr>
<td>(Mastrandrea et al. 2008)</td>
<td>37 (37/0)</td>
<td>16.2±1.8</td>
<td></td>
<td>DXA</td>
<td>TB,LS,FN</td>
<td>↔ all BMD.</td>
<td>Adjusted for age, BMI and OC use. Trend of lower TB BMD in T1D. No correlation to HbA1c or disease duration.</td>
</tr>
<tr>
<td>Author, year</td>
<td>n (F/M)</td>
<td>Age (yrs)†</td>
<td>Disease duration (yrs)†</td>
<td>Modality</td>
<td>Site</td>
<td>Bone density findings</td>
<td>Additional findings of bone-related parameters/Other comments</td>
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<tr>
<td>(Moyer Mileur et al. 2008)</td>
<td>11 (11/0)</td>
<td>12.9±1.0</td>
<td>5.9±3.7</td>
<td>pQCT</td>
<td>Tibia</td>
<td>↓ Cortical vBMD</td>
<td>Adjusted for height for age, BMC to BA. Lower IGF-1 noted in poorer HbA1c and younger age at diagnosis. Cortical thickness positively assoc with age at diagnosis.</td>
</tr>
<tr>
<td>(Kaire Heilman et al. 2009)</td>
<td>30 (11/19)</td>
<td>4.7-18.6</td>
<td>5.4±3.4</td>
<td>DXA</td>
<td>LS, TB</td>
<td>↓ TB BMC and LS vBMD</td>
<td>Adjusted for age, height and BMI. Only present in boys. Inverse association to urinary markers of oxidative stress, plasma ICAM-1 levels and HbA1c.</td>
</tr>
<tr>
<td>(Saha et al. 2009a)</td>
<td>48 (26/22)</td>
<td>12-17.8</td>
<td>6.8 (1-13.5)</td>
<td>pQCT</td>
<td>Radius/ Tibia LS, FN</td>
<td>↔ Trabecular/vBMD, FN BMC</td>
<td>Adjusted for age, weight and height. Smaller cortical areas at radius and tibia. Boys affected more than girls.</td>
</tr>
<tr>
<td>(Inge Roggen et al. 2013)</td>
<td>56 (23/33)</td>
<td>17.9 (17.2-24.8)</td>
<td>M 10.6±3.9</td>
<td>pQCT</td>
<td>Radius</td>
<td>↔ Trabecular vBMD,</td>
<td>Smaller CSA in T1D, esp males. In girls, the CSA SDS correlated negatively with the BMI SDS and positively with the height SDS. No correlation to HbA1c.</td>
</tr>
<tr>
<td>(Loureiro et al. 2014)</td>
<td>75 (29,46)</td>
<td>12 (6-20)</td>
<td>5.0±3.5</td>
<td>DXA</td>
<td>LS (L1-L4)</td>
<td>↓ LS BMD</td>
<td>Inverse association to HbA1c. Adjusted for age, height, weight, puberty.</td>
</tr>
<tr>
<td>(de Souza et al. 2016)</td>
<td>86 (46,40)</td>
<td>12.3±4.2</td>
<td>5.6±4.3</td>
<td>DXA</td>
<td>LS (L1-L4)</td>
<td>↓ LS BMD</td>
<td>Adjusted for age and sex. No association to HbA1c but inverse association with disease duration. Lower IGF1 mRNA expression between cases and controls.</td>
</tr>
<tr>
<td>(Tsentidis et al. 2016)</td>
<td>40 (22/18)</td>
<td>13.0±3.5</td>
<td>5.2±3.3</td>
<td>DXA</td>
<td>TB, LS</td>
<td>↓ TB and LS BMD</td>
<td>Adjusted for age and sex. No difference in sclerostin levels between cases and controls. Higher DKK-1 in T1D; inverse association with LS BMD.</td>
</tr>
<tr>
<td>(Tsentidis et al. 2017)</td>
<td>50 (24,26)</td>
<td>11.6±2.2</td>
<td>6.0±2.7</td>
<td>DXA</td>
<td>LS (L2-L4)</td>
<td>↔ LS BMD</td>
<td>Adjusted for age and sex but not size corrected. Higher urinary Pyr and DPyr.</td>
</tr>
<tr>
<td>(Sav et al. 2018)</td>
<td>95 (36/59)</td>
<td>16.2±1.2</td>
<td>7.8±4.8</td>
<td>pQCT</td>
<td>Tibia</td>
<td>↓ Trabecular vBMD</td>
<td>↓ cortical thickness but ↑ cortical vBMD. No association with HbA1c. Reduced bone strength. Reduced muscle power; inversely associated with disease duration.</td>
</tr>
</tbody>
</table>

BMC: bone mineral content, BMD: Bone mineral density, vBMD volumetric BMD, TB total body, LS lumbar spine, FN femoral neck, BMI body mass index; CSA cross sectional area; GH/IGF-1: growth hormone/insulin-like growth factor-I; mRNA messenger ribonucleic acid, DKK Dipkoff-1, Pyr pyridinoline, DPyr deoxypyridinoline.†(median/range or means± SD
1.5.6.1.2 Bone size and microarchitecture

The most crucial finding from the meta-analysis by Vestergaard et al. is that the marginal reduction in BMD in individuals with T1D, as assessed by DXA, only generated an expected relative fracture risk of 1.09 at the spine and 1.42 at the hip, which is significantly less than the 3-7-fold increased risk seen clinically as detailed in section 1.5.4.1. This, and the overlap in the BMD values of subjects with and without osteoporotic fracture (Schuit et al. 2004), highlighted that bone fragility in not only dependent on bone mineral mass but also alterations in bone macro- and microstructure. Historically, the only technique to assess bone microarchitecture required bone biopsy but the advance in imaging techniques have opened up many non-invasive options.

High resolution(HR)-pQCT studies have documented smaller cross sectional radial and tibial bone area in T1D compared to controls (Heap et al. 2004, Bechtold et al. 2006, Saha et al. 2009b, Roggen et al. 2013), especially during childhood (Saha et al. 2009b) with normalisation before entering adulthood (Bechtold et al. 2007). In addition, HR-pQCT has enabled the distinction between the bone compartments demonstrating not only reduction in trabecular/cortical volumetric BMD in distal radius or tibia of individuals with T1D (Forst et al. 1995, Lettgen et al. 1995, Heap et al. 2004, Bechtold et al. 2006, Danielson et al. 2009, Saha et al. 2009b), but also lower cortical thickness with resultant decrease in estimated bone strength calculated using finite element analysis, which is a computerised numerical method to study such simulation. (Shanbhogue et al. 2015). These alterations had also been shown to be associated with glycaemic control and the presence of other microvascular diseases (Heap et al. 2004, Shanbhogue et al. 2015). T1D patients with microvascular complications were found to have lower trabecular thickness (radius \( p<0.01 \)), greater trabecular separation (radius \( p<0.01 \), tibia \( p<0.01 \)) and network inhomogeneity (radius \( p<0.01 \), tibia \( p<0.01 \)) in comparison to T1D patients without microvascular complications (Shanbhogue et al. 2015). There was no difference noted in the cortical porosity between T1D, even in those with microvascular complications, compared to healthy controls (Shanbhogue et al. 2015).

Magnetic resonance imaging (MRI) confirmed trabecular deficits with reduced trabecular bone volume and trabecular number with greater trabecular separation in young adults with T1D and further implicated the presence of microvascular complications, in particular retinopathy, with diabetic osteopathy (Abdalrahaman et al. 2015). Furthermore, using magnetic resonance spectroscopy (MRS), Granke et al found that the fracture toughness of
bone was negatively associated with pentosidine levels (Granke et al. 2015), a well-known AGE found in diabetic patients.

Interestingly, bone histomorphometry and micro-CT of the biopsied specimens showed a reduction in trabecular bone volume, trabecular thickness and trabecular number with greater trabecular separation in T1D adults compared to healthy age and gender matched controls, with even more exaggerated aberrations in these parameters in the diabetics who have fractured, although these did not reach statistical significance (Armas et al. 2012). This could be due to the small sample size of the study (n=18 in each arm), but more importantly the selection bias of a cohort of T1D patients with very good glycaemic control [median HbA1c 6.8% (6.3-8.3), or 51mmol/mol].

1.5.6.1.3 Bone marrow adiposity

Given the association between marrow adiposity and morphometric vertebral fractures (Schwartz et al. 2013), the study of diabetic osteopathy and fracture risk will not be comprehensive without assessing the bone marrow compartment as well. There are only two studies to date looking at marrow adiposity in individuals with T1D, which demonstrated higher vertebral bone marrow fat in cases compared to healthy controls, although both were not statistically significant most likely due to the small sample sizes (Abdalrahman et al. 2015, Abdalrahaman et al. 2017). However, Abdalrahaman et al. demonstrated good correlation between glycaemic control and % fat fraction in individuals with T1D; those who had poorer glycaemic control had significantly higher vertebral bone marrow fat. In addition, the same group also showed that bone marrow adiposity also correlated significantly with total body and lumbar spine BMD.

1.5.6.2 Bone imaging studies in T2D

Contrary to T1D, adults with T2D have been found to have increased BMD, in the range of 5-10% above an age-matched non-diabetic population (Bonds et al. 2006), with a reported pooled estimated LS BMD Z-score of 0.41 ± 0.01 and FN BMD Z-score 0.27 ± 0.01 (Vestergaard 2007). A more recent meta-analysis substantiated this increased BMD ranging from 25-50% higher compared to non-diabetic controls at the hip and spine but not the forearm (Ma et al. 2012). Meta-regression from both meta-analyses showed that body mass index (BMI) was a major determinant for BMD. One meta-analysis found longer duration of disease to be associated with reduced BMD at the hip but not spine (Vestergaard 2007) with no association with glycaemic control, contradicted by the later meta-analyses which
showed a rather counterintuitive positive correlation between HbA1c and BMD levels (Ma et al. 2012). The authors proclaimed considerable heterogeneity which may have influenced the associations found, stemming from large variation in types of study design, diagnostic definitions and individual characteristics including ethnicity. Importantly, BMD remains a significant predictor of fracture risk in T2D, that is independent of diabetes mellitus itself (Leslie et al. 2013).

Interestingly, HR-pQCT showed that individuals with T2D have lower cortical vBMD and higher cortical porosity in distal radius compared with controls (Burghardt et al. 2010, Farr et al. 2014b). Trabecular vBMD was 10% higher in T2D patients with significantly increased trabecular thickness in the tibia compared to the non-diabetics (Burghardt et al. 2010). This suggests that in T2D the cortical bone compartment may be more affected and the higher trabecular mineralisation could account for the higher BMD seen in this population as assessed by DXA. However, two other studies did not find any cortical deficits in patients with T2D compared to non-diabetics except in the subgroup with microvascular complications (Shanbhogue et al. 2016) and in those with T2D who fractured (Patsch et al. 2013a). MRI further confirmed increased cortical porosity in T2D patients (Pritchard et al. 2012, Pritchard et al. 2013) with no difference in bone marrow adiposity between cases and controls (Baum et al. 2012, Patsch et al. 2013b, Kim et al. 2017). HbA1c was positively correlated with vertebral bone marrow adiposity (Baum et al. 2012).

In summary, the available data suggests that T1D and T2D may have differential effects on the structural quality of bone although data are still scarce and somewhat controversial. There are limitations such as small sample sizes, different techniques to measure and interpret data and inadequate adjustment of confounders such as age, disease duration and metabolic status which may have affected the findings.
1.6 Approaches to investigating bone health

There are a wide variety of methods for assessing bone properties. Non-invasive methods such as qualitative ultrasounds (QUS), dual energy X-ray absorptiometry (DXA), computed tomography (CT) and magnetic resonance imaging (MRI) are more commonly used, although the more invasive methods of bone turnover studies and histomorphometry can provide more functional information.

1.6.1 Bone quantitative ultrasound (QUS)

Quantitative ultrasound is a very attractive technique for the evaluation of skeletal status in a large population such as diagnosis of osteoporosis, as it is low cost, highly portable and free from radiation exposure (Gluer 1997). It is highly suitable for screening and follow-up in children as it is painless, fast to execute and completely free of contraindications which is ideal for compliance in the paediatric population (Baroncelli et al. 2001, McDevitt and Ahmed 2007). The velocity and degree of penetration of the sound waves provides two important measures in QUS; speed of sound (SOS, m/s) and broadband attenuation (BUA, dB/MHz). These measures do not directly measure bone mass, but provide information on apparent bone density, trabecular structure and bone elasticity (Gluer et al. 1994). The major limitation of this technique, however, is reproducibility with inferior precision compared to DXA bone densitometry (Naessen et al. 1995, Gluer 1997) as it is highly technician dependent and restricted to use only in superficial peripheral bones such as phalanges, metacarpals and calcaneus (Naessen et al. 1995, Baroncelli et al. 2001).

1.6.2 Dual X-ray Absorptiometry (DXA)

DXA has been available for clinical use since 1987 and is by far the most widely used technique for measuring bone density in clinical trials and epidemiological studies. This is done through the projection of two x-ray beams of different peak voltage over the bone area of interest which allows the soft tissue component to be subtracted generating the bone density values. It is a well standardised and easy to use technique with high precision (maximum acceptable precision error, 2-2.5%) and low radiation dose (0.5-35 μSv). Importantly, DXA BMD correlates well with biochemically determined bone strength (Ammann and Rizzoli 2003) and fracture risk with approximately 1.6 fold increase for every SD decrement in BMD irrespective of gender (Johnell et al. 2005, Cummings et al. 2006, Rivadeneira et al. 2007). DXA, however, has some pertinent disadvantages: Firstly, it is a 2-dimensional measurement which gives areal BMD (g/cm²) rather than true volumetric
BMD ($g/cm^3$). This is particularly crucial in growing children, when DXA will overestimate fracture risk in a child with smaller size who will have lower areal BMD than a normal sized-individuals. Secondly, it does not distinguish between cortical and trabecular bone, hence provides limited information on bone quality; the reason why DXA BMD only explains approximately 70% of bone strength. In addition, DXA has limitations in measuring BMD in obese patients, older patients with bony degenerative changes or vascular calcifications (Tothill et al. 1997, Diederichs et al. 2011).

### 1.6.3 Quantitative computed tomography (QCT)

More advanced radiological modalities have enabled the assessment of bone macro- and microarchitecture using high resolution micro-computed tomography ($\mu$QCT), synchrotron radiation computed tomography (SRCT), peripheral quantitative computed tomography (pQCT) and magnetic resonance imaging (MRI).

QCT is a three-dimensional non-projection imaging technique of assessing bone. The main advantages of QCT over DXA are that it provides (a) true volumetric BMD (mg hydroxyapatite /cm$^3$), which is independent of body size hence better prediction of fragility fracture risks (Yu et al. 1995, Bergot et al. 2001) and (b) information on bone architecture distinguishing between cortical and trabecular bones (Black et al. 2003). As multidetector CT (MDQCT) can specifically detect changes in the more metabolically active trabecular bone, it is a more sensitive discriminator of BMD changes than DXA (Heuck et al. 1989, Graeff et al. 2007). The incorporation of finite element analysis (FEA) modelling into non-invasive bone further improved QCT estimation of bone strength and stiffness, differentiating the degree of cortical versus trabecular loss providing insight into the pathophysiology of vertebral fractures with aging (Christiansen et al. 2011). The major limitations of QCT is the high radiation exposure (60-2900 μSv) (Table 1.7), rendering it unsuitable for the paediatric population or any longitudinal studies limiting its current use to *ex-vivo* or pre-existing *in vivo* cohort studies only. Specifically in relation to microarchitecture, MDQCT scanners are restricted by the spatial resolution of 250-300 μm, which is larger than the thickness of a trabecula 50-200 μm (Issever et al. 2010).

High resolution peripheral QCT (HR-pQCT) confers better spatial resolution (~120 μm) and a smaller effective radiation dose (<3 μSV). With improved spatial resolution, the trabecular spacing (400-800 μm) and trabecular thickness can be more accurately assessed (Boutroy et al. 2005), although the latter is still subjected to a degree of partial volume averaging. In
addition, pQCT can also give information on bone geometry and muscle cross sectional area, enabling the study of the interaction between muscle and bone systems (Schoenau et al. 2000, Rauch et al. 2001, Bajaj et al. 2015). The main limitations of HR-pQCT are that it requires a dedicated scanner and is limited to studying the peripheral skeleton such as radius and tibia. This is however particularly useful for examining cortical bone changes in metabolic bone disorders as the distal radius contains more cortical bone than the vertebral body. As HR-pQCT scanners have high-resolution, these examinations require a longer scanning time (3-10mins) than QCT (in seconds) and are more prone to motion artifacts, especially in children.

Table 1.7 Radiation dose exposure based on different X-ray based imaging techniques

<table>
<thead>
<tr>
<th>Test</th>
<th>Effective Dose (µSv)</th>
<th>Equivalent Period of Background Radiation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chest X-ray (single anterior posterior)</td>
<td>20</td>
<td>3 days</td>
</tr>
<tr>
<td>DXA (hip and spine)</td>
<td>3</td>
<td>&lt;1 day</td>
</tr>
<tr>
<td>2D QCT spine, scout image and 3 slices of 10mm thickness</td>
<td>60</td>
<td>1 week</td>
</tr>
<tr>
<td>3D multidetector QCT spine, L1-L2, pitch 1</td>
<td>1500</td>
<td>6 months</td>
</tr>
<tr>
<td>3D multidetector QCT hip, pitch 1</td>
<td>2900</td>
<td>1 year</td>
</tr>
<tr>
<td>HR-pQCT</td>
<td>&lt;3</td>
<td>&lt; 1 day</td>
</tr>
<tr>
<td>Roundtrip transcontinental airplane flight</td>
<td>60</td>
<td>1 week</td>
</tr>
</tbody>
</table>

*Based on the calculation of natural background radiation at sea level of 3000 µSv per year (rounded up to the nearest day). DXA dual absorptiometry, QCT quantitative CT, HR-pQCT high resolution peripheral quantitative CT, 2D two-dimensional, 3D three-dimensional

[Modified from (Damilakis et al. 2010)]

1.6.4 Magnetic resonance imaging (MRI)

MRI is emerging as a more comprehensive tool in the assessment of bone health given its capability of quantifying all the various bone compartments including bone macro- and microarchitecture without the burden of ionising radiation. This lends itself well for repeatability in longitudinal studies, especially in the paediatric population. It can directly acquire images in any plane de novo and has the potential to derive functional information to better understand patho-mechanisms of impaired bone health. From a set of contiguous image slices the 3-dimensional structure of bone can be reconstructed and measures of the cortical and trabecular bone microarchitecture can be obtained, including topology (eg, plate vs rod character of the trabeculae) of the latter (Wehrli 2007). The spatial resolution achievable is 200-300 µm, making it impossible to delineate individual trabeculae. This
inferiority to HR-pQCT however, is definitely outweighed by the ability of MRI to image more proximal and larger joints such as proximal tibia and femur; commonly implicated in fragility fractures. With the addition of FEA as described above, the mechanical properties of bone such as stiffness, elasticity and predicted strength can be assessed (Wehrli 2007, Magland et al. 2012). Bone mineral density can also be measured which strongly correlates to the BMD obtained from QCT (Hong et al. 2000, Ho et al. 2013).

The current main attraction of MRI is its ability to evaluate the bone marrow compartment, specifically looking at the bone marrow fat content with or without spectroscopy (Li et al. 2011, Bandirali et al. 2015). There has been a growing interest in the effects of bone marrow adiposity on bone health because of the shared stem cell lineage between osteoblasts and adipocytes (Paccou et al. 2019). Importantly, vertebral bone marrow adiposity has been shown to be an independent predictor of fracture in osteoporotic women (Wehrli et al. 2000). Furthermore, MRI is also the only modality available for imaging bone matrix, the collagenous non-mineralised component of bone onto which the mineralised component of bone is deposited (Cao et al. 2008, Cao et al. 2010, Wu et al. 2010). Its major drawbacks include the high cost, longer scanning time (20-30mins) and specific contraindications.

1.6.5 Bone histomorphometry

Bone biopsy can provide both qualitative and quantitative information on bone tissue, the latter referred to as bone histomorphometry. It is the only available method to study bone cell function within the in vivo structural content to understand the patho-mechanisms of metabolic bone diseases. Standard histomorphometric analyses measure structural parameters, static bone formation and resorption parameters, and dynamic formation parameters, as summarised in Table 1.7 (Rauch 2009). The bone specimens should be horizontal, full thickness biopsy sample of uncrushed bone containing two cortices separated by a trabecular component. This can be theoretically from any sites although transiliac samples are most commonly used, due to limited reference data for other sites in children (Glorieux et al. 2000, Rauch 2003). This technique is also of particular importance for paediatric use as the histomorphometric results are not directly influenced by the growth process (Parfitt et al. 2000). The major drawbacks of histomorphometry are the invasive procedure with prior bone labelling, labour intensive process and need for special equipment and expertise (Rauch 2009).
Remodelling activity is indeed elevated in young children, decreases until the age of 8 or 9 years, and increases again during puberty. After the age of puberty, remodelling activity declines into the much lower adult range (Parfitt et al. 2000).

Table 1.8 Commonly used bone histomorphometric parameters

<table>
<thead>
<tr>
<th>Structural parameters</th>
<th>• Core width (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Cortical width (µm)</td>
</tr>
<tr>
<td></td>
<td>• Bone volume/tissue volume (%)</td>
</tr>
<tr>
<td></td>
<td>• Trabecular thickness (µm)</td>
</tr>
<tr>
<td></td>
<td>• Trabecular number (mm⁻¹)</td>
</tr>
<tr>
<td>Static formation parameters</td>
<td>• Osteoid thickness (µm)</td>
</tr>
<tr>
<td></td>
<td>• Osteoid surface/bone surface (%)</td>
</tr>
<tr>
<td></td>
<td>• Osteoid volume/bone volume (%)</td>
</tr>
<tr>
<td></td>
<td>• Osteoblast surface/bone surface (%)</td>
</tr>
<tr>
<td></td>
<td>• Wall thickness (µm)</td>
</tr>
<tr>
<td>Dynamic formation parameters</td>
<td>• Mineralising surface/bone surface (%)</td>
</tr>
<tr>
<td></td>
<td>• Mineral apposition rate (%)</td>
</tr>
<tr>
<td></td>
<td>• Mineralisation lag time (days)</td>
</tr>
<tr>
<td></td>
<td>• Bone formation rate/bone surface</td>
</tr>
<tr>
<td>Static resorption parameters</td>
<td>• Eroded surface/bone surface (%)</td>
</tr>
<tr>
<td></td>
<td>• Osteoclast surface/bone surface (%)</td>
</tr>
</tbody>
</table>

[Modified from (Rauch 2009)]

1.6.6 MicroRNAs

In addition to biochemical markers of bone turnover, recent studies have found a crucial role for miRNAs in bone development and homeostasis (Kim and Lim 2014). MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at a post-transcriptional level, either by suppressing translation or inducing mRNA degradation (Rodriguez et al. 2004). They are important regulators and modulators of cell differentiation, proliferation and apoptosis as a single miRNA can target up to 100 distinct mRNAs, hence controlling the expression of entire gene networks (Baek et al. 2008). Dysregulation of miRNA have been associated with osteoporosis and shown to be discriminative of fragility fractures (Li et al. 2009, Seeliger et al. 2014). Heilmeier et al. discovered specific miRNAs involved in osteogenesis, adipogenesis, or both and demonstrated that these circulating miRNAs in combination were able to differentiate the fracture status in post-menopausal osteoporosis and T2D women (Heilmeier et al. 2016).
1.7 Metformin

Metformin is a biguanide class of drug, well known as the most frequently prescribed first line anti-diabetic medication for Type 2 diabetes (T2D) (Nathan et al. 2009). It maintains glucose homeostasis by suppressing liver glucose production and increase peripheral glucose uptake (Bailey and Turner 1996). Metformin is considered an insulin-sensitizer as it lowers glycaemic levels without increasing insulin secretion (Garber and Samson 2004). In addition, metformin has also been shown to have cardiovascular benefits including improvement in lipid profile, improved endothelial dysfunction with lower intravascular thrombotic risk. However, despite being around for over 50 years, the precise mechanism of action of metformin remains unclear.

1.7.1 Metformin action in the liver

Several studies have shown that metformin can act via activation of the enzymes tyrosine kinase (Dominguez et al. 1996) and AMP-activated protein kinase (AMPK) (Zhou et al. 2001, Musi et al. 2002). AMPK activation phosphorylates the enzymes involved in several hepatic biosynthetic pathways such as acetyl-CoA carboxylase, hydroxymethylglutaryl-CoA reductase, glycogen synthase and endothelial nitric oxide synthase, which results in a decrease in glucose production, cholesterol and triglyceride synthesis from the liver (Zhou et al. 2001). This has been the widely accepted mechanism of action until genetic loss of function experiments with AMPK deficient hepatocytes (Foretz et al. 2010) challenged it. Since then, studies have put forward different mechanisms of action by which metformin suppresses gluconeogenesis in hepatocytes through inhibition of (i) the mitochondrial respiratory chain complex I (El-Mir et al. 2000) and (ii) a specific mitochondrial enzyme glycerophosphate dehydrogenase (mGPD) (Madiraju et al. 2014), which consequently decrease hepatic glucose output. Miller et al also discovered another novel action of metformin in glucose homeostasis by blocking glucagon-dependent glucose output from hepatocytes by reducing production of cyclic AMP and protein kinase A activity (Miller et al. 2013).

1.7.2 AMPK

AMPK has been a widely studied subject in the last two decades owing to its importance as a master sensor of cellular energy status. It is a heterotrimeric serine/threonine kinase comprising of α catalytic and regulatory β and γ subunits (Hardie et al. 1998). The α catalytic subunit contains the conventional serine/threonine protein kinase domain as well as the
autoinhibitory sequences, the β subunit contains a glycogen-binding domain and a tethering domain for α and γ subunits, while the γ subunit contains four cystathionine-β-synthase sequence repeats, which are responsible for the binding of regulatory nucleotides (McBride and Hardie 2009, Oakhill et al. 2009). Mammals express two α (α1, α2), two β (β1, β2) and three γ subunits (γ1, γ2, γ3) in 12 possible combinations of AMPK heterotrimer in a tissue specific manner, allowing for different subcellular localisation and regulation (Hardie 2007). The predominant isoforms in most cells are α1, β1 and γ1, although hepatocytes significantly expresses α2 (Woods et al. 1996), whereas skeletal and cardiac muscles also express α2, β2, γ2 and γ3 (Stapleton et al. 1996, Thornton et al. 1998, Cheung et al. 2000). The degree of AMP-dependence is highly reliant on the α and γ subunits, with stimulation varying from only 50% for the α1γ3 complexes to more than 5-fold for the α2γ2 combination (Cheung et al. 2000). Also the α2 complexes are enriched within the nucleus whilst α1 complexes are largely cytoplasmic (Salt et al. 1998, Turnley et al. 1999). As the name suggests, AMPK is activated when AMP binds to the regulatory γ subunit, which promotes phosphorylation of Thr-172 by the upstream kinases (e.g. LKB1, CAMKKβ) within the α catalytic subunit and protects Thr-172 from dephosphorylation by protein phosphatases (Shaw et al. 2004, Hawley et al. 2005).

In mammals, AMPK is functionally important as a regulator of cellular energy homeostasis, akin to a thermostat for temperature. Increase in cellular AMP to ATP ratio, a signal of cellular energy compromise, will activate AMPK to switch on energy-generating (catabolic) pathways and switch off energy-consuming (anabolic) pathways that are not essential for short term cell survival in order to restore the AMP/ATP ratio (Kahn et al. 2005, Hardie 2007). AMPK is switched on by cellular stresses that either interfere with ATP production (e.g. hypoxia, glucose deprivation or ischaemia) (Kudo et al. 1995, Marsin et al. 2000) or by stresses that increase ATP consumption (e.g. muscle contraction and exercise) (Winder and Hardie 1996, Fujii et al. 2000). Specifically, in pancreatic β-cells low glucose activates AMPK in the same range of concentrations over which it inhibits insulin release (Salt et al. 1998). The AMPK activation by exercise is dependent on both the duration and the intensity of the exercise (Rasmussen and Winder 1997, Stephens et al. 2002). It is also activated by hormones that act via Gq-coupled receptors, and by leptin and adiponectin. Hence, AMPK is able to regulate many metabolic pathways in peripheral tissues by phosphorylating different metabolic enzymes involved in lipid (Hardie and Pan 2002), glucose and glycogen metabolisms.
Pharmacologically, several chemical compounds are known AMPK activators, such as 5-aminooimidazole-4-carboxamide riboside (AICAR) and metformin. AICAR is taken up into cells and converted to monophosphorylated nucleotide 5-aminooimidazole-4-carboxamide-1-D-ribofuranosyl-5’-monophosphate (ZMP), which mimics all the activating effects of AMP on the AMPK system without disturbing the cellular levels of AMP, ADP or ATP (Corton et al. 1995). Metformin has also been found to activate AMPK (Zhou et al. 2001) by a mechanism that involves phosphorylation of the upstream kinase but interestingly, with no alteration in cellular AMP:ATP ratio (Fryer et al. 2002, Hawley et al. 2002). There are not many AMPK inhibitors. Compound C (also called dorsomorphin) is the only available cell-permeable AMPK inhibitor, which has been shown to rescue the proliferative actions of AICAR and metformin (Isakovic et al. 2007). It is however, a non-selective (low specificity) AMPK inhibitor as it has also been found to inhibit BMP (Yu et al. 2008) and several other kinases (Vogt et al. 2011).

1.7.3 The role of AMPK and metformin in bones

In the last decade, there have been novel findings that AMPK signalling pathway plays a role in bone physiology. On the cellular level, AMPK subunit isoforms are expressed in bones. The α1 subunit is the most dominant isoform in primary osteoblasts, osteoblastic cell lines, osteoclasts and bone tissue (Kim et al. 2008, Kasai et al. 2009, Quinn et al. 2010, Shah et al. 2010), in contrast to the very low α2 subunit expressions. Both β1 and β2 subunits are equally expressed, whilst the γ1 is the preferential γ form with minimal/non-existent γ2 and γ3 expressions.

Many in vitro and in vivo studies have shown a positive effect of AMPK activation on bone cell activities. AMPK activation by AICAR and metformin on mouse pre-osteoblasts MC3T3-E1 showed a dose-dependent increase in cell proliferation, type 1 collagen production, alkaline phosphatase activity and mineral deposition (Cortizo et al. 2006, Kanazawa et al. 2007). Similarly, AMPK activation in rat primary osteoblasts with AICAR and metformin demonstrated increases in cell proliferation (Zhen et al. 2010), AP activity (Shah et al. 2010, Zhen et al. 2010), increase in mineralisation of bone matrix and bone nodule formation (Utting et al. 2006, Shah et al. 2010, Zhen et al. 2010), with similar dose-dependency. The role of AMPK activation in bone formation is further reinforced when co-treatment of pre-osteoblast with AICAR and compound C demonstrated suppression of the stimulatory effect of AICAR on bone formation (Shah et al. 2010). Molinuevo et al. demonstrated that metformin also has positive osteogenic effect on in vitro and in vivo rat
bone marrow progenitor cells, mediated through AMPK activation (Molinuevo et al. 2010). This study also suggested for the first time that metformin may have some effect on suppressing adipogenesis. There are however studies with contradictory findings of decreased AMPK activity during osteoblast differentiation (Kasai et al. 2009, Chen et al. 2017, Chava et al. 2018).

Through AMPK signalling pathway, adiponectin was demonstrated to suppress bone resorption by inhibiting TNFα-induced osteoclastogenesis (Yamaguchi et al. 2008). AMPK activation via metformin also stimulated OPG and inhibited RANKL mRNA and protein expression in osteoblasts, reducing osteoclast differentiation and activity (Mai et al. 2011).

The most compelling evidence for the role of AMPK signalling in bone mass regulation came from genetic studies. Mice with deletion of AMPKα2 subunit demonstrated no change in tibial bone mass, in line with the low expression of α2 subunit in bone. In contrast, AMPKα1 knock-out (KO) mice showed both significant cortical and trabecular bone deficits on imaging, with 40% less trabecular volume, 30% less trabecular number and 12% reduction in trabecular thickness and compromised cortical indexes (Shah et al. 2010) compared to wild-type (WT) mice. Similarly with AMPKβ1 or β2 KO mice, trabecular bone density and mass were reduced although no differences were noted in osteoblast and osteoclast numbers (Quinn et al. 2010), suggesting a more significant impact of AMPK activation on bone cell activities than numbers alone.

The complex signalling pathways involved in the commitment and differentiation of MSC towards adipocytes or osteocytes have been eluded to in section 1.3.2.3., but the mechanisms linking AMPK activation to bone cell differentiation and bone mass however remain poorly understood. Studies have shown a possible cross talk with Wnt/β-catenin signalling in the regulation of osteoblast differentiation (Zhao et al. 2011) where activation of AMPK by AICAR/metformin promoted β-catenin transcription (Zhao et al. 2011) and marked increase in Runx2 expression with no effects on PPARγ (Molinuevo et al. 2010). The involvement of melavonate pathway for both osteoblast and osteoclast differentiation (Kanazawa et al. 2009) has also been shown. Further studies to investigate the differentiation of MSCs and better define the underlying molecular mechanisms involved would be needed, in particular the link between AMPK and PPARγ.
1.8 Aims of the thesis

This thesis was designed to understand the effects of T1D on the bone health of children with the condition, given the current knowledge of diabetic osteopathy in adults.

The primary hypothesis is that:

1. Children with T1D have deficit in bone microarchitecture, as determined by high resolution MRI, compared to children without T1D

The secondary hypotheses included:

1. Children with T1D have increased bone marrow adiposity, as determined by magnetic resonance spectroscopy, compared to children without T1D
2. Poorer diabetes control, as measured by HbA1c, is positively correlated to bone microarchitecture deficit and increase in bone marrow adiposity
3. Metformin can promote osteogenesis and prevent adipogenesis by acting on mesenchymal stem cells.

By studying the problem at a cellular and clinical level, the intention is to be able to better define the problem, if any, in children and suggest a mechanistically sound therapeutic approach.

The principle aims of the thesis are to:

1. Determine if bone health is impaired in children with T1D
2. Study the bone microarchitecture and bone marrow adiposity of children with T1D
3. Identify the mechanisms of action of metformin, and its potential use as a bone therapeutic agent
2 Methodology

2.1 Introduction

This chapter details the methodology adopted for the multi-modality assessment of bone health in the clinical study in Chapter 4 of this thesis. The modalities involved are MRI of the right knee, MRS of the lumbar spine, DXA scan of total body, hip and lumbar spine, and the biochemical assays of bone markers of turnover. Information on the image acquisition and analysis protocols are provided for all imaging studies. Additionally, the methodology used in the laboratory studies in Chapter 5 of this thesis is also described.

Clinical studies: Imaging and Biochemistry

2.2 MRI

2.2.1 Principles of MRI

The early concept of MRI scanning first appeared in medical use in the late 1960s when Raymond Damadian discovered that malignant tissue had different nuclear magnetic resonance (NMR) parameters to normal tissue. The ability to tissue characterise using a magnetic scanner allowed him to produce the image of a rat tumour in 1974 (Damadian and Cope 1974) before pioneering the first super conducting magnetic scanner in the late 1970s to produce the first image of the human body (Goldsmith et al. 1977). In its most basic, an MRI scanner comprises a couch for the patient to lie on, a large magnet, a radiofrequency (RF) coil to transmit and receive the RF waves and a processor which converts the received RF signals into an MRI image.

Fundamentally, MRI is based on the intrinsic magnetic properties of atomic nuclei. All atomic nuclei consist of protons and neutrons which spin about its own axis. In nuclei with an even number of protons and neutrons, half spin in one direction and the other half in the opposite direction resulting in no net spin. Nuclei with an odd number of protons (positively charged) and neutrons, such as $^1$H, $^{13}$C, $^{19}$F, $^{23}$Na, $^{31}$P, however, have a net spin (motion), making them important in MRI as they produce a small magnetic field (Westbrook et al. 2011, Brown et al. 2014).
Hydrogen nuclei are ideal in clinical MRI as they are the most abundant element in the human body present within water (H2O) and fat (CH3 etc.) and their solitary protons give them the largest relative magnetic moment. MRI uses a strong external magnetic field (B₀) to align these magnetic moments in the tissues of the body. In the absence of an applied magnetic field, the magnetic moments of these hydrogen nuclei are randomly orientated (Figure 2.1A). When placed in a strong magnetic field, two things happen to the hydrogen nuclei:

1. They align with the magnetic field, in one of two ways: parallel or anti-parallel (Figure 2.1B). The factors affecting the direction of alignment are determined by the strength of the external magnetic field (in unit Tesla) and the thermal energy of the nuclei. Hydrogen nuclei which do not possess enough energy to oppose the magnetic field known as the low-energy nuclei align parallel to the magnetic field, whilst the high-energy nuclei with sufficient energy to oppose the magnetic field align anti-parallel. There are always fewer higher energy nuclei than low-energy nuclei, resulting in a small excess lined up parallel to B₀ constituting the net magnetisation vector (NMV) (Figure 2.1B). The magnitude of NMV is larger at higher field strengths as fewer nuclei possess enough energy to oppose the magnetic field hence more align parallel to B₀, resulting in improved signal.

2. They rotate or “precess” around the axis of the magnetic spin at a special frequency called the Larmor frequency, which is dependent on the strength of the external magnetic field.
Figure 2.1 Motions in the atoms (hydrogen nuclei in clinical MRI). The magnetic moments of the spinning hydrogen nuclei are normally randomly aligned as shown in A. When an external magnetic field is applied ($B_0$), the magnetic moments of the hydrogen nuclei will do one of two things: i) *Align* parallel, or anti-parallel to $B_0$ depending on the strength of $B_0$ applied, as shown in B. Hydrogen nuclei which absorb enough energy to oppose the magnetic field will lie anti-parallel to $B_0$. There are usually more low energy than high energy nuclei resulting in a net magnetic moments, also known as net magnetisation vector (NMV) marked in red arrow. ii) *Precess* around $B_0$ (marked with blue arrow). The application of $B_0$ accords the already spinning hydrogen nucleus an additional spin along the precessional path as shown in C. [Adapted from (Westbrook et al. 2011)]

The next pre-requisite to obtain an MRI image is a radiofrequency (RF) pulse which is where the resonance comes from in the name magnetic resonance imaging. When a RF pulse with a frequency matching exactly the precessional frequency of the MR active nuclei is delivered in a perpendicular direction to $B_0$, the nucleus gains energy from the RF pulse and resonates. The application of a RF pulse that causes resonance is termed excitation, which results in the net magnetisation (NMV) moving out of alignment from $B_0$, lying at an angle to it (flip angle) (Figure 2.2). The magnitude of the flip depends on the amplitude and duration of the RF pulse. The flip angle is usually 90 degrees, resulting in the NMV deviating from the longitudinal plane into the transverse plane (Figure 2.2).

Figure 2.2 Excitation phase. Transfer of net magnetisation vector (NVM) from the longitudinal plane (Y) to the transverse plane (X) when RF pulse is applied during the excitation phase.
When the RF pulse is switched off, the hydrogen nuclei give up the absorbed energy and the NMV returns, or ‘relaxes’ back to the longitudinal plane (B0). The relaxation process can be divided into 2 parts called T1 and T2 relaxation, which are two independent processes which occur simultaneously (Blink 2004). T1 relaxation, also known as the spin-lattice relaxation, describes the recovery of the longitudinal magnetisation when the nuclei lose their energy to the surrounding environment (lattice). T2 relaxation, also known as the spin-spin relaxation, describes the decay of transverse magnetisation due to the magnetic fields of the neighbouring nuclei interacting with each other (Figure 2.3).

**Figure 2.3 T2 relaxation (or spin-spin relaxation) phase.** A) Immediately after the 90° RF pulse, the net magnetisation vector all lie in the transverse plane, X, with all hydrogen nuclei vectors rotating in the X-Z plane around the Y-axis. The vectors are in-phase as they all point in the same direction. B) Once the RF pulse is switched off, T2 relaxation begins with decay of the transverse magnetisation as the magnetic fields of each hydrogen nuclei affect one another. The nuclei will start to rotate at different speeds and so the vectors no longer all point in the same direction. This is called ‘dephasing’. C) Over time, T2 decay progresses and more dephasing happens until there is no phase coherence left, i.e. not one vector is pointing in the same direction anymore as seen in D [Adapted from (Blink 2004)].

The time taken for T1 and T2 relaxation to occur varies between tissues, and so at a given time $t$, the signal intensity available to create the MRI image will be different for each tissue (Figure 2.4). T1 relaxation time is the time it takes 63% of the longitudinal magnetisation to recover in the tissue, whilst T2 relaxation time is the time it takes for 63% of the transverse magnetisation to be lost, *i.e.* 37% remain (Figure 2.4A and Figure 2.4B, respectively). Fat has a short T1 and T2 time, respectively, due to its inherent low energy state and large lipid molecules which are closely packed together (Figure 2.4C and Figure 2.4D). Water, on the other hand, has a high inherent energy so does not easily give up energy into its lattice from the hydrogen nuclei so the T1 time is long (Figure 2.4C). The T2 time of water is also long,
compared to fat and muscle, as the molecules are spaced apart and hence spin-spin interaction is less likely to occur (Figure 2.4D).

![Figure 2.4 T1 and T2 relaxation times and their respective curves for different tissues. A) T1 relaxation time is the time taken for 63% of the longitudinal magnetisation (M_Y) to recover whilst B) T2 relaxation time is the time taken for 63% of the coherent transverse magnetisation to be lost (i.e. 37% remaining). T1 and T2 times vary for the different tissues with the T1 times for fat, muscle and fluid shown in C) whilst the T2 times shown in D) [Adapted from (Ridgway 2010, Westbrook et al. 2011)]](image)

The magnitude and timings of the RF pulses constitutes a pulse sequence. Figure 2.5 shows a typical MRI pulse sequence, specifically a gradient echo pulse sequence (http://www.revisemri.com/questions/pulse_sequences/se_ge_differences/). The repetition time (TR) is the time between two RF pulses which determines the amount of longitudinal relaxation (T1) that is allowed to occur before the next excitation. The echo time (TE) is the time from the application of the RF pulse to the peak of the signal induced in the coil which determines the amount of decay of transverse magnetisation (T2 relaxation) prior to the signal being read. The signal which is the energy shed by the nuclei during the relaxation processes are retrieved by a receiver coil (some transmit coil can also act as receiver). In order to orientate the signals received into a coherent 3-dimensional image, a field of view (FOV) is placed over the body part to be imaged and this is ‘divided’ into volume element, also known as voxels. Three gradient wire coils are placed within the magnet to create additional graduated magnetic fields which are activated at different times to encode the
signals: slice-encoded axially head-to-toe ($G_s$), phase-encoded anterior-posteriorly ($G_p$) and frequency-encoded to determine left-right ($G_f$) (Figure 2.5) into an image (Blink 2004).

**Figure 2.5 Example of an MRI pulse sequence diagram.** The RF pulses are shown on the top line, and the magnetic field gradients are shown on the lines marked $G_s$, $G_p$, and $G_f$, where the subscripts refer to the slice, frequency and phase directions. The important timing parameters shown in the pulse sequence diagram are the repetition time (TR) and the echo time (TE). TR is the time between subsequent applications of the pulse sequence, and TE is the time between the application of the first RF pulse and the measurement of the signal in the transverse plane [Adapted from (http://www.revisemri.com/questions/pulse_sequences/se_ge_differences.)].

Manipulation of the pulse sequence parameters together with the magnetic field gradients used forms the basis of contrast generation in MRI scanning. The factors that affect image contrast in diagnostic imaging are usually divided into intrinsic or extrinsic parameters, the former of which cannot be changed as they are inherent to the biological tissues (eg T1 and T2 times). Extrinsic parameters are those that can be altered such as the pulse sequence parameters (eg TR, TE, flip angle etc) which can be optimised by varying the acquisition parameters. T1-weighted imaging have short TR, characterised by bright fat and dark water. T2-weighted imaging have long TE and is characterised by bright water and dark fat. Other tissues will have an intensity of somewhere in between in either.
An optimal image quality, is dependent not only on good image contrasts but also on several other factors, including:

- signal-to-noise (SNR) ratio
- spatial resolution, and
- scan time.

The signal-to-noise ratio and spatial resolution are both dependent on voxel volume which itself is controlled by: 1) slice thickness, 2) image matrix and 3) field of view (FOV). Care needs to be exercised as one trades off the other. Increasing slice thickness will increase voxel volume resulting in enhanced SNR but this will compromise spatial resolution. Larger voxel makes it harder to resolve small structures well as the individual signal intensities are averaged together resulting in a partial volume effect. Scan time should be as short as possible to reduce movement artefacts which inevitably degrades image quality.

Conventionally, MRI is seen as the imaging modality for soft tissues but technical advances in the last 10-15 years have significantly improved the utility of magnetic resonance in the musculoskeletal system with high resolution MRI and specialised pulse sequence development (Young and Bydder 2003). Escalation from 1.5T to 3.0T static magnetic field strength has improved the signal-to-noise ratio with better image quality, as with innovation in the array of detector coils available. The development of high resolution MRI has also markedly facilitated imaging of solid structures, especially bone, where RF with rapid switch from excitation of spins to detection of signals allow high signal levels to be obtained with short T2 (Gatehouse and Bydder 2003). An example MRI image of the tibia is shown in Figure 2.6, which illustrates the differences in signal intensities between bone, bone marrow, muscle and subcutaneous fat. The signal intensities of bone and fatty marrow are very different, which makes MRI ideal for imaging trabecular structure.
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Figure 2.6 Sagittal image of MRI tibia. The difference in signal intensities between bone, bone marrow, muscle and subcutaneous fat is demonstrated.

2.2.2 Proximal Tibia Imaging

Imaging was performed on a 3 Tesla (T) Siemens MAGNETOM Prisma MRI scanner (Siemens, Erlangen, Germany) using a transmit/receive (Tx/Rx) extremity coil for the knee. The MR images were then pre-processed, coded and analysed using MATLAB (Mathworks Inc, San Mateo, CA, USA) software.

2.2.2.1 Acquisition

The participants were positioned supine with legs extended throughout the scan. Ear plugs were provided for noise minimisation and comfort. MRI images of the proximal tibia were acquired using an extremity coil positioned over the right knee. The knee was further immobilised with additional paddings placed within the coil itself. Three localiser scans were initially performed in the sagittal, coronal and axial planes to identify the region of interest (ROI) using a T1-weighted spin-echo sequence (Figure 2.7A-C). Twenty axial micro-MRI images (0.4mm thickness) of the metaphysis were collected, with the first slice positioned immediately distal to the epiphyseal growth plate, and subsequent slices positioned distal to that. The TrueFISP (true fast imaging with steady state precession) pulse sequence with the parameters shown in

Table 2.1, was used to provide a 3D volumetric data set of isotropic resolution in the axial plane (Figure 2.7D). The in-plane spatial resolution achievable at 200µm can resolve larger trabeculae (usually 78-200µm), while the lower spatial resolution in the slice direction at 400µm will contribute significantly to the partial volume effect.
Figure 2.7 Image acquisition of proximal tibia MRI. Initial localiser scans in sagittal (A), coronal (B) and axial (C) views and a representative slice of the axial images obtained (D).

Table 2.1 TrueFISP Pulse sequence parameters for imaging of the proximal tibia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (mm$^3$)</td>
<td>0.2 x 0.2 x 0.4 (slice thickness)</td>
</tr>
<tr>
<td>Echo time [TE] (ms)</td>
<td>4.69</td>
</tr>
<tr>
<td>Repetition time [TR] (ms)</td>
<td>10.83</td>
</tr>
<tr>
<td>Flip angle ($^\circ$)</td>
<td>60</td>
</tr>
<tr>
<td>Number of averages</td>
<td>15</td>
</tr>
<tr>
<td>Field of View (mm$^2$)</td>
<td>100 x 100</td>
</tr>
<tr>
<td>Matrix size</td>
<td>448 x 448</td>
</tr>
<tr>
<td>Number of slices</td>
<td>20</td>
</tr>
<tr>
<td>Bandwidth (Hz/pixel)</td>
<td>189</td>
</tr>
<tr>
<td>Scan time (mins)</td>
<td>10</td>
</tr>
</tbody>
</table>
2.2.2.2 Image Pre-Processing

Prior to analysis, images were imported into and pre-processed in MATLAB to improve the image quality and enable better quantification.

a) Sharpening Filter

A sharpening filter was applied to the trabecular bone image to increase the contrast along the edges where the different colours meet, resulting in a sharper image. The ‘unsharp masking’ technique in the analysis software sharpened the image by subtracting a blurred (unsharp) version of the image from the original one. The use of a sharpening filter, however, does not compensate for movement artefact. An example of an image before and after the use of a sharpening filter is shown in Figure 2.8.

![Figure 2.8 MRI axial image before (A) and after (B) application of sharpening filter.](image)

b) Low-pass filter

A low-pass filter was then applied to correct for bone marrow inhomogeneity in paediatric images, due to the physiological conversion of yellow to red marrow in children (see Chapter 1, section 1.2.4). This step is crucial as without which the darker regions of the marrow can be wrongly assigned as bone by the analysis software in the binarisation stage (see section 2.2.2.3a) which will affect the quantitative results. Figure 2.9 shows an image before and after correction for bone marrow inhomogeneity, and the corresponding binarised images.
Figure 2.9 Paediatric MRI image before (A) and after (B) application of a low-pass filter to correct for bone marrow inhomogeneity, and the corresponding binarised images (C and D). Binarisation is where the image is being assigned to either ‘bone’ or ‘marrow’ before quantification.

2.2.2.3 Image Analysis

After filtering, the images were coded and analysed using MATLAB based on a method previously described by Majumdar et al., to give measures for apparent bone volume to total volume ratio (appBV/TV), apparent trabecular number (appTbN), apparent trabecular thickness (appTbTh) and apparent trabecular separation (appTbSp) (Majumdar et al. 1997). Validation of the software was performed using a custom-made phantom as described previously, with high intra-operator, inter-operator and inter-scan repeatability (McComb et al. 2014b).

Five image slices (Slice 9, 10, 11, 12 and 13 out of the 20 slices) per participant were analysed and the mean taken, based on a separate study carried out by our group to determine the feasibility of partial image analysis in yielding a representative estimate of trabecular bone microarchitecture by high-resolution MRI (detailed in Chapter 3).

a) Image Segmentation/Binarisation

In the filtered MRI images, bone appeared dark (low signal intensity) while fatty marrow was bright (high signal intensity) (Figure 2.10B). Using a graphics cursor, the region of interest (ROI) in each axial slice was manually drawn along the trabecular-cortical boundary
to ensure the analysed ROI consisted only of the trabecular bone and bone marrow (Figure 2.10C). Once defined, the ROI was displayed in reverse grey scale for ease of visualisation, where trabecular bone has a high signal intensity as the cortical rim whilst bone marrow has a low intensity. Muscle tissue has an intermediate signal intensity.

The image was then thresholded and binarised, where by each pixel was assigned as either ‘bone’ or ‘marrow’ (binarisation) using a standardised method of image thresholding (Figure 2.10D) (Majumdar et al. 1995, Majumdar et al. 1997). A histogram of the signal intensities in the ROI was plotted and the mean signal intensity ($I_{ROI}$) of the ROI was obtained (Figure 2.10E). Due to the thickness of the image slices exceeding the dimension of trabecular bone, each pixel in the image may not correspond to only one kind of tissue i.e., either bone or marrow but may contain a mixture of the two tissues in varying degree (partial volume effect). Consequently, the histogram of the signal intensities, did not have two individual peaks but a single peak and an asymmetric tail for the lower signal intensities. The intensity of the trabecular bone ($I_{Bone}$) was obtained based on that of the thick cortical rim. The peak of the histogram represents the most frequently occurring pixel intensity value in the ROI.

---

**Figure 2.10 Sequence of image analysis for a representative MRI axial image.** Raw image acquired (A), filtered to correct marrow inhomogeneity (B), region of interest (ROI) manually drawn in red and then image binarised (D) resulting in a histogram depicting all the signal intensities in the ROI. $I_{ROI}$ is the mean signal intensity of the region of interest and $I_{Low}$ is the signal intensity of bone marrow, also known as $I_{Marrow}$.
This peak intensity, since it is the mixture of the two phases, should have a value that is lower than the high intensity trabecular intensity, $I_{\text{Marrow}}$, was set at the lower signal intensity at which the histogram reached half its peak value (Figure 2.11). This is an empirical level and was adopted for the purpose of standardisation as it is very difficult to identify a trabecular space which contains solely bone marrow. Applying these intensity thresholds and taking into the spatial resolution of the slice thickness, the image is then binarised using a clustering method of similarity measures known as fuzzy c-mean clustering (Folkesson et al. 2010).

![Histogram with Intensity Levels](image)

**Figure 2.11 Assignment of marrow intensity ($I_{\text{Marrow}}$), which is set at the lower intensity where the histogram reached half its peak value.** This is an empirical method for standardisation due to difficulty in identifying a trabecular space with solely bone marrow.
**Calculation of Apparent Trabecular Bone Volume/Total Volume (appBV/TV)**

Based on the values from the histogram, the appBV/TV (i.e. the number of bone pixels/total number of pixels) can be calculated from the following equation (Majumdar *et al.* 1997):

$$I_{ROI} = (appBV/TV)I_{bone} + (1 - appBV/TV)I_{marrow}$$

$$\Rightarrow appBV/TV = \frac{I_{ROI} - I_{marrow}}{I_{bone} - I_{marrow}}$$

Equation 1

The intensity value at which the fractional trabecular bone content in the ROI corresponded to the calculated appBV/TV was selected as the threshold, and the image was binarised into a bone and a marrow phase.

**b) Image quantification of trabecular structure**

Standard stereological method was extended to quantify the trabecular structures in the binarised image (Majumdar *et al.* 1995). A grid of parallel lines, each one pixel thick and separated by 10 pixels, was used as a mask, so that only the sections of the image which lie along the lines were considered and the rest of the image was set to zero. For each line in turn, a starting value corresponding to the value of the first pixel in the line was established. Each pixel along the line is then examined in turn, and the number of times that a change from bone to marrow (1 to 0) or marrow to bone (0 to 1) occurs was counted, and defined as $P_L(\theta)$. The number of pixels which correspond to bone, $P_P$ are also counted.

Based on these, the mean intercept length (MIL) was calculated from the following equation (Majumdar *et al.* 1995, Majumdar *et al.* 1997):

$$MIL(\theta) = 2 \cdot \frac{P_P}{P_L(\theta)}$$

Equation 2
Calculation of Apparent Trabecular Thickness (appTbTh)

The MIL is calculated for all angles between 0° and 360° in steps of 10°. The apparent trabecular thickness (in mm) is then calculated using the following equation (Majumdar et al. 1996):

$$appTbTh(mm) = 0.5 \times \text{mean}(\text{MIL}(\theta),\theta=0,360) \times R$$

Equation 3

where R is the pixel resolution in mm.

Calculation of Apparent Trabecular Number (appTbN)

Apparent trabecular number (in mm⁻¹) is calculated using the following equation (Majumdar et al. 1996):

$$appTbN(mm^{-1}) = \frac{appBV}{TV} \times \frac{1}{appTbTh}$$

Equation 4

Calculation of Apparent Trabecular Spacing (appTbSp)

Apparent trabecular spacing (in mm) is calculated using the following equation (Majumdar et al. 1996):

$$appTbSp(mm) = \frac{1}{appTbN} - appTbTh$$

Equation 5
2.3 MRS

2.3.1 Principles of MRS

Magnetic resonance spectroscopy (MRS) can be used in conjunction with MRI for non-invasive *in vivo* quantification of a certain metabolite of interest. Instead of producing an image, $^1$H MRS measures the abundance of hydrogen (1H) in the different elements (including water and fat) within the tissue to produce a spectra of signal intensity versus chemical shift. Based on the Larmor equation similar in MRI, the resonant frequency of a hydrogen nucleus is determined by the strength of the external magnetic field. However, the shielding effect of the orbiting electron cloud around the hydrogen nuclei, causes a chemical shift, expressed in unit parts per million (ppm), which results in identical nuclei resonating at different frequencies within the tissue (Westbrook *et al.* 2011). This generates well resolved signal peaks which enable the different metabolites to be distinguished (Figure 2.12).

Radiofrequency (RF) coils are used to transmit the RF magnetic induction field ($B_1$) and to detect the resulting signal using a transmit/receive coil or a separate dedicated receiver coil. Using a point-resolve spatially localised spectroscopy pulse sequence (PRESS), a spectrum of the different metabolites present can be obtained from a single well-defined spatial volume (single voxel spectroscopy) (Jansen *et al.* 2006). The spectrum displays the metabolites as separate peaks due to the difference in their resonance frequency with their characteristic line widths,

![Figure 2.12](image)

*Figure 2.12* $^1$H-MR spectroscopy of a normal brain. $^1$H spectra of a normal brain (A) with the volume of interest (marked with a square) placed in the white matter of the left frontal cortex as seen in the corresponding T2-weighted MR image (B) The spectra exhibit the various different metabolites present including $N$-acetyleaspartate (NAA), total choline (Cho), glutamate plus glutamine (Glx) and Creatine (Cr). [Adapted from (Ricci *et al.* 2007)]
line shapes, phase and area. Quantification of the concentration of the metabolite of interest can be done by accurately calculating the area under the peak. Figure 2.12 demonstrates the MR image and spectra of the metabolites in the brain, which is the organ where this technique is most widely used clinically.

### 2.3.2 Lumbar spine imaging

Imaging was performed on a 3 Tesla (T) Siemens MAGNETOM Prisma MRI scanner (Siemens, Erlangen, Germany) using a transmit/receive (Tx/Rx) extremity coil for the knee. The MR images were then pre-processed, coded and analysed using MATLAB (Mathworks Inc, San Mateo, CA, USA) software.

#### 2.3.2.1 Acquisition

Both MRI and MRS imaging were performed on the same 3T-MRI scanner during a single scanning session for each participant. Once the knee coil was removed, the participant was positioned further into the MRI scanner to image the lumbar spine at the level of L3. Ideally, measurement of marrow adiposity would be done at the same site as the measurements for the trabecular bone microarchitecture in the lower limb but this is technically impossible. Localiser and T2 scans (sagittal and axial) were performed to allow positioning of the voxel within the vertebral body of L3 (Figure 2.13A). A Point-REsolved Spectroscopy Sequence (PRESS), as summarised in Table 2.2, with no water suppression and a short echo time (TE) was used to enable detection of lipids.

| Table 2.2 PRESS Pulse sequence parameters for imaging of the lumbar spine (L3) |
|---------------------------------|---|
| **Parameter**                  | **Value**       |
| Resolution (mm$^3$)            | 20 x 20 x 20   |
| TE (ms)                        | 30             |
| TR (ms)                        | 2000           |
| Flip angle (°)                 | 90             |
| Number of averages             | 80             |
| Bandwidth (Hz/pixel)           | 1200           |
| Scan time (mins)               | 2.5            |
2.3.2.2 Image Analysis

MRS analysis was performed using the Java-based magnetic resonance user interface (jMRUI) software package to obtain measures for the calculation of percentage fat fraction (%FF) within the bone marrow (Vanhamme et al. 1997, Naressi et al. 2001). The resulting spectrum shows peaks corresponding to water and fat as shown in Figure 2.13B. A best-fit Gaussian model was applied to the acquired spectra, and the area under the resulting water peak ($I_{\text{Water}}$) and lipid peak ($I_{\text{Lipid}}$) were measured to work out the lipid-to-water ratio (LWR).

\[
LWR = \frac{I_{\text{Lipid}}}{I_{\text{Water}}}
\]

The percentage fat fraction (FF) was then calculated, as a measure of bone marrow adiposity, using the following equation (Schellinger et al. 2004):

\[
%FF = \left( \frac{LWR}{LWR + 1} \right) \times 100
\]
2.4 Dual Energy X-ray Absorptiometry (DXA)

2.4.1 Principles of DXA

Conventional X-ray images are insensitive to bone density changes with at least 30% of bone mineral losses required before they may be visually detected. This has led to the development of, initially single and now dual energy, radiographic absorptiometry devices specifically designed to quantitate bone density. DXA scanners usually consist of a couch for the patient, an x-ray source underneath which moves together with a mobile radiation detector arm placed directly above. The patient is placed on the couch in the path of the radiation beam from the source, and the radiation detector is then scanned back and forth over the measurement site. Fundamentally, DXA is reliant on the difference in the attenuation of the dual x-ray beams (high energy and low energy) when they pass through soft tissue and bone. Both bone and soft tissue will attenuate the dual beams as they pass through the body, with the low energy attenuated more than the high energy beam, but the difference is much greater in bone (Faulkner 2001). The attenuation values of the soft tissues and that of bone at the respective high and low energies are used to compute the mass of the bone (or BMC, g) at each point in the X-ray beam based on the following equations:

\[
I^L = I_0^L \left[ \exp \left( -\frac{L}{\mu_{b}^L} m_b - \frac{L}{\mu_{t}^L} m_t \right) \right]
\]

\[
I^H = I_0^H \left[ \exp \left( -\frac{H}{\mu_{b}^H} m_b - \frac{H}{\mu_{t}^H} m_t \right) \right]
\]

\[
\frac{m_b}{m_t} = \frac{\left( \frac{\mu_{b}^L}{\mu_{b}^H} \right) \log \left( \frac{I^H}{I_0^H} \right) - \log \left( \frac{I^L}{I_0^L} \right)}{\mu_{b}^L - \mu_{b}^H \left( \frac{\mu_{t}^L}{\mu_{t}^H} \right)}
\]

Equation 6

where: \( I_0 \) = incident radiation intensity

\( I \) = transmitted radiation intensity

\( \mu \) = mass attenuation value for the attenuating material (cm\(^2\)/g)

\( m \) = mass of the attenuating material (g)
and subscripts b and t refer to bone and tissue, respectively, while L and H for high and low energies, respectively. The computer then evaluates the bone area in the coronal plane of the area scanned and the BMD (measured in g/cm²) is determined as the total bone mass (BMC, measured in g) divided by the bone area (BA, measured in cm²).

\[
\text{BMD (g/cm}^2\text{)} = \frac{\text{BMC (g)}}{\text{BA (cm}^2\text{)}}
\]

Equation 7

Since the densitometry is a projectional technique providing a two-dimensional image of the bone being measured, the BMD derived is therefore the mass of the bone tissue per unit area, not per unit volume. It is therefore often referred to as areal BMD (aBMD) to emphasize that it is not a true volumetric density. Additionally, as the dual beam allows for the delineation of soft tissue versus bone, DXA can also be used to assess body composition (fat mass, lean body mass, and percentage body fat), on both total body (TB) and regional basis (Korkusuz 2015).

The clinical utility of DXA falls into three broad categories:

1. confirmation of low bone mass by comparison with normative data
2. assessment of fracture risk
3. monitoring skeletal change in response to therapy or due to aging

DXA can measure effectively any skeletal site, but its clinical use in the assessment of fracture risk has been commonly concentrated on the lumbar spine, hip (femoral neck and total hip) and total body. For BMD measurements to be clinically useful, they need to be compared to established normative data and expressed in two standard deviations (SD) of BMD, categorised as T-score and Z-score. The T-score compares the patient’s BMD to the young-adult reference population of the same gender and has been adopted as the WHO diagnostic classification of osteoporosis. It is, however, restricted to postmenopausal women and men age 50 and older, and not applicable for children and young people. On another hand, the Z-score is applicable for children, premenopausal women and men younger than 50 years, as it is compared to the BMD of an age-, sex-, and ethnicity-matched reference population. Its measurement of -2.0 or lower is indicative of significantly decreased bone density for chronological age (Cosman et al. 2014).
Care must be exercised to avoid erroneous BMD measurements. Most of these pitfalls can be broadly categorised into technical and interpretation errors (Peh 2014). Over time, the scanner system can be expected to drift due to hardware deterioration such as X-ray tube aging, environmental changes and other factors resulting in technical errors. Daily scanning of the calibration standard supplied with the system needs to be carried out to ensure accurate BMD measurements (Faulkner 2001, Peh 2014). The scanner can self-adjust to bring it back into the original calibration if a small drift is detected, without the need for technical assistance. Periodically, scanning of a quality control phantom (simulated bone material encased in plastic designed to mimic soft tissues) is also carried out to monitor for scanner stability as well as allowing for cross-calibration between different scanners if necessary. The positioning of patient and the placement of ROIs, both crucial for proper image acquisition, can be technically difficult due to patient cooperation and under developed skeletal landmarks in younger children (Lenchik et al. 1998).

The greatest challenge in paediatric densitometry, however, is in the interpretation of DXA measurements. This is much more complex in young growing patients, necessitating a validated adjustment method to take into account the altered growth, stages of puberty and the effects of chronic disease on bone size. The revised ISCD Official Position in 2013 recommended that in children with short stature or growth delay, spine BMC and aBMD results should be adjusted using either bone mineral apparent density (BMAD) or the height Z-score (Crabtree et al. 2014). TBLH BMC and aBMD, on the other hand, should be adjusted using the height Z-score. When considering adjustments of DXA measurements for bone size, height, LBM, skeletal age, or pubertal stage in growing children, it is relevant to consider the goal of the exercise as there is no single adjustment ideal for all. Crucially, size adjustment techniques have been shown to improve the DXA predictive ability for fracture (Crabtree et al. 2013). The paucity of large representative normative databases can add to dilemma in interpretation as many manufacturer reference databases are often small in numbers and not representative of the individual population being studied (Crabtree et al. 2014). A well-trained and experienced DXA technologist is required to avoid most of the errors in acquisition and analysis.
2.4.2 Whole Body Imaging

All imaging were performed systematically by one practitioner (SS) using a Lunar Prodigy densitometer (GE Medical Systems, Wisconsin, USA) to assess the bone and body composition parameters and analysed using the Encore software (Version 13.0). Following height and weight measurements, the participants were positioned supine on the scanner couch and the mobile X-ray source moved from in a cranio-caudal manner to obtain whole body (TB) and lumbar spine (LS) measurements. Total scan time was 10-15mins per participant. Bone parameters obtained include bone mineral content (BMC) [g], bone area (BA) [cm\(^2\)] and mean areal bone mineral density (BMD) [g/cm\(^2\)]. Body composition parameters obtained include whole body lean mass (LM) [g] and fat mass (FM) [g].

2.4.2.1 Analysis

As outlined in previous studies, the predicted and percentage predicted bone area (ppBA) for age and sex were calculated based on our reference data (Warner et al. 1998, Ahmed et al. 2004). This allowed for a comparison of the actual bone mineral content (BMC) of the individual participant with the predicted BMC of a subject of the same sex and bone area from which the percentage predicted BMC (ppBMC), expressed as an SDS (BMC SDS) could be calculated. The coefficient of variation (%CV) calculated on repeated DXA measurement of a phantom was <1% of BMC and <2.5% \textit{in vivo}. Body composition parameters were adjusted for height to minimise the size-related effects of DXA.
2.5 Biochemical assessment

2.5.1 Blood sampling

Non-fasting blood samples were obtained from all participants in the morning to avoid any bias from diurnal variation. Serum markers for bone formation (bone alkaline phosphatase [BAP], osteocalcin [OC]), bone resorption (C terminal telopeptide of Type I collagen [CTX]), adiposity (pre-adipocyte factor-1 [PREF-1]), growth hormone axis (IGF-1, IGF-BP-3, the acid labile subunit [ALS]), sclerostin and other regulator markers of bone turnover (PTH, AP, 25-Hydroxy-vitamin D [25OHD]) were measured.

2.5.2 Biochemical assays

All samples of whole blood were centrifuged on the same day to obtain the serum, which was aliquoted and stored at -80°C until the assays were run. The samples were analysed in duplicate concurrently and samples from each patient were analysed in a single run to minimise analytical variation. Osteocalcin (OC), bone-specific alkaline phosphatase (BAP), C terminal telopeptide of Type I collagen (CTX) were analysed by ELISA (Immunodiagnostic systems, Boldon, UK). The intra-assay variation for OC, BAP and CTX was 1.4%, 9.6% and 3.1%, respectively. Plasma IGF-1 and its binding proteins, IGFBP-3, and the acid labile subunit (ALS) were analysed by ELISA (Mediagnost GmbH, Reutlingen, Germany). Intra-assay variabilities were 16.0%, 2.9%, and 10.9%, respectively. Sclerostin (SOST) was also analysed by ELISA (TECO, Pathway Diagnostics, Surrey, UK) with intra-assay variation of 14.1%. Parathyroid hormone (PTH) and alkaline phosphatase (AP) were measured using chemiluminescent microparticle immunoassay (Abbott Diagnostics, IL, USA). Intra-assay variations were <6.3% and <8.0% respectively. 25-hydroxy-Vitamin D was measured by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) based on our in-house previously published method, with intra-assay variation of <10% (Knox et al. 2009). Reference data for the respective assays were obtained from the literature to calculate the age- and gender-specific SDS values (Rauchenzauner et al. 2007, Ertl et al. 2014).
Laboratory-based experiments: Material and Methods

2.6 Cell culture

2.6.1 Principle of cell culture: proliferation and differentiation

Cell culture is a technique whereby prokaryotic, eukaryotic or plant cells are grown under controlled condition. Most commonly it refers to the culturing of cells derived from animals or humans. Harrison was thought to be the father of cell culture as he successfully cultivated frog neuroblasts in a lymph clot in vitro for several weeks in 1907 (Harrison et al. 1907). There are several milestones in the development of cell culture technology, the first being the discovery of trypsin in the 1950s which enabled the removal of intact adherent cells allowing cell subcultures (Dulbecco 1952) Trypsinisation also facilitated the generation of a single cell suspension, further advancing single cell cloning. Secondly, the introduction of antibiotic use in cell culture inhibited the growth of contaminant, which facilitated long-term cell line propagation (Cruickshank and Lowbury 1952). The third major milestone was the use of chemically defined culture media to maintain the cells at an appropriate stage of maturation to retain their proliferative capacity instead of favouring differentiation, and vice versa. (Eagle 1955). This led ultimately to serum-free media (Ham 1965) which can facilitate the selective growth of a particular type of cell, eg. Dulbecco’s Modified Eagle’s Medium (DMEM) for embryonic mouse cells (Dulbecco and Freeman 1959). Crucially, all these are carried out in vitro in an aseptic environment, working within a hood with particulate air filter with principals of laminar flow, and key sterile manipulation techniques.

Primary culture is the cultivation of surgically or enzymatically removed cells from an organism, in a suitable culture environment (Freshney 2005). These cells can be passaged to create further subcultures, also called cell lines. Subcultures provide the opportunity to expand a cell population, apply further selective pressure with a selective medium and achieve a higher growth fraction and also the generation of replicate cultures for characterisation, preservation by freezing or experimentalisation. Briefly, subculture involves the dissociation of the cells from one another and the substrate to be reseeded at a reduced concentration into a flask to generate a secondary culture and tertiary culture and so on. Each time a cell is subcultured, it will proliferate and multiply back to previous density by going through the growth cycle: lag period of no growth to recover from trypsinisation and cytoskeleton reconstruction enabling them to re-enter cell cycle, log phase of exponential growth and plateau or stationary phase. Some cells readily differentiate once in plateau phase, others exit the cell cycle into G0 but retaining viability. Reduced proliferation in the
stationary phase is due partly to exhaustion of growth factors in the medium as well as contact inhibition from cells when they are in high density.

**Figure 2.14 Evolution of a Cell Line.** The vertical (Y) axis represents total cell growth (assuming no reduction at passage) for a hypothetical cell culture. Total cell number (cell yield) is represented on this axis on a log scale, and the time in culture is shown on the X-axis on a linear scale. Although a continuous cell line is depicted arising at 14 weeks, with different cells it could arise at any time. Likewise, senescence may occur at any time, but for human diploid fibroblasts it is most likely to occur between 30 and 60 cell doubling time. [Adapted from (Hayflick and Moorhead 1961)]

Cell proliferation is regulated by signals from the environment, either contact-mediated or molecular-signalling (Freshney 2005). Low cell density is conducive for cell proliferation giving them room for spreading, which permits entry into cell cycle in the presence of mitogenic growth factors. Most cell lines have limited life span becoming senescent after several passages. With subsequent passages, cells also lose their ability to differentiate. Cell differentiation is the process where a cell changes from one cell type to another, usually into a more specialised cell type. The condition required for differentiation, however, is often antagonistic to that of proliferation. Cell differentiation favours a high cell density where there is enhanced cell-cell and cell-matrix interaction together with the presence of various specific differentiation factors (Freshney 2005). Based on these, it is often necessary in cell
culture to define two sets of culture conditions – one to optimise cell proliferation and one to optimise cell differentiation.

2.6.2 Cell Culture experiments

AMPK α1/α2 knockout mouse embryonic fibroblasts (MEFs), C3H10T1/2 mouse mesenchymal stem cells (Clone 9; ATCC CCL-226) and 3T3-L1 preadipocytes were maintained in DMEM (41965-039, Sigma-Aldrich Ltd, Gillingham, Dorset, UK) containing 10% (v/v) FCS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/ml streptomycin. Differentiation experiments were undertaken at 80-90% confluence. To promote adipogenic differentiation, cells were cultured in the standard media supplemented with either 10 μM pioglitazone alone or in combination with 100 nM insulin, 500 µM 3-isobutyl-1-methylxanthine (IBMX) and 10 μM dexamethasone (IID medium). For osteogenic differentiation, cells were cultured in standard media supplemented with 284 µmol/L ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone (AGD medium). Differentiation media was changed every 3 days.

2.6.3 Preparation of Cell Extracts

C3H10T1/2 MSCs were harvested and nuclear extracts prepared using the Nuclear Extraction kit from Active Motif, Belgium. Briefly, the media was aspirated and cells were harvested in ice-cold PBS containing phosphatase inhibitors and centrifuged (200 x g, for 5 minutes at 4°C) to obtain a cell pellet. The cell pellet was re-suspended in complete lysis buffer containing 10 mM DTT and phosphatase inhibitor cocktail and then centrifuged (14000 x g, for 10 minutes at 4°C) to obtain nuclear and supernatant fractions.

2.7 Electrophoresis and Immunoblotting

2.7.1 Principles of electrophoresis

Electrophoresis is a technique used for separation of proteins in vertical slab gels to identify/investigate a target protein of interest or to purify proteins for further applications (Sambrook and Russell 2001, Ausubel et al. 2003). The separation of proteins is dependent on protein characteristics: surface features, bioproperties, molecular size, and net charge, of which electrophoresis exploits the latter two. Typically, a constant electric field is applied to the electrophoresis chamber (10-20v per cm gel length) loaded with the protein samples, which causes migration of negatively charged molecules through the gel sieve in the
direction of the positively charged anode. Small proteins migrate relatively easier through the gel than larger proteins, hence allowing the separation of protein by molecular size. The separated proteins are then transferred from the gels onto a membrane such as polyvinylidene difluoride (PVDF) or nitrocellulose membranes to allow further staining and quantification.

SDS-PAGE, or sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is a discontinuous one-dimensional electrophoresis system conceived by Ulrich K Laemmli, which is most commonly used to separate proteins with molecular masses between 5 and 250kDa (Laemmli 1970). SDS acts as a surfactant, covering the proteins’ intrinsic charges and conferring them very similar charge-to-mass ratios, negating the intrinsic charges of the proteins, allowing for precise protein separation by mass. The SDS-PAGE method is composed of gel preparation, sample preparation, electrophoresis, protein staining or western blotting, which is detailed in section 1.7.2. and 1.7.3.

2.7.1.1 Gel preparation

Typically, the separating gels are 14cm x14cm in size, although smaller mini-gels 6cm x 8cm are also often used, with thickness of up to 0.75mm. Mini gels provide faster separation at the expense of lower resolution (Ausubel et al. 2003). The separating and stacking gels are cast and hand-poured into special moulds with combs inserted to create little wells for the protein samples (Figure 2.15). The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated, in particular the one of interest. Polymerisation of the polyacrylamide with cross-linking formed from bisacrylamide results in gels of different porosity, crucial for its sieving function. A general rule of thumb, of 5% gels for 60-200kDa and 10% gels for 16-70kDa and 15% gels for 12-45kDa. The composition for making up the separating and stacking gels, with varying amounts of acrylamide and bisacrylamide, are available from most laboratory manuals (Sambrook and Russell 2001), with one example shown in Figure 2.16, although pre-cast gels are available commercially.
Figure 2.15 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). (A) Schematic representation of an SDS-PAGE set up, with the stacking and running/separating gels, and (B) a Bio-rad model of the apparatus [Images from www.chemistry.gatech.edu and www.biorad.com]

**SEPARATING GEL**

<table>
<thead>
<tr>
<th>Stock solution*</th>
<th>Final acrylamide concentration in separating gel (%)</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>12</th>
<th>13</th>
<th>15</th>
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<tr>
<td>30% acrylamide/</td>
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<td>3.00</td>
<td>3.50</td>
<td>3.75</td>
<td>4.00</td>
<td>4.50</td>
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<td>6.00</td>
<td>6.50</td>
<td>7.50</td>
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<tr>
<td>0.8% bisacrylamide</td>
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<tr>
<td>H₂O</td>
<td>8.75</td>
<td>8.25</td>
<td>7.75</td>
<td>7.50</td>
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<td>6.75</td>
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<td>5.25</td>
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<tr>
<td>10% (w/v)</td>
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<td>ammonium</td>
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<td>TEMED</td>
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</tbody>
</table>

Figure 2.16 Recipes for polyacrylamide separating gel with volume in millilitres (mls) of each solution needed depending on percentage (%) of gel required. Polymerisation of the polyacrylamide with cross-linking formed from bisacrylamide results in gels of different porosity, crucial for its sieving function [Adapted from (Ausubel et al. 2003)]
2.7.1.2 Sample preparation

The protein sample is diluted 1:1 (v/v) with 2x SDS buffer and heated to 95°C for 3-5 mins in a sealed microcentrifuge tube. The comb is removed and the wells are carefully filled with the protein samples using pipettes. By convention, the far left lane is filled with a protein-molecular weight standards (or protein marker) mixture.

2.7.1.3 Electrophoresis and protein transfer

Once the protein samples are loaded into the wells, the electrophoresis running chamber is filled with a running buffer solution before an electrical field is passed through the chamber until the bromphenol blue dye in the protein marker lane has reached the bottom of the gel. During this stage, the proteins are separated by their molecular sizes vertically, with larger proteins which travel slower through the gel isolated at the top closest to the loading point, whilst smaller proteins further below in molecular mass order Figure 2.17

![Molecular sizes of the protein of interests](image)

**Figure 2.17** Molecular sizes of the protein of interests (A) PPARγ, (B) Runx2 and (C) phospho-ACC dictates the corresponding choice of polyacrylamide gel use. Protein of bigger molecular mass require higher gel porosity to allow better capture ideally in the middle of the separating gel, hence lower polyacrylamide/bisacrylamide content.

Two-dimensional gel electrophoresis separates the proteins in the first dimension by isoelectric focusing and in the second dimension in the presence of SDS, with incredible resolving power.
2.7.2 Principles of immunoblotting

Immunoblotting (also referred to as Western blotting) is a technique used to identify specific antigens or target proteins among unrelated protein species, by employing the function of polyclonal or monoclonal antibodies (Sambrook and Russell 2001, Ausubel et al. 2003). It involves identification of the protein of interest using an antigen-antibody (or protein-ligand) specific reactions. Proteins are typically solubilised using sodium dodecyl sulfate (SDS) then separated by electrophoresis, as described in section 2.7.1.

The first step to immunoblotting is to electrophoretically transfer these proteins from the SDS-polyacrylamide gels onto membranes to allow subsequent quantification. The gel is removed from the electrophoresis running chamber into a new set up (Figure 2.18) containing a transfer buffer solution termed as the electrophoresis transfer chamber in this thesis. For transfer from SDS gels, the membrane must be placed on the side of the gel facing the anode in a very specific ‘sandwich’ arrangement (Figure 2.18) for an effective horizontal transfer to occur. The choice of membrane, from nitrocellulose to nylon and PVDF membranes is largely dependent on the binding efficiency of the protein of interest to them. Nitrocellulose remains a standard membrane used in immunoblotting, although PVDF membranes have an approximately 6-fold stronger binding affinity which can ensure the proteins are retained more efficiently for subsequent steps (Van Oss et al. 1987).

![Figure 2.18 Electrophoresis transfer chamber set-up. Protein transfer from SDS-polyacrylamide gels onto PVDF/nitrocellulose membranes.](www.mitosciences.com)
At this stage, removable Ponceau staining can be used to check how well the proteins have transferred across (Muilerman et al. 1982). The transferred protein sits on the surface of the membrane, allowing access for reaction with immunodetection reagents. Additional bindings sites are blocked by immersing the membrane in a solution containing protein (e.g. milk) or detergent blocking agent solution (e.g. TBST). The membrane can then be probed with a primary antibody (usually raised in mouse, rabbit or sheep) to target a specific antigen/protein and then with a secondary antibody (usually raised in donkey) labelled, with either enzymes (e.g. horseradish peroxidase, or alkaline phosphatase), or radioisotopes to enable detection and quantification, as shown in Figure 2.19. Conveniently, the membranes can be stripped and re-probed as needed.

Figure 2.19 Principles of immunoblotting (also known as Western blotting), using antibodies to detect a specific antigen or target protein (in grey filled circle).

Immunoblotting is now widely used in conjunction with two-dimensional polyacrylamide gel electrophoresis, not only for traditional goals, such as the immunoaffinity identification of proteins and analysis of immune responses but also as a genome-proteome interface technique.

2.7.3 Western blotting experiments

Protein samples were separated by SDS-PAGE and then transferred electrophoretically onto nitrocellulose membranes. During electrophoresis, gels were run for 80 mins at 120V and then transferred for 120mins at a constant current of 400mA. Membranes were blocked with 5% (w/v) Marvel milk powder in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) for 1 hour. Membranes were then incubated with primary antibodies overnight at 4°C. Following washes with TBST (TBS supplemented with 0.5% (v/v) Tween-20), membranes were incubated with secondary antibodies for 1 hour at room temperature. Antibodies were diluted to the required concentration in 50% (v/v) Sea Block (Thermo Scientific) and 50% (v/v)
TBST. The primary antibodies used were to peroxisome proliferator-activated receptor gamma (PPARγ; marker for adipogenesis, Cell Signalling Technologies (CST), Danvers, MA), Runt-related transcription factor 2 (Runx2; marker for osteogenesis, CST, Danvers, MA), phosphorylated-ACC (P-ACC (Ser79); marker for AMPK activity, CST, Danvers, MA) and phosphorylated-p70S6K (P-p70S6K (Thr389); upstream regulator of mTOR signalling, CST, Danvers, MA). Antibodies were detected using a LI-COR® Odyssey Infrared Imaging systems and densitometric analysis was carried out using ImageJ software (National Institute of Health, UK) software Version 1.47.

2.8 Oil Red O staining

2.8.1 Principles of Oil Red O staining

Oil red O staining (ORO) is a histological technique used in the visualisation of fat cells and neutral fat (Mehlem et al. 2013). Under light microscopy, lipid-laden adipocytes that are stained with ORO appear bright red/orange, leaving the remainder of the cellular constituents non-stained (nuclei can be counterstained). The ORO dye can be made from stock (needs to be protected from UV light) or purchased ready-made. Isopropanol, propylene glycol and triethyl phosphate (TEP) can be used as solvent-carriers for ORO.

2.8.2 Oil Red O staining experiments

C3H10T1/2 cells were incubated with adipogenic IID media in the presence or absence of either 10 µM pioglitazone, 500 µM metformin, 100 µM of the AMPK-activator, A769662, or 10 µM of the p70S6K-inhibitor, rapamycin. Following differentiation, media was aspirated and cells were fixed to cell culture plates with 10% (v/v) neutral buffered formalin for 30 minutes. The formalin was then aspirated and staining was carried out with the addition of 0.3% (w/v) ORO in isopropanol:water (60:40) for 5 minutes in room temperature. The ORO was then aspirated and wells washed with distilled water four times. Imaging was carried out using a Zeiss Axiovert 25 microscope with QImaging camera and supporting software.
2.9 Transfection and Luciferase reporter assays

2.9.1 Principles of transient transfection and luciferase reporter assay

Luciferase reporter assay is a bioluminescence technique used to study gene regulation, based on the interaction of the enzyme luciferase, with a luminescent substrate. Common commercially available luciferases are from the firefly (Photinus pyralis) and Renilla (Renilla reniformis), which is a sea pansy, both of which have different substrates D-luciferin and coelenterazine respectively (Cormier et al. 1975, Allard and Kopish 2008). Using genetic reporters (known as reporter genes), gene expression and cellular events coupled to gene expression can be examined. Typically, a DNA reporter construct is prepared, where the reporter gene is attached to a regulatory sequence of the gene of interest and cloned into an expression vector (plasmid) that is then transfected into cells. The Renilla luciferase gene is commonly used as an internal control to normalise the values of the experimental reporter genes for variations that could be caused by effectiveness of transfection and sample handling (Shifera and Hardin 2010). It is the control of choice as Renilla is constitutively expressed in transfected cells and its expression is not up- or down-regulated by the experimental factors. More crucially, it does not have any effect on the substrate of firefly luciferase due to its action on an entirely different substrate (Cormier et al. 1975, Shifera and Hardin 2010). Once the cells are harvested, the luciferase and Renilla detection agents are added and the DNA of the gene of interest can be quantified. The advantages of bioluminescent reporters include: i) almost instantaneous results, ii) typically no endogenous activity in host cells to interfere with quantification, and iii) exceptional sensitivity, 10- to 1000-fold higher assay sensitivity than fluorescent reporters such as the green fluorescent protein (GFP) which is highly useful in detecting target protein in vanishingly small quantity enveloped within an intricate biological environment (Allard and Kopish 2008).

2.9.2 Transient transfection and luciferase assay experiments

C3H10T1/2 cells were transfected with the indicated plasmids in 6-well plates, with 1.125 μg/well PPRE (PPARγ reporter, purchased from Adgene) and 6xOSE reporter constructs (Runx2 reporter, supplied by Jian Huang, Rush Medical Centre, Chicago, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and then treated with metformin, A769662 or rapamycin with and without adipogenic differentiation media. Cells were then harvested 48 hours after transfection and assayed using the Luciferase reporter assay system (Promega, Madison, WI) according to manufacturer’s instructions. As a transfection control,
the Renilla plasmid 0.125 μg/well was co-transfected in each transfection experiment, and the luciferase activity was normalised to the Renilla activity.

2.10 Statistical analyses

2.10.1 Clinical imaging studies

All data were analysed using SPSS for Windows software program, Version 22 (SPSS, Chicago, IL, USA). Group differences were compared by Mann-Whitney test for continuous variables and Chi-square test used for categorical variables. Spearman’s rank correlation coefficient was used to assess the association between variables. Specific sub-analyses were performed and factors significant on univariate analysis were included in a multivariate logistic regression. All continuous data were presented as median (ranges) with \( p<0.05 \) considered as significant. Further information about the statistical analyses are detailed in the relevant individual chapters.

2.10.2 Laboratory studies

All experiments were performed in triplicate and statistical analysis was performed using Student’s t-test or one-way ANOVA in SPSS for Windows software program, Version 22 (SPSS, Chicago, IL, USA). Results are expressed as mean ± standard error (SEM) and differences with \( p<0.05 \) were considered statistically significant.
3 The Precision of Partial Image Analysis of Trabecular Bone Microarchitecture in High-Resolution Magnetic Resonance Imaging

3.1 Abstract

High-resolution magnetic resonance imaging (HR-MRI) can assess trabecular bone microarchitecture but the number of image slices required for reliable assessment is unclear. The feasibility of partial image analysis was determined on the proximal tibia MRI images of 20 healthy controls (all female; median age 21 years (range 18,35) and 10 cases (3M:7F; median age 19.5 years (range 16,48) with known bone abnormalities including osteogenesis imperfecta and other endocrinopathies. Images were analysed using Matlab to generate the trabecular bone microarchitecture parameters, including apparent trabecular volume to total volume (appBV/TV), trabecular thickness (appTbTh), trabecular number (appTbN) and trabecular separation (appTbSp). The mean values obtained from twenty images (20IM) of the total image set were compared to that for 10 images (10IM), 5 images (5IM) and one image (1IM) from the centre of the total image set using Bland-Altman analysis. Co-efficient of variations (CV) within subjects were compared for the total and partial image set and significance level analysed with Levene’s test and Mann-Whitney U-test. The results demonstrated that partial image analyses of 5IM or 10IM were quantitatively as reliable as analysis of the full image set of 20IM, for both cases and controls. The mean intra-subject CV (±SD) for appBV/TV in healthy controls was 2.6±1.1% for 20IM, 3.0±1.5% for 10IM and 3.1±1.5% for 5IM. Cases had higher mean appBV/TV CV (SD) at 3.7±2.1% for 20IM, 4.7±3.0% for 10IM and 4.3±3.1% for 5IM; all p>0.05 when compared to that of controls. However, sub-analysis of the 4 cases with osteogenesis imperfecta, a more severe osteopathy, demonstrated even higher mean CV (±SD) at 4.6±2.7% for 20IM, 7.1±3.1% for 10IM and 5.9±3.6% for 5IM (p=0.183, p<0.005 and p=0.157, respectively). In conclusion, these findings indicate that partial image sets can reliably represent a larger complete set of images when assessing trabecular bone microarchitecture parameters. Given that the time taken to analyse 5 images per participant is half that of analysing 10 images, this Chapter has objectively informed the final analyses in Chapter 4 for using 5 image partial image set analysis.
3.2 Introduction

High-resolution magnetic resonance imaging (HR-MRI) can be used to assess trabecular bone microarchitecture. Each scan, however, generates a high number of image slices which can lead to a laborious and time-consuming analysis process. The minimum number of image slices required to be analysed for a meaningful result is unclear. The main aim of this chapter was to determine the number of images that needed to be analysed to yield representative estimates of the trabecular bone parameters.

3.3 Methods

Retrospective analyses of the MRI images obtained in a previous study cohort of 20 healthy adult controls (all female; median age 21 years (range 18,35) and 10 adult cases (3M:7F; median age 19.5 years (range 16,48) with known childhood-onset bone abnormalities including osteogenesis imperfecta and other endocrinopathies were undertaken (McComb et al. 2014b). Each participant had 30 slices of MR images taken of the right proximal tibia, with the first slice placed immediately distal to the growth plate as a point of reference. The first five (slices 1-5) and last five slices (slices 26-30) were not suitable for analysis due to the low signal-to-noise ratio of these images.

Figure 3.1 Partial image set analysis. Thirty MR image slices were taken per participant from the right proximal tibia, with the first slice placed immediately distal to the growth plate, as shown in the red rectangular box. All 30 slices were analysed per participant and the partial image set involved analysis of slice 16 as the central most slice (1 IM), slices 14-18 (5 IM), slices 11-20 (10 IM) and slices 6-25 (20 IM).
Using MATLAB software, the 20 most central images (20 IM; slices 6-25), which represented the total image set, were analysed to generate the trabecular bone microarchitecture parameters, including apparent trabecular volume to total volume (appBV/TV), trabecular thickness (appTbTh), trabecular number (appTbN) and trabecular separation (appTbSp). The same analyses were conducted for partial image sets of 10 images (10 IM; slices 11-20), 5 images (5 IM; slices 14-18) and one image (1 IM; slice 16) from the centre of the total image set (Figure 3.1). Bland-Altman analysis was used to determine the agreement between the partial image set analysis to that of the total image set. The Shapiro-Wilk test was conducted on the distribution of the differences between the partial and total image sets to confirm normality. Analysis of variance (ANOVA) was used to compare the means between groups and Levene’s tests used to assess the significance of the CV within subjects. All the images were analysed by a single investigator (myself) to ensure consistency. There was no blinding carried out in the analysis.

3.4 Results

The mean trabecular bone microarchitecture parameters for 20IM, 10IM, 5IM and 1IM were summarised in Table 3.1. There were no significant differences between trabecular bone microarchitecture parameters from 10IM, 5IM or 1IM vs 20IM (p>0.05).

<table>
<thead>
<tr>
<th>Measure of trabecular bone microarchitecture parameters</th>
<th>20IM*</th>
<th>10IM*</th>
<th>5IM*</th>
<th>1IM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>appBV/TV</td>
<td>0.442 ± 0.024</td>
<td>0.441 ± 0.024</td>
<td>0.442 ± 0.025</td>
<td>0.447 ± 0.027</td>
</tr>
<tr>
<td>appTbTh (mm)</td>
<td>0.771 ± 0.049</td>
<td>0.769 ± 0.049</td>
<td>0.764 ± 0.048</td>
<td>0.787 ± 0.051</td>
</tr>
<tr>
<td>appTbN</td>
<td>0.576 ± 0.043</td>
<td>0.578 ± 0.043</td>
<td>0.584 ± 0.044</td>
<td>0.569 ± 0.045</td>
</tr>
<tr>
<td>appTbSp (mm)</td>
<td>0.979 ± 0.104</td>
<td>0.976 ± 0.105</td>
<td>0.966 ± 0.104</td>
<td>0.981 ± 0.114</td>
</tr>
</tbody>
</table>

appBV/TV = apparent trabecular bone volume/total volume; appTbTh = apparent trabecular thickness; appTbN = apparent trabecular number; appTbSp = apparent trabecular separation; IM = number of images analysed from the total or partial image sets; Values in mean±SD. *Between group comparisons all p>0.05

The differences between the total (20IM) and partial image analyses (10IM, 5IM and 1IM) became more apparent when partial image analyses of fewer image slices were used, with more values scattered away from the mean difference, as shown in Figure 3.2 and Figure 3.3. Figure 3.2 demonstrated that partial image set analyses of 10IM, 5IM and 1IM agreed well with the total image set analysis for 20IM in healthy controls, as most of the values laid
between the upper and lower limits of agreement. The differences in appBV/TV values for partial image set of 1IM compared to 20IM, however, were far more dispersed from the mean, with significantly more values lying outwith 95% confidence interval of the mean difference. Similar agreements were found for partial image set analyses in cases with childhood-onset bone abnormalities, as shown in Figure 3.3. Partial image analyses of 10IM, 5IM and 1IM agreed well with total image analysis for 20IM, although most values outwith the 95% confidence interval of the mean difference.

The reproducibility of the image analysis was determined in the cases and controls with the intra-subject CV for apparent bone volume/total volume summarised in Table 3.2. Although the mean CV for appBV/TV were higher in cases than controls, these did not reach statistical significance (all \( p > 0.05 \)). Further sub-analysis of only the cases with osteogenesis imperfecta (OI), which is a more severe osteopathy, demonstrated significantly higher mean CV compared to healthy controls \( (p < 0.05) \) for all total and partial image sets.

<table>
<thead>
<tr>
<th>Image sets</th>
<th>Controls (n=20)</th>
<th>Cases (All) (n=10)</th>
<th>p</th>
<th>Cases (OI only) (n=4)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>20IM</td>
<td>2.6 ± 1.1</td>
<td>3.7 ± 2.1</td>
<td>&gt;0.05</td>
<td>4.6 ± 2.7</td>
<td>0.037</td>
</tr>
<tr>
<td>10IM</td>
<td>3.0 ± 1.5</td>
<td>4.7 ± 3.0</td>
<td>&gt;0.05</td>
<td>7.1 ± 3.1</td>
<td>0.028</td>
</tr>
<tr>
<td>5IM</td>
<td>3.1 ± 1.5</td>
<td>4.3 ± 3.1</td>
<td>&gt;0.05</td>
<td>5.9 ± 3.6</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Table 3.2 Measures of intra-subject coefficient of variation (CV) for apparent bone volume total volume (appBV/TV).

IM = number of images analysed from the total or partial image sets; Values in mean±SD.
Figure 3.2 The agreement between partial and total image sets estimates of apparent trabecular bone volume/total volume (appBV/TV) in healthy subjects, using Bland-Altman analysis. Partial image sets of (A) 10 images (10IM), (B) 5 images (5IM) and (C) 1 image (1IM), agreed well with the total image set (20IM), although 1IM had most values dispersed outwith the 95% confidence interval of the mean difference. Mean difference marked with solid blue line whilst upper and lower limits of agreement (LOA) marked with dashed red lines. Dashed blue lines indicate the 95% confidence interval of the mean difference.
Figure 3.3 The agreement between partial and total image sets estimates of apparent trabecular bone volume/total volume (appBV/TV) in cases with childhood-onset bone abnormalities, using Bland-Altman analysis. Partial image sets of (A) 10 images (10IM), (B) 5 images (5IM) and 1 image (1IM), agreed well with the total image set (20IM), although 1IM had most values dispersed outwith the 95% confidence interval of the mean difference. Mean difference marked with solid blue line whilst upper and lower limits of agreement (LOA) marked with dashed red lines. Dashed blue lines indicate the 95% confidence interval of the mean difference.
3.5 Discussion

Although laborious, this exercise in Chapter 3 has been instrumental in informing the final decision with regards to the minimum number of image slices needed in partial image set analysis to be representative of the total image set. Based on the results, partial image analyses of 10 images, 5 images and even 1 central image only were quantitatively as reliable as analysis of the full image set of 20 images, for both cases and controls. Although remaining within the upper and lower limits of agreement, the values of the differences between 1IM and 20IM were mostly scattered closer to the extremes of the limits with most sitting outwith the 95% confidence interval of the mean difference. This made the partial image analysis of 1IM not ideal.

The principal advantage of using fewer images to assess trabecular bone microarchitecture is a substantial reduction in time for analysis. The time taken to analyse each slice was approximately 1.5 minutes. Therefore, partial image analysis of 5 images vs 20 images reduced the analysis time from approximately 30 minutes to 7.5 minutes per participant. Correspondingly, the analysis time for 10 images were 15 minutes per participant. Given that the partial image analyses for 5 and 10 images corresponded well with the total image analysis of 20 images, it is concluded that partial image analysis of 5 images per participant was the most time-efficient, yet accurate representative of the total image set. The final MRI analyses of Chapter 4 were, therefore conducted with partial image analysis of 5 images per participant as detailed in 2.2.2.3.

The reproducibility of the image analysis method was also studied in this Chapter as the region of interest per image per participant had to be manually drawn each time. Based on the result, the within subject CV compared well for partial and total images analyses. More importantly, the within subject CV between cases and controls were not significantly different. Further sub-analysis of the cases with osteogenesis imperfecta, a more severe osteopathy, demonstrated significantly higher mean CV suggesting that in cases with severe abnormalities of bone health, a larger set of images may need to be analysed to improve precision. This, however, should be interpreted with caution due to the very small sample size (n=4). The very precise nature of bone microarchitecture estimates seen in this Chapter 3, even in individuals who were older, would confidently allow extrapolation for the younger cohort studied in Chapter 4, except for measures of trabecular thickness which was limited by partial volume effect based on the current imaging sequence.
In conclusion, these findings indicate that partial image sets can reliably represent a larger complete set of images when assessing trabecular bone microarchitecture parameters, reducing the overall time taken for image analysis.
4 The Assessment of Bone Health in Children with Established Type 1 Diabetes

4.1 Abstract

To understand the effects of T1D on bone health, 32 affected children at a median (range) age of 13.7 years (10.4, 16.7), were recruited for multimodality assessment of bone health. Serum bone alkaline phosphatase (BAP) and c-terminal telopeptide of type 1 collagen (CTX) as well as DXA total body (TB) and lumbar spine (LS) bone mineral content (BMC) adjusted for bone area were converted to SDS. 3T MRI of the proximal tibia was performed to assess bone volume/total volume (appBV/TV), trabecular number (appTbN) and vertebral bone marrow fat fraction (%) and compared to 26 healthy controls. In T1D, median BAP SDS and CTX SDS were -0.6 (-2.5, +2.1) and -1.1 (-2.5, +0.5) and median TB and LS BMC SDS were -0.1 (-1.1, 0.9) and -0.3 (-1.0, 1.8), respectively. Median appBV/TV in cases and controls was 0.55 (0.47, 0.63) and 0.59 (0.47, 0.63) (p=0.024) and median appTbN was 1.67 (1.56, 1.93) and 1.82 (1.56, 1.99); p=0.004), respectively. The median bone marrow fat fraction in cases and controls was similar at 23% (11, 66) and 20% (8, 61), respectively (p=0.25). Median BAP SDS in T1D cases with HbA1c>75mmol/mol was -0.79 (-2.5, -0.54) compared to 0.5 (-0.64, 2.10) in those with HbA1c<58mmol/mol (p=0.009). Children who presented in DKA and those treated with insulin via MDI had significantly lower BAP SDS compared to those not in DKA, and those on insulin pump, with p=0.017 and p=0.025 respectively. Fractures were encountered in 10/32 (31%) cases after diagnosis of T1D and in 5/26 (19%) controls (p<0.001). Median HbA1c in those cases with and without fracture was 72mmol/mol (49, 100) and 62mmol/mol (27, 87), respectively (p=0.007) whilst median TB BMC SDS was -0.5 (-1.1, 0.0) and 0.0 (-0.5, +0.9), respectively (p<0.001). There was no significant difference in bone microarchitecture or bone marrow adiposity between these fracture groups. Children with T1D display a low bone turnover state associated with reduced bone mineralisation and poorer bone microarchitecture. Fractures were associated with poorer glycaemic control and bone mineral status.
4.2 Introduction

Adults with T1D have a significantly increased risk of fracture compared to the general population (Vestergaard 2007, Hothersall et al. 2014). Indeed, recent studies also confirm that this increased fracture risk affects children and young people with T1D (Weber et al. 2015, Vavanikunnel et al. 2019). New presentation of T1D peaks between the ages of 9 and 14 years (Scottish Diabetes Survey Monitoring Group NHS Scotland 2014, Health Quality Improvement Partnership and Royal College of Paediatrics and Child Health 2015) and given that childhood and adolescence are important physiological periods for optimal bone development (Baxter-Jones et al. 2011), it is possible that these young people with T1D may be especially susceptible to abnormalities of bone health.

Earlier studies suggested that the deficit in bone mass in T1D may be present quite early on, with evidence of osteopenia as early as two years after the diagnosis (McNair et al. 1978, Gunczler et al. 1998, Gunczler et al. 2001). Children with T1D have also been reported to have lower lumbar spine bone mineral density (BMD) within the first few months of diagnosis (Gunczler et al. 2001). This reduction worsens in children with disease duration of more than three months but stabilises with no further correlation between longer disease duration and BMD, suggesting that there may be a degree of reversibility in the process. It is unclear whether the duration of the condition or any other clinical parameters of diabetes or its control are associated with adverse skeletal morbidity. As the significantly increased fracture risk is disproportionate to the marginal reduction in bone mineral density in adults with T1D (Vestergaard et al. 2007), there is an increasing realisation that a comprehensive assessment of bone requires an assessment of bone microarchitecture as well as bone densitometry (McComb et al. 2014b).

Metabolic conditions that may affect bone health, such as diabetes, are also associated with marked alterations in body composition, adiposity and bone marrow adiposity (Baum et al. 2012, Patsch et al. 2013b, Sheu et al. 2017). Glycaemic control in diabetes has been shown to be positively related to lumbar spine marrow adiposity (Baum et al. 2012, Yu et al. 2017). MRI with its ability to study microarchitecture as well as bone marrow adiposity promises to be a useful imaging modality. Indeed, recent MRI-based studies in young women with childhood-onset T1D revealed a deficit in trabecular bone microarchitecture (Abdalrahaman et al. 2015). The current study was performed to better understand the effects of T1D on bone health in children, and specifically to determine whether these MRI-based findings in trabecular bone microarchitecture were present at an earlier age in children with T1D. The
detailed multimodality analysis of bone health performed in these children also provided a mechanistic insight into the effect of diabetes on the developing skeleton.

4.3 Methods

4.3.1 Subjects

Children and adolescents aged between 10-18 years old with T1D for at least three years were recruited from the Paediatric Diabetes Service at the Royal Hospital for Children, Glasgow during the period August 2014 and January 2016. The diagnosis of T1D was based on clinical presentation and ongoing insulin requirement with autoantibody confirmation in uncertain cases. For the MRI-based studies, age and sex-matched children and adolescents without T1D were recruited from general paediatric clinics, siblings of the index cases and children of staff members to provide a reference group. Exclusion criteria included other chronic diseases or medications known to affect bone health and children with metallic implants and pacemakers incompatible with MRI. Children with autoimmune conditions such as hypothyroidism and Coeliac disease were included if they had good compliance with the respective treatments as determined biochemically.

4.3.2 Design

Information on personal health, medication history and lifestyle habits, including dairy intake, use of vitamins or calcium supplementation, cigarette smoking, and a family history of early osteoporosis was collected. History of fractures was also ascertained, and only those radiologically-confirmed (sustained after diagnosis of T1D) were included. Age-appropriate questionnaires were used to collect information on level of physical activity (Kowalski et al. 1997a, Kowalski et al. 1997b). In children with T1D, additional information was also collected on age at diagnosis, severity of initial presentation, disease duration, glycaemic control, as measured by glycosylated haemoglobin (HbA1c) over the last 12 months, and presence of microvascular complications. Glycaemic control was categorised as good (HbA1c <7.5% or <58 mmol/mol), moderate (HbA1c 7.5-9.0% or 58-75mmol/mol) or poor (HbA1c >9.0% or >75mmol/mol) (Rewers et al. 2009). Children with T1D had assessments of bone biochemical markers, DXA, MRI and MRS whilst healthy controls only underwent MRI and MRS. The study protocol was approved by the West of Scotland Research Ethics Committee and informed consent was obtained from the study participants.
4.3.3 Anthropometry and puberty staging

All children had height measured using a stadiometer without footwear and weighed barefooted with only light clothing on. Age- and sex-adjusted Z scores for height, weight and BMI were calculated using the LMS method based on UK population reference data (Cole 1990). Puberty assessment was undertaken and categorised according to Tanner stages for girls and boys, with the additional option of self-assessment in those who did not want to be examined (Morris and Udry 1980). For analysis, pubertal status was defined by genital status in male and breast development in female. Based on the assessment, all children were categorised into pre-pubertal (Tanner Stage 1), early puberty (Tanner Stage 2 and 3) and late puberty (Tanner Stage 4 and 5).

4.3.4 Biochemical Markers of Bone Metabolism & GH/IGF-1 Axis

In all 32 cases, non-fasting blood samples were collected in the morning to coincide with clinic visit, centrifuged and the supernatant stored at -80°C. Details of the specific assays are as described in Chapter 2. Markers of bone formation (bone alkaline phosphatase [BAP], osteocalcin [OC]), bone resorption (C terminal telopeptide of Type I collagen [CTX]), adiposity (pre-adipocyte factor-1 [PREF-1]), growth hormone axis (IGF-1, IGFBP-3, the acid labile subunit [ALS]), sclerostin and other regulator markers of bone turnover (PTH, AP, 25-Hydroxy-vitamin D [25OHD]) were measured.

4.3.5 Dual energy X-ray Absorptiometry (DXA)

Lumbar spine (LS, L2-L4) and total body (TB) measurements were acquired by a Lunar Prodigy densitometer (GE Medical Systems, Waukesha, Wisconsin, USA) and analysed using Encore software (Version 13.0). Details of the image acquisition and analysis are as described in Chapter 2. As outlined in previous studies, reference data were used to calculate a predicted and a percentage predicted bone area (BA) for age and sex (Warner et al. 1998, Ahmed et al. 2004). The reference data allowed for a comparison of the actual bone mineral content (BMC) of the individual with the predicted BMC of a subject of the same sex and bone area from which the percentage predicted BMC (ppBMC), expressed as an SDS (BMC SDS) could be calculated. The coefficient of variation (%CV) calculated on repeated DXA measurement of a phantom was <1% of BMC and the in vivo %CV was <2.5%. DXA was also used to perform thoracolumbar vertebral fracture assessment, as previously described (Kyriakou et al. 2016).
4.3.6 Magnetic Resonance Imaging (MRI) Assessment of Microarchitecture

All 32 children with T1D and 26 healthy controls were scanned. Twenty slice images were acquired at the proximal tibia using a 3T Siemens MAGNETOM Prisma MRI scanner (Siemens, Erlangen, Germany) and analysed using MATLAB software (Mathworks Inc, San Mateo, CA, USA) to obtain measures for apparent bone volume ratio (appBV/TV), apparent trabecular number (appTbN), apparent trabecular thickness (appTbTh), and apparent trabecular separation (appTbSp). Details of the image acquisition and analysis are as described in Chapter 2. The repeatability of microMRI is high with an average CV of 1%. The images were of sufficiently good quality to be analysed in 30 of the 32 cases and in all 26 control participants.

4.3.7 Magnetic Resonance Spectroscopy (MRS) Assessment of Bone Marrow Adiposity

All 32 children with T1D and 22 healthy controls were scanned. 1H-MRS was performed using an 18-channel body array (anterior) and a 32-channel spine coil (posterior) to acquire images at the lumbar spine (L3) position. Analysis was performed using the Java-based magnetic resonance user interface (jMRUI) software to calculate the percentage fat fraction (%FF) within the bone marrow as a measure of bone marrow adiposity (Schellinger et al. 2004). Details of the image acquisition and analysis are as described in Chapter 2. The repeatability of MRS is also high with an average CV of 2.5%. The images were of sufficiently good quality to be analysed in 29 of 32 children with T1D and 21 of 22 healthy controls.

4.3.8 Sample Size Calculation and Statistical Analyses

The primary hypothesis was that children with T1D would have a lower appBV/TV compared to healthy children. The estimated coefficient of variation (CV) for appBV/TV was less than 5% in our previous work (McComb et al. 2014b, Abdalrahaman et al. 2015) and based on this a 7.5% difference at $p<0.05$ with a power of 0.8 would require at least 25 subjects in each arm.

Data were analysed using IBM SPSS software (Version 22, SPSS, Chicago, IL, USA) and described as medians (ranges). Group differences between cases and controls were initially compared by Mann-Whitney test for continuous variables and Chi-square test for categorical
variables, and then adjusted for multiple testing using false discovery rates (FDR). Specifically, biochemical markers of bone turnover and DXA parameters were compared against 0, using the above tests depending on the variables. Spearman’s rank correlation coefficient was used to assess the association between variables. Further analysis was performed to compare the characteristics of those children with T1D with a history of fractures with those without fractures. Factors significant on univariate analysis (HbA1c, TB BMC and physical activity score) were included in a multivariate logistic regression for comparison between the fracture and non-fracture groups. All continuous data were presented as median (ranges) with \( p < 0.05 \) considered as significant, unless otherwise specified.

4.4 Results

4.4.1 Clinical characteristics

Thirty-two children with T1D, median (range) age 13.7 years (10.4, 16.7), were recruited (16 boys) (Table 4.1). The median age at diagnosis was 5.9 years (1.3, 10.8) and median duration of disease was 7.2 years (3.1, 12.4). The median average HbA1c in the preceding 12 months was 8.1% (4.6, 11.3) or 65mmol/mol (27, 100); glycaemic control was good in seven, moderate in 19 and poor in six. Of the 32 children, 3 were pre-pubertal, 17 in early puberty (Tanner stages 2 and 3) and 12 in late puberty (Tanner stages 4 and 5). Ten presented with diabetic ketoacidosis (DKA) at the time of diagnosis. The median insulin dose was 1.0unit/kg/day (0.6, 1.8) with 10 out of 32 on continuous subcutaneous insulin infusion (CSII) via pump. Of the 24 eligible children with T1D, 16 had undergone retinopathy screening and 19 had assessment of microalbuminuria. Mild background retinopathy was present in 3 (19%) and microalbuminuria was absent in all.

4.4.2 Bone Metabolism & GH/IGF-1 Axis

Children with T1D had reduced BAP SDS -0.57 (-2.50, 2.10), with median absolute value 59.2mcg/L (14.4, 118.5) (Table 4.2). Those with poor control had a lower BAP SDS of -0.79 (-2.5, -0.54) compared to 0.50 (-0.64, 2.10) for children with good glycaemic control \((p=0.009)\) and -0.62 (-1.62, 1.11) for children with moderate control \((p=0.03)\) (Figure 4.1A). Serum osteocalcin did not show any relationship to glycaemic control, age or duration of diagnosis. Although median CTX SDS was also reduced compared to controls \((p<0.001)\) (Table 4.2) this did not show the same relationship to glycaemic control as BAP \((p=0.954)\) (Figure 4.1C). However, median CTX SDS \((r, -0.44, p=0.012)\) and sclerostin were inversely
related to age of diagnosis ($r = -0.38, p=0.038$) (Figure 4.1D and Figure 4.1F). Although serum IGF-1 was not significantly lower in children with T1D, the latter had a lower ALS SDS ($p<0.001$) and a higher IGFBP-3 SDS ($p<0.001$) (Table 4.2). Children with T1D who were in DKA at initial presentation had significantly lower BAP SDS than those who were not acidotic ($p<0.017$) (Figure 4.2A). Further hospital admissions with DKA subsequent to the initial presentation, however, did not appear to have an effect on BAP ($p=0.734$) (Figure 4.2B). The T1D children on continuous subcutaneous insulin infusion (CSII; pump therapy), did not only have significantly lower HbA1c ($p=0.005$) and lower daily insulin requirement for weight ($p=0.04$), but also had higher BAP SDS ($p=0.025$) compared to those on MDI (Figure 4.2C-E).
Table 4.1 Clinical characteristics of the whole cohort, with sub-categories of children with T1D based on glycemic control as per HbA1c

<table>
<thead>
<tr>
<th></th>
<th>Whole Cohort</th>
<th>T1D Cases Based on Glycemic control (HbA1c)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1D (n=32)</td>
<td>Control (n=26)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>13.7 (10.4,16.7)</td>
<td>13.8 (10.2,17.8)</td>
<td>0.994</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>16/16</td>
<td>13/13</td>
<td>0.999</td>
</tr>
<tr>
<td>Height SDS</td>
<td>0.3 (-1.5,2.5)</td>
<td>-0.1 (-1.6,2.7)</td>
<td>0.173</td>
</tr>
<tr>
<td>Weight SDS</td>
<td>0.8 (-1.3,3.2)</td>
<td>0.6 (-1.2, 3.1)</td>
<td>0.569</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>0.5 (-0.6,2.9)</td>
<td>1.0 (-1.6,2.7)</td>
<td>0.798</td>
</tr>
<tr>
<td>Age at diagnosis (yr)</td>
<td>5.9 (1.3,10.8)</td>
<td>6.3 (4.2,10.3)</td>
<td>0.810</td>
</tr>
<tr>
<td>Disease duration (yr)</td>
<td>7.2 (3.1,12.4)</td>
<td>7.3 (3.1,10.1)</td>
<td>0.639</td>
</tr>
<tr>
<td>HbA1c average in last 12m (%)</td>
<td>8.1 (4.6,11.3)</td>
<td>7.0 (6.6,7.3)</td>
<td>0.173</td>
</tr>
<tr>
<td>HbA1c average in last 12m (mmol/mol)</td>
<td>65 (27,100)</td>
<td>53 (49,56)</td>
<td>0.801</td>
</tr>
<tr>
<td>HbA1c at diagnosis (%)</td>
<td>10.7 (7.3,17.2)</td>
<td>9.5 (7.3,9.9)</td>
<td>0.069</td>
</tr>
<tr>
<td>Insulin dose (unit/kg/day)</td>
<td>1.0 (0.6,1.8)</td>
<td>0.89 (0.60,1.03)</td>
<td>0.001</td>
</tr>
<tr>
<td>DKA at initial presentation (Y/N) (n=31)</td>
<td>21/10</td>
<td>0/5</td>
<td>0.187</td>
</tr>
<tr>
<td>Insulin pump/injections</td>
<td>10/22</td>
<td>6/0</td>
<td>0.001</td>
</tr>
<tr>
<td>25-OH Vitamin D (nmol/L)</td>
<td>48 (18,75)</td>
<td>53 (48,60)</td>
<td>0.283</td>
</tr>
<tr>
<td>Physical activity score</td>
<td>2.4 (1.3,4.1)</td>
<td>2.0 (1.4,3.7)</td>
<td>0.425</td>
</tr>
<tr>
<td>Previous fracture(s)(Y/N)</td>
<td>10/22</td>
<td>1/5</td>
<td>0.130</td>
</tr>
</tbody>
</table>

Values are presented as median (range). a n=25, b n=27, c n=24, d n=19
The median 25-OH vitamin D level was 48nmol/L (18, 75) and PTH 3.6mmol/L (1.5, 6.6) in the children with T1D. There were no intergroup differences in 25-OH vitamin D levels in the children with [47nmol/L (25,70)] and without [49nmol/L (18,75)] DKA at initial presentation (p=0.555) or the children on continuous subcutaneous insulin infusion (CSII; pump therapy) [49nmol/L (37,60)] compared to those on multiple daily injections (MDI) [47nmol/L (18,75)] of insulin (p=0.602).

4.4.3 Bone Densitometry by DXA

Median BMC SDS for TB and LS was marginally but significantly lower than 0 at -0.1 (-1.1, 0.9) and -0.3 (-1.0, 1.8), respectively (Table 4.2). TB and LS BMC SDS did not show any correlation to glycaemic control (p=0.236, p=0.256, respectively), age at diagnosis (p=0.235, p=0.896), or disease duration (p=0.956, p=0.757). TB and LS BMC SDS were also not significantly different whether the children were acidotic or not at initial presentation (p=0.145 and p=0.260 respectively) (Figure 4.3A, B, D-F). TB BMC SDS, however, was inversely related to CTX SDS (r, -0.5, p=0.003) (Figure 4.3C).

4.4.4 Bone microarchitecture by MRI

Comparison of bone microarchitecture variables revealed that appBV/TV and appTbN were significantly lower and appTbSp higher in T1D children than healthy controls (Table 4.2, Figure 4.4A, C-D). Serum ALS showed an inverse association to appBV/TV (r, -0.54, p=0.002), appTbTh (r, -0.42, p=0.02) and appTbSp (r, 0.46, p=0.01) in the T1D children; however, removal of two cases with high values of ALS SDS resulted in an absence of this association except for appBV/TV where an inverse association was still present at r, -0.44 (p=0.02) (Figure 4.5Figure 4.4A). In the T1D cases, there was no association of appBV/TV, appTbN, or app TbSp with HbA1c, age at diagnosis or duration of T1D. The bone microarchitecture parameters were also not significantly different whether the children were acidic at the time of presentation for appBV/TV, appTbTh, appTbN, or app TbSp with p=0.795, p=0.962, p=0.620, p=0.925 respectively. However, daily insulin dose corrected for weight were positively associated with TbN (r= 0.41, p=0.036) (Figure 4.5B). There was no evidence of an association between any of the bone microarchitecture variables and BMC SDS for TB or LS.
### Table 4.2 Biochemical markers, DXA and MRI-based Measures of Bone Microarchitecture and Vertebral Bone Marrow Adiposity

<table>
<thead>
<tr>
<th>Parameters</th>
<th>T1D (n=32)</th>
<th>Controls (n=26)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biochemical markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-OH Vitamin D (nmol/L)</td>
<td>48 (18,75)a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH (mmol/L)</td>
<td>3.6 (1.5,6.6)b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sclerostin (ng/ml)</td>
<td>0.22 (0.01,0.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteocalcin (ng/ml)</td>
<td>56.5 (13.8,262.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAP SDS</td>
<td>-0.57 (-2.60,2.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX SDS</td>
<td>-1.05 (-2.49,0.51)</td>
<td>&lt;0.001c</td>
<td></td>
</tr>
<tr>
<td>IGFBP1 SDS</td>
<td>-0.24 (-3.64,1.48)</td>
<td>0.364c</td>
<td></td>
</tr>
<tr>
<td>IGFBP3 SDS</td>
<td>1.66 (0.13,3.81)</td>
<td>&lt;0.001c</td>
<td></td>
</tr>
<tr>
<td>ALS SDS</td>
<td>-0.70 (-1.67,0.76)</td>
<td>&lt;0.001c</td>
<td></td>
</tr>
<tr>
<td><strong>DXA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB BMC for BA SDS</td>
<td>-0.1 (-1.1,0.9)</td>
<td></td>
<td>0.018c</td>
</tr>
<tr>
<td>LS BMC for BA SDS</td>
<td>-0.3 (-1.0,1.8)</td>
<td></td>
<td>0.011c</td>
</tr>
<tr>
<td><strong>MRI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>appBV/TV</td>
<td>0.55 (0.47,0.63)a</td>
<td>0.59 (0.47,0.63)</td>
<td>0.024</td>
</tr>
<tr>
<td>appTbN (mm⁻¹)</td>
<td>1.67 (1.56,1.93)a</td>
<td>1.62 (1.56,1.99)</td>
<td>0.004</td>
</tr>
<tr>
<td>appTbSp (mm)</td>
<td>0.27 (0.21,0.32)a</td>
<td>0.24 (0.20,0.33)</td>
<td>0.001</td>
</tr>
<tr>
<td>appTbTh (mm)</td>
<td>0.32 (0.27,0.39)a</td>
<td>0.32 (0.25,0.38)</td>
<td>0.954</td>
</tr>
<tr>
<td>Marrow FF (%)</td>
<td>23.1 (11.0,66.0)b</td>
<td>20.0 (8.0,61.1)</td>
<td>0.250</td>
</tr>
</tbody>
</table>

Values are presented as median (range). PTH = parathyroid hormone, BAP = bone alkaline phosphatase, CTX = c-terminal telopeptide of collagen 1, IGFBP = insulin-like growth factor binding protein, ALS = acid labile subunit, DXA = dual energy xray absorptiometry, TB = total body, LS = lumbar spine, BMC = bone mineral content, BA = bone area, SDS = standard deviation score, MRI = magnetic resonance imaging, appBV/TV = apparent bone volume to total volume, appTbN = apparent trabecular number, appTbSp = apparent trabecular separation, appTbTh = apparent trabecular thickness. FF = fat fraction.

a n=30, b n=29, c compared to 0
Figure 4.1 The relationships between T1D disease characteristics and markers of bone turnover (as assessed by bone formation marker BAP, bone resorption marker CTX and sclerostin). Children with poor glycaemic control had significantly lower bone formation marker compared to children with moderate and good controls. Children diagnosed younger with T1D had significantly higher bone resorption and sclerostin levels.
Figure 4.2 The relationships between acidosis (DKA) and methods of insulin delivery (MDI vs CSII) and T1D disease characteristics, bone formation marker (BAP) and bone density (as assessed by DXA). Children with T1D who were acidotic at initial presentation had significantly lower BAP SDS than those who were not acidic (A). This relationship did not stand with total number of acidotic events requiring hospital attendance (B). Children with insulin delivered by CSII (pump) did not only have lower HbA1c (C) and lower total daily insulin dose (D), but also has higher bone formation marker (E). [DKA=diabetic ketoacidosis, WW=Walking wounded, or non-DKA, MDI=multiple daily injection, CSII (continuous subcutaneous insulin infusion, DXA=dual x-ray absorptiometry, BAP=bone alkaline phosphate]
Figure 4.3 The relationships between bone mineral density status (as assessed by DXA) and markers of bone turnover (as assessed by bone formation marker BAP, bone resorption marker CTX and sclerostin) in children with T1D. TB BMC SDS was inversely related to CTX SDS. [BAP bone alkaline phosphatise; CTX c-terminal telopeptide of type 1 collagen]
Figure 4.4 Bone microarchitecture parameters comparing children with T1D and healthy controls. Children with T1D have significantly altered bone microarchitecture, including (A) lower bone volume to total volume, (C) lower trabecular number and (D) higher trabecular separation. [appBV/TV=apparent bone volume to total volume, appTbTh=apparent trabecular thickness, appTbN=apparent trabecular number, appTbSp=apparent trabecular separation]
Figure 4.5 The relationships between bone microarchitecture parameters with markers of GH/IGF-1 axis and insulin dose in children with T1D. Serum ALS was inversely associated with appBV/TV whilst insulin dose is positively associated with appTbN. [ALS=acid labile subunit, appBV/TV= apparent bone volume to total volume, appTbN=apparent trabecular number]

4.4.5 Bone Marrow Adiposity by MRI

Median vertebral bone marrow adiposity, expressed as percentage fat fraction (%FF), was higher in cases than controls, but this did not reach statistically significant difference (Table 4.2, Figure 4.6). In the cases, there was no association between %FF and markers of GH/IGF-1 axis or bone turnover, BMC SDS for TB or LS. Combined analysis of the cases and controls showed an inverse association between %FF and appTbN (r, -0.40, p=0.004) and a positive association with appTbSp (r, 0.33, p=0.021) (Figure 4.7).

Figure 4.6 Vertebral bone marrow adiposity, expressed in fat fraction % in T1D children and healthy controls. Children with T1D had higher fat fraction compared to healthy controls, although this did not reach statistical significance.
Figure 4.7 The relationships between percentage fat fraction (%FF) and bone microarchitecture parameters in the whole cohort. %FF is inversely associated with trabecular number and positively associated with trabecular separation.

4.4.6 Fracture data

Children with T1D were significantly more likely to fracture compared to healthy controls ($p<0.001$) (Table 4.1). Of the 32 children with T1D, 10 (31%) had radiologically confirmed fracture following the diagnosis of T1D, as summarised in Table 4.3. Two out of these 10 sustained a lower extremity fracture (lower leg/ankle, or foot), whilst the rest had upper extremity limb fractures (humerus, elbow, forearm, hand). The children with lower extremity fractures were younger when diagnosed with T1D. None of the children had any abnormalities identified on vertebral fracture assessment by DXA.

The clinical characteristics of the fracture and non-fracture group were comparable for age, gender, puberty, anthropometric measurements, insulin doses and delivery systems (Table 4.4). The median age of diagnosis was 4.4 years (1.3, 10.8) in the fracture group and 6.5 years (3.8, 10.8) in the non-fracture group, although this did not reach statistical significance between groups ($p=0.077$) (Figure 4.8A, Table 4.4). The median duration of disease was 8.6 years (3.1,12.4) in the fracture group and 7.0 years (3.1,10.9) in the non-fracture group ($p=0.204$). The median HbA1c was higher in the fracture group at 8.7% (6.6, 11.3) or 72mmol/mol (49, 100) compared to the non-fracture group of 7.8% (4.6, 10.1) or 62mmol/mol (27, 87) ($p=0.005$ and $p=0.007$ respectively) (Table 4.4, Figure 4.8B).
Table 4.3 Age, distribution, sites of fracture and the events preceding in the fracture cohort

<table>
<thead>
<tr>
<th>Age at diagnosis (year)</th>
<th>Sex</th>
<th>Age at fracture (year)</th>
<th>Duration of disease to fracture time (year)</th>
<th>Fracture distribution (UL/LL/Other)</th>
<th>Site of Fracture</th>
<th>Event preceding fracture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>M</td>
<td>12.3</td>
<td>11.0</td>
<td>LL</td>
<td>Left tibia + metatarsals</td>
<td>Hyperextension injury during football</td>
</tr>
<tr>
<td>13.1</td>
<td></td>
<td>11.8</td>
<td></td>
<td>LL</td>
<td>Left calcaneum</td>
<td>Fell off skateboard at low speed</td>
</tr>
<tr>
<td>13.2</td>
<td></td>
<td>11.9</td>
<td></td>
<td>UL</td>
<td>Left 5th proximal phalange</td>
<td>Hyperextension injury during football</td>
</tr>
<tr>
<td>2.2</td>
<td>F</td>
<td>4.9</td>
<td>2.7</td>
<td>LL</td>
<td>Right fibula</td>
<td>Fell in park from standing height</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.1</td>
<td>4.5</td>
<td>UL</td>
<td>Left supracondyle</td>
<td>Fell off motorcycle with protective gear</td>
</tr>
<tr>
<td>2.8</td>
<td>M</td>
<td>5.9</td>
<td>3.1</td>
<td>Other</td>
<td>Right clavicle</td>
<td>Fell in park from standing height</td>
</tr>
<tr>
<td>3.7</td>
<td>M</td>
<td>6.4</td>
<td>2.7</td>
<td>UL</td>
<td>Left radius and ulnar</td>
<td>Fell on outstretched hand</td>
</tr>
<tr>
<td>4.3</td>
<td>M</td>
<td>10.9</td>
<td>6.6</td>
<td>UL</td>
<td>Right radius and ulnar</td>
<td>Fell in school from standing height</td>
</tr>
<tr>
<td>4.5</td>
<td>F</td>
<td>7.4</td>
<td>2.9</td>
<td>UL</td>
<td>Right radius</td>
<td>Trip over schoolbag</td>
</tr>
<tr>
<td>5.0</td>
<td>M</td>
<td>8.1</td>
<td>3.1</td>
<td>UL</td>
<td>Left supracondyle</td>
<td>Fell onto left side from standing height</td>
</tr>
<tr>
<td>7.3</td>
<td>M</td>
<td>10.4</td>
<td>3.1</td>
<td>UL</td>
<td>Left radius</td>
<td>Slipped and fell during football</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.0</td>
<td>4.7</td>
<td>UL</td>
<td>Left radius</td>
<td>Hyperextension injury during football</td>
</tr>
<tr>
<td>10.3</td>
<td>F</td>
<td>11.5</td>
<td>1.2</td>
<td>UL</td>
<td>Left radius</td>
<td>Fell on outstretched hand</td>
</tr>
<tr>
<td>10.8</td>
<td>M</td>
<td>11.2</td>
<td>0.4</td>
<td>UL</td>
<td>Left scaphoid</td>
<td>Fell on outstretched hand</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.8</td>
<td>1.0</td>
<td>UL</td>
<td>Left humeral supracondyle</td>
<td>Fell off bicycle</td>
</tr>
</tbody>
</table>

M = Male, F = Female, UL = upper limb, LL = lower limb
### Table 4.4 Clinical characteristics of all children with T1D by sub-categories of fracture and non-fracture

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No Fracture (n=22)</th>
<th>Fracture (n=10)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>13.5 (10.7,16.7)</td>
<td>13.8 (10.4,16.4)</td>
<td>0.920</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>9/13</td>
<td>7/3</td>
<td>0.127</td>
</tr>
<tr>
<td>Height SDS</td>
<td>0.3 (-1.5,2.5)</td>
<td>0.4 (-1.4,2.1)</td>
<td>0.795</td>
</tr>
<tr>
<td>Weight SDS</td>
<td>0.8 (-0.4,2.4)</td>
<td>0.5 (-1.3,3.2)</td>
<td>0.366</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>0.6 (-0.6,2.9)</td>
<td>0.5 (-0.6,2.4)</td>
<td>0.411</td>
</tr>
<tr>
<td>Tanner stage (Pre/Early/Late) (n)</td>
<td>3/10/9</td>
<td>0/7/3</td>
<td>0.316</td>
</tr>
<tr>
<td>Age at diagnosis (yr)</td>
<td>6.5 (3.8,10.8)</td>
<td>4.4 (1,3.10.8)</td>
<td>0.077</td>
</tr>
<tr>
<td>Disease duration (yr)</td>
<td>7.0 (3.1,10.9)</td>
<td>8.6 (3.1,12.4)</td>
<td>0.204</td>
</tr>
<tr>
<td>HbA1c average in last 12m (%)</td>
<td>7.8 (4.6,10.1)</td>
<td>8.7 (6.6,11.3)</td>
<td>0.005</td>
</tr>
<tr>
<td>HbA1c average in last 12m (mmol/mol)</td>
<td>62 (27,87)</td>
<td>72 (49,100)</td>
<td>0.007</td>
</tr>
<tr>
<td>HbA1c at diagnosis (%)</td>
<td>10.8 (7.3,17.2)</td>
<td>10.3 (8.8,13.3)</td>
<td>0.574</td>
</tr>
<tr>
<td>HbA1c at diagnosis (mmol/mol)</td>
<td>95 (56,164)</td>
<td>89 (73,122)</td>
<td>0.574</td>
</tr>
<tr>
<td>Severity at diagnosis</td>
<td>14/7/1</td>
<td>6/4/0</td>
<td>0.740</td>
</tr>
<tr>
<td>- Not DKA/DKA/unknown (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin dose (unit/kg/day)</td>
<td>1.0 (0.6,1.8)</td>
<td>1.2 (0.8,1.7)</td>
<td>0.264</td>
</tr>
<tr>
<td>Insulin pump/injections</td>
<td>7/15</td>
<td>3/7</td>
<td>0.921</td>
</tr>
<tr>
<td>TB BMC for BA SDS</td>
<td>0.0 (-0.5,0.9)</td>
<td>-0.5 (-1.1,0.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LS BMC for BA SDS</td>
<td>-0.3 (-1.0,0.8)</td>
<td>-0.5 (-1.0,1.8)</td>
<td>0.617</td>
</tr>
<tr>
<td>25-OH Vitamin D (nmol/L)</td>
<td>48 (18,70)</td>
<td>47 (25,75)</td>
<td>0.884</td>
</tr>
<tr>
<td>PTH (mmol/L)</td>
<td>3.7 (1.8,6.6)</td>
<td>3.4 (1.5,5.7)</td>
<td>0.475</td>
</tr>
<tr>
<td>Sclerostin (ng/ml)</td>
<td>0.21 (0.01,0.70)</td>
<td>0.25 (0.05,0.50)</td>
<td>0.734</td>
</tr>
<tr>
<td>Osteocalcin (ng/ml)</td>
<td>44.3 (16,1204.4)</td>
<td>60.6 (13,9,262.8)</td>
<td>0.190</td>
</tr>
<tr>
<td>BAP SDS</td>
<td>-0.62 (-2.50,1.11)</td>
<td>-0.55 (-1.62,2.10)</td>
<td>0.764</td>
</tr>
<tr>
<td>CTX SDS</td>
<td>-1.17 (-2.49,0.29)</td>
<td>-0.73 (-1.67,0.51)</td>
<td>0.047</td>
</tr>
<tr>
<td>IGF-1 SDS</td>
<td>-0.30 (-3.64,1.14)</td>
<td>-0.06 (-2.33,1.48)</td>
<td>0.734</td>
</tr>
<tr>
<td>IGFBP-3 SDS</td>
<td>1.78 (0.13,3.81)</td>
<td>1.37 (0.16,3.26)</td>
<td>0.952</td>
</tr>
<tr>
<td>ALS SDS</td>
<td>-0.67 (-1.67,0.76)</td>
<td>-0.79 (-1.63,-0.12)</td>
<td>0.434</td>
</tr>
<tr>
<td>Physical activity score</td>
<td>2.2 (1.3,3.7)</td>
<td>2.8 (1.7,4.1)</td>
<td>0.043</td>
</tr>
<tr>
<td>appBV/TV</td>
<td>0.55 (0.47,0.63)</td>
<td>0.54 (0.50,0.61)</td>
<td>0.571</td>
</tr>
<tr>
<td>appTbN (mm⁻¹)</td>
<td>1.71 (1.56,1.93)</td>
<td>1.64 (1.57,1.84)</td>
<td>0.512</td>
</tr>
<tr>
<td>appTbSp (mm)</td>
<td>0.26 (0.21,0.32)</td>
<td>0.27 (0.23,0.32)</td>
<td>0.389</td>
</tr>
<tr>
<td>appTbTh (mm)</td>
<td>0.32 (0.27,0.39)</td>
<td>0.32 (0.27,0.37)</td>
<td>0.587</td>
</tr>
<tr>
<td>%FF</td>
<td>26.4 (13.5,66)</td>
<td>20.0 (11.0,45.7)</td>
<td>0.451</td>
</tr>
</tbody>
</table>

Values are presented as median (range). PTH = parathyroid hormone, BAP = bone alkaline phosphatase, CTX = c-terminal telopeptide of collagen 1, IGF = insulin-like growth factor, IGFBP = insulin-like growth factor binding protein, ALS = acid labile subunit, DXA = dual energy xray absorptiometry, TB = total body, LS = lumbar spine, BMC = bone mineral content, BA = bone area, SDS = standard deviation score, MRI = magnetic resonance imaging, appBV/TV = apparent bone volume to total volume, appTbN = apparent trabecular number, appTbSp = apparent trabecular separation, appTbTh = apparent trabecular thickness. FF = fat fraction.
Figure 4.8 Sub-analysis of children with T1D depending on fracture status. Children with T1D who fractured had poorer glycaemic control (with higher HbA1c) and lower TB BMC-for-BA SDS than those without fractures. The physical activity score was higher in the fracture cohort.

The children with a history of fracture had lower TB BMC SDS ($p<0.001$) with a median of -0.5 (-1.1, 0.0) (Figure 4.8C), but similar LS BMC SDS ($p=0.617$) (Table 4.4). Both groups had similar BAP SDS, IGF-1 SDS and CTX SDS (Table 4.4). The median physical activity score (1=least, 5=most active) for the fracture group was 2.8 (1.7, 4.1) and 2.2 (1.3, 3.7) in the non-fracture group ($p=0.043$) (Figure 4.8D). There was no significant difference in bone microarchitecture or bone marrow adiposity between these fracture groups (Table 4.4). On multivariate analysis, taking into account HbA1c, TB BMC, and physical activity score, the only significant variable was TB BMC (adjusted OR 0.002, 95% CI 0.0 - 0.765, $p=0.04$).
4.5 Discussion

This is the first study to look in detail and specifically at the association between T1D disease characteristics, bone microarchitecture and marrow adiposity by MRI and fracture incidence in children. The group of children studied had an overall diabetes control which was better than expected for adolescents (Cameron and Wherrett 2015) but similar to that described recently in a population based study of skeletal morbidity in young people with T1D (Weber et al. 2015). In addition, contrary to other studies of bone health in children and adolescents with T1D (Parthasarathy et al. 2016), the current cohort were well nourished and were also vitamin D sufficient.

Previous studies on bone turnover status of children and adolescents with T1D have produced variable results (Valerio et al. 2002, Hamed et al. 2011, Tsentidis et al. 2016). Given that these markers vary markedly in growing children, the values for the two commonly studied markers of bone formation and bone resorption, BAP and CTX, were adjusted for age and sex in the current study. The existence of a low bone turnover state, characterised by low circulating levels of both BAP and CTX, is important to highlight in a group of well-controlled children with T1D. This biochemical picture has also been described in young adults with T1D (Starup-Linde et al. 2014, Abdalrahman et al. 2015). Whilst, it is possible that this low bone turnover state may also be a reflection of functional growth hormone resistance which may exist in people with diabetes (Baroncelli et al. 2000), circulating IGF-1 was not particularly low in the current cohort. Given that there was a clear inverse association between bone formation and glycaemia and between bone resorption and the age at diagnosis of diabetes, it is possible that the mechanisms that influence bone turnover in T1D are mediated through several pathways including those that may be dependent on chronic hyperglycaemia or insulinopenia (Abdalrahman et al. 2015). For instance, the inverse association between circulating sclerostin and age at diagnosis further supports the notion of a sclerostin-mediated signalling pathway inhibition of bone turnover (Gennari et al. 2012).

Marked reduction in diabetes complications have been reported in T1D adolescents with a shift towards a more physiologic insulin replacement using continuous subcutaneous insulin infusion (CSII) via pump (Downie et al. 2011). The higher levels of BAP in T1D children on CSII compared to those on multiple daily injections (MDI) suggests that this form of replacement may indeed be advantageous for bone health preservation. However, given that this group also had a lower HbA1c could suggest a degree of selection bias. The lower levels
of BAP in children who presented in DKA at initial diagnosis is very interesting and suggests the possibility of an additional programming effect of acidosis or sustained hyperglycaemia prior to initial presentation on skeletal development. Given that the effects of acidosis on bone homeostasis may be independent of calcitropic hormones (Topaloglu et al. 2005) and it is possible that acidosis may have an effect on mesenchymal stem cell differentiation (Guntur et al. 2018), a long lasting effect on bone health that may result from the initial acidotic insult deserves further exploration. Alternatively, as DKA at T1D presentation is more likely to occur in younger children, the lower BAP may be an indication of a more aggressive autoimmune process leading to more marked insulin deficiency or shorter honeymoon period.

In the current study, children with T1D had marginally lower bone mineral density when corrected for size and this is consistent with previous studies demonstrating that children and adolescents with T1D have lower bone mineral density status (Leger et al. 2006, Saha et al. 2009b, Loureiro et al. 2014). Although bone microarchitecture studies have been performed in T1D and some have reported deficits (Maratova et al. 2018, Samelson et al. 2019) the current study is the first study in children with T1D that has shown a deficit in MRI-based assessment of trabecular bone microarchitecture, a finding which is similar to what has been described before in young women with T1D (Abdalrahaman et al. 2015). It is interesting to note that that the two studies (Maratova et al. 2018, Samelson et al. 2019) that have shown a deficit and the one (I. Roggen et al. 2013) which did not show a deficit examined the tibia and radius, respectively and the possibility of a selective effect on a weight bearing site needs further exploration in adequately powered studies.

The inverse association of bone microarchitecture parameters with bone marrow adiposity in children confirms our previous reports of this finding in young women (Abdalrahaman et al. 2017) and not only does this further reinforce the hypothesis that the observed skeletal deficit in T1D may have its origins in a shift of mesenchymal stem cell differentiation towards adipogenesis rather than osteogenesis but it also highlights the unique benefit of MRI based assessment for providing mechanistic insight when studying bone health. Furthermore, the positive association between insulin dose and trabecular number substantiates the anabolic effect of insulin on bone (Thomas et al. 1996a, Fulzele et al. 2010). The lack of an association between bone microarchitecture parameters and DXA-based measures of bone density has also been previously noted in young women with T1D (Abdalrahaman et al. 2017) and highlights the point that bone microarchitecture assessment provides additional information on bone health that cannot be obtained from bone
densitometry. Recent histomorphometric and microCT based studies of trabecular bone microarchitecture have shown an inverse association with HbA1c (de Waard et al. 2018, Karim et al. 2018) but this was not observed in the current study or our previous study in young women (McComb et al. 2014a) and perhaps these studies were not sufficiently powered to investigate this association. An association between the deficits in trabecular features and fractures was also not observed and this may also require a larger cohort as well as a study of cortical parameters, as described recently in adults (Samelson et al. 2019). In the previous study performed in young women with T1D, we had reported that bone microarchitectural deficits were more likely in those who had retinopathy (Abdalrahaman et al. 2015). However, given the low prevalence of microvascular complications in children (Sauder et al. 2019), the current study was not sufficiently powered to investigate this finding in this age group.

Approximately a third of the current cohort of children with T1D had suffered a radiologically confirmed fracture before the median age of 13.8 years. Fracture rates in children with T1D have rarely been reported but the current rate of fractures was higher than previously reported in children and young people with T1D (Cameron and Wherrett 2015, Parthasarathy et al. 2016, Sauder et al. 2019) and the control group. In the UK, approximately one third of children are reported to suffer a fracture before the age of 17 years, with a fracture incidence of 3% in boys and 1.5% in girls at their peak (Cooper et al. 2004). Therefore, it is possible that the control group had a relatively lower incidence of fractures. In hindsight, one of the limitations of the study is the lack of data on the rate of fracture in the children before diagnosis of T1D, as this could exclude the remote possibility that by chance the T1D subgroup had individuals with increased bone fragility unrelated to the diabetes.

There were some notable differences between the groups of children with T1D who suffered a fracture compared to those who had not suffered a fracture. Children who fractured had lower total body bone mineral density and poorer glycaemic control compared to the non-fracture group. Abnormalities in cortical bone mineral density with reduced muscle cross-sectional area have been previously reported in T1D and are consistent with an anabolic bone deficit (Bechtold et al. 2006). This is further supported by our finding of significantly lower total body, but not lumbar, bone mineral density in T1D children with fracture. Given that bone microarchitecture at the proximal tibia was not significantly different between the fracture and the non-fracture group, this reinforces the need to study cortical bone architecture in more detail in this condition. Although the combination of lower bone mineral
density, poorer glycaemic control and an increased risk of fractures could have been explained by reduced physical activity (Goulding 2007), this confounder was discarded as both groups had comparable levels of physical activity.

In summary, children and adolescents with relatively well controlled T1D are more likely to have a history of fractures and show deficits in trabecular bone microarchitecture, BMD and bone formation. Although, the association of these markers of bone health to clinical markers of T1D and its management require further exploration through prospective clinical trials, we would recommend that assessment of bone health should become routine in all therapeutic trials in the field of diabetes.
5 The Effects of Anti-Diabetic Drugs on Mesenchymal Stem Cell Differentiation and the Molecular Mechanism of Metformin on Adipogenesis Suppression

5.1 Abstract

It is possible that people with diabetes mellitus (DM) have altered mesenchymal stem cell (MSC) differentiation in the bone marrow, leading to a shift in the balance of differentiation away from bone formation (osteogenesis) in favour of fat cell development (adipogenesis) and a predisposition to fragility fractures. Different classes of antidiabetic drugs have been shown to affect bone health differently, with negative effects from thiazolidinediones whilst metformin is associated with clinical reduction in fracture risk. The effects of metformin on the differentiation of mouse C3H10T1/2 mesenchymal stem cells into fat and bone cells were investigated, with particular interest in the reciprocity of the bone-fat balance. The underlying molecular signalling involved, specifically in the adipogenesis effect of metformin, was also studied, focussing on the AMPK signalling given that metformin is a known AMP-activated protein kinase (AMPK) activator. Metformin was found to exert reciprocal control over the osteogenic transcription factor Runx2, and the adipogenic transcription factor, PPARγ with increase in Runx2 transcriptional activities, independently on Runx2 protein expression and suppression of PPARγ - suggesting a bone protective mechanism through promotion of osteogenesis and suppression of adipogenesis. The suppression of adipogenesis by metformin appeared to be independent of AMPK activation but rather through the suppression of the mTOR/p70S6K signalling pathway. Basal AMPK and mTOR/p70S6K activity did appear to be required for adipogenesis, as demonstrated by the use of the AMPK inhibitor, compound C. It appears, therefore, that basal AMPK activity is required for adipogenesis and that metformin can inhibit adipogenesis through AMPK-dependent or -independent mechanisms, depending on the cellular context.
5.2 Introduction

Adults with Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D) have a significantly increased risk of fractures compared to the general population (Vestergaard 2007, Hothersall et al. 2014). Studies have shown that different classes of antidiabetic drug used in T2D have different effects on the bone. Thiazolidinediones (TZDs), such as pioglitazone and rosiglitazone, have been irrefutably shown to increase fracture risk (Schwartz et al. 2006, Kahn et al. 2008, Loke et al. 2009) whilst the effects of metformin remain inconclusive. Several large population-based case control studies have associated metformin with a reduction in fracture risk (Vestergaard et al. 2005, Melton et al. 2008, Borges et al. 2011) although others did not show any convincing benefit (Kahn et al. 2008). It would be conveniently advantageous if metformin, which is also low cost and safe, confers a bone protective role as it is increasingly used in T1D to improve insulin resistance with a modest effect on glycaemic control (Vella et al. 2010).

There is a growing body of evidence supporting the bone-fat balance and its important role in the pathophysiology of bone loss. Osteoblasts and adipocytes share a common precursor, both derived from the pluripotent MSCs located in the bone marrow, suggesting a mutually exclusive and reciprocal lineage selection of one or the other (Figure 5.1).

![Figure 5.1. Bone-fat balance.](image)

**Figure 5.1. Bone-fat balance.** Osteogenesis and adipogenesis are proposed to be mutually exclusive given that osteoblasts and adipocytes originate from the common progenitor mesenchymal stem cells. PPARγ2 is the key regulator of adipogenesis whilst Runx2 activation determines osteogenesis [Adapted from (Yang et al. 2008)].

The differentiation of MSCs are dependent on two main lineage-specific transcription factors, namely runt-related transcription factor 2 (Runx2) for osteogenesis and the peroxisome proliferator-activated receptor γ (PPARγ) for adipogenesis. By reason that TZDs are agonists of the PPAR family of nuclear transcription factors, particularly the PPARγ isoform (Yki-Jarvinen 2004), their use is detrimental to bone (negative skeletal consequences) as it activates PPARγ and promotes adipogenesis at the expense of
osteogenesis (Rzonca et al. 2004). This is evident is both mice (Botolin et al. 2005) and men (Schwartz et al. 2006, Grey et al. 2007).

On the contrary, the mechanism of action of metformin on skeletal health is less clear. Metformin is a known AMP-activated protein kinase (AMPK) activator as detailed in section 1.7. Mice with deleted AMPK subunits demonstrated a reduction in bone volume and trabecular number supporting the role of AMPK in bones (Shah et al. 2010). AMPK functions to inhibit ATP consuming pathways and at the same time activate catabolic pathways to re-establish cellular energy homeostasis. Osteoblast differentiation has been proposed to be dependent on the cellular energy sensor AMP-activated protein kinase (AMPK), as the expression of various osteogenic genes has been shown to be inhibited by compound C, a chemical inhibitor of AMPK, and a dominant negative form of AMPK (Banerjee et al. 1997).

Indeed, metformin was found to have direct osteogenic effect in vitro by stimulating proliferation and differentiation of osteoblasts, through various signalling pathways including the PI3K/Akt pathway (Ma et al. 2010b), ERK-1,2 (Cortizo et al. 2006) and AMPK activation (Kanazawa et al. 2008, Jang et al. 2011). Based on the bone-fat balance theory, the main aims of the current study are to: (i) determine the effects of metformin on mesenchymal stem cell differentiation in particular its effects on adipogenesis, to examine the suggested bone-fat reciprocity and (ii) understand the underlying molecular mechanisms involved, in particular the role of the AMPK signalling pathway in these processes.

5.3 Materials and Methods

5.3.1 Cell culture and induction of differentiation

Commercially available C3H10T1/2 mouse mesenchymal stem cells (Clone 9; ATCC CCL-226) were chosen as they do not undergo spontaneous differentiation under normal culture conditions and show a more homogenous colony-forming rates during cell expansion as compared to human cell lines, as detailed in section 1.3.3. The C3H10T1/2 cells were maintained in DMEM (41965-039, Sigma-Aldrich Ltd, Gillingham, Dorset, UK) containing 10% (v/v) foetal calf serum (FCS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/ml streptomycin. Cells were kept to 80-90% sub-confluence before further subculture and plating.
At confluence, cells in plates were treated for 5 days with specific differentiation media and treatments for 5 days. To promote adipogenic differentiation, cells were cultured in the standard media supplemented with either 10 μM pioglitazone (PIO) alone or in combination with 100 nM insulin, 500 μM 3-isobutyl-1-methylxanthine (IBMX) and 10 μM dexamethasone (IID medium). Insulin is widely used to induce proliferation and differentiation of pre-adipocytes, as it mimics insulin-like growth factor-1 at high concentration and activates the mitogen-activated protein kinase pathways. IBMX is a competitive, nonselective phosphodiesterase inhibitor, which together with dexamethasone, promotes transcriptional activation of PPARγ through protein kinase A signalling. They also induce C/EBPδ and C/EBPβ which are both transcription factors for growth and differentiation. For osteogenic differentiation, cells were cultured in standard media supplemented with 284 μmol/L ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone (AGD medium). The culture media was changed every 3 days. Cells were also incubated with 10% FCS alone, as negative control for differentiation. The specific treatments included 500 μM metformin, 100 μM of the AMPK-activator (A769662), or 10 μM of the p70S6K-inhibitor (rapamycin), as used previously (Molinuevo et al. 2010, Moreno et al. 2008, Yarwood et al. 1999). Cells were also incubated with 10% FCS alone, as negative control for differentiation.

5.3.2 Preparation of cell extracts

C3H10T1/2 MSCs were harvested and nuclear extracts prepared using the Nuclear Extraction kit from Active Motif, Belgium. Briefly, the media was aspirated and cells were harvested in ice-cold PBS containing phosphatase inhibitors and centrifuged (200 x g, for 5 minutes at 4ºC) to obtain a cell pellet. The cell pellet was re-suspended in complete lysis buffer containing 10 mM DTT and phosphatase inhibitor cocktail and then centrifuged (14000 x g, for 10 minutes at 4ºC) to obtain nuclear and supernatant fractions. Protein concentration of the nuclear fraction was calculated by bicinchoninic acid assay (BCA) (Smith et al. 1985) using prepared BCA kit reagents from Thermo Fisher before loading of samples for electrophoresis.

5.3.3 Western blotting

Protein samples were separated by SDS-PAGE and then transferred electrophoretically onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) Marvel milk powder in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) for 1 hour. Membranes were then incubated with primary antibodies overnight at 4ºC. Following washes with TBST (TBS supplemented
with 0.5% (v/v) Tween-20, membranes were incubated with secondary antibodies for 1 hour at room temperature. Antibodies were diluted to the required concentration in 50% (v/v) Sea Block (Thermo Scientific) and 50% (v/v) TBST. The primary antibodies used were to peroxisome proliferator-activated receptor gamma (PPARγ; marker for adipogenesis, Cell Signalling Technologies (CST), Danvers, MA; dilution 1:1000), Runt-related transcription factor 2 (Runx2; marker for osteogenesis, CST, Danvers, MA; dilution 1:1000), phosphorylated-ACC (P-ACC (Ser79); marker for AMPK activity, CST, Danvers, MA; dilution 1:1000) and phosphorylated-p70S6K (P-p70S6K (Thr389); upstream regulator of mTOR signalling, CST, Danvers, MA; dilution 1:1000). Antibodies were detected using a LI-COR® Odyssey Infrared Imaging systems and densitometric analysis was carried out using ImageJ software (NIH, UK) software Version 1.47.
5.3.4 Oil Red O staining

After 5 days of cell differentiation and treatment (as described in section 5.3.1), the media was aspirated and cells were fixed to cell culture plates with 10% (v/v) neutral buffered formalin for 30 minutes. The formalin was then aspirated and staining was carried out with the addition of 0.3% (w/v) Oil Red O (ORO) in isopropanol:water (60:40) for 5 minutes in room temperature. The ORO was then aspirated and wells washed with distilled water four times. Imaging was carried out using a Zeiss Axiovert 25 microscope with QImaging camera and supporting software.

5.3.5 Transient transfection and luciferase assay

C3H10T1/2 cells were transfected with the indicated plasmids in 6-well plates, with 1.125 μg/well PPRE (PPARγ reporter, purchased from Adgene) and 6xOSE reporter constructs (Runx2 reporter, supplied by Jian Huang, Rush Medical Centre, Chicago, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and then treated with metformin, A769662 or rapamycin with and without adipogenic differentiation media. Cells were then harvested 48 hours after transfection and assayed using the Luciferase reporter assay system (Promega, Madison, WI) according to manufacturer’s instructions. As a transfection control, the Renilla plasmid 0.125 μg/well was co-transfected in each transfection experiment, and the luciferase activity was normalised to the Renilla activity.

5.3.6 Statistical analysis

All experiments were performed in triplicate and statistical analysis was performed using Student’s t-test or one-way ANOVA. Results are expressed as mean ± standard error (SEM) and differences with $p<0.05$ were considered statistically significant.
5.4 Results

5.4.1 Effects of antidiabetic drugs pioglitazone and metformin on murine MSC (C3H10T1/2) differentiation

In confluent C3H10T1/2 MSCs cells (Figure 5.2A and Figure 5.2B), IID-containing medium promoted lipid accumulation (Figure 5.2C), an effect that was further enhanced by co-treatment of cells with PIO (Figure 5.2D) as seen on Oil red O staining. In addition, treatment of cells with either metformin or A769662 suppressed adipogenesis promoted by IID alone (Figure 5.2E and Figure 5.2G, respectively) or by a combination of IID plus PIO (Figure 5.2F and Figure 5.2G). Metformin was a more effective inhibitor of lipid accumulation and less so with A769662, possibly as A769662 is only a selective activator of AMPK β1-containing complexes.

Figure 5.2 Effects of pioglitazone and metformin on lipid accumulation in C3H10T1/2 MSCs. Confluent C3H10T1/2 MSCs (A and B) were induced to differentiate by addition 10% foetal calf serum (FCS) in the presence or absence of adipogenic IID medium (insulin, isobutylmethylxanthine (IBMX) and dexamethasone and/or 10 μM pioglitazone (PIO), 500 μM metformin or 100 μM A769662. After 5 days cells were fixed with formalin and stained with Oil Red O to detect neutral lipid accumulation (red). IID and pioglitazone promoted lipid accumulation (C and D) whilst metformin and the AMPK-activator, A769662, suppress adipogenesis (E-H). Representative micrographs from an experiment carried out on three separate occasions with similar results are shown.
The western blot experiments demonstrated that treatment with IID or PIO induced a significant increase in the two PPARγ splice variants, PPARγ1 and PPARγ2 (Figure 5.3A and Figure 5.3B), which correlated with a significant increase in PPARγ transcriptional activity, as determined by luciferase assay (Figure 5.4A). In agreement with the ORO experiments in Figure 5.2, treatment of cells with either metformin or A769662 effectively suppressed IID- and PIO-stimulated increases in PPARγ protein levels (Figure 5.3A and Figure 5.3B), as well as IID- and PIO-stimulated PPARγ activity (Figure 5.4A).

![Western Blot](image)

**Figure 5.3 Effects of Pioglitazone, Metformin and the AMPK-activator, A769662, on early markers of differentiation in C3H10T1/2 cells.** (A) Confluent CH3H10T1/2 cells were stimulated for 5 days with 10 µM pioglitazone (PIO) or an adipogenic (IID) or osteogenic (AGD) cocktail, in the presence or absence of 500 µM metformin or 100 µM A769662. Cell extracts were then prepared and immunoblotted with antibodies to PPARγ, Runx2 and AMPK. The phosphorylation-dependent electrophoretic mobility shift of Runx2 induced by AGD treatment is indicated (pRunx2). Representative immunoblots from an experiment carried out on three separate occasions with similar results are shown. (B) Densitometric analysis of PPARγ1 (i) and PPARγ2 (ii) levels are shown as means±SEM. Significant increases (*, p<0.05) relative to control, and significant decreases relative to PIO-stimulated cells (#, p<0.05), are indicated (n=3). Non-significant changes are also indicated (ns).
Treatment of cells with AGD did not significantly affect PPARγ protein levels (Figure 5.3A and Figure 5.3B) or activity (Figure 5.4B). AGD treatment alone did promote a noticeable phosphorylation band-shift of Runx2 protein compared to control (Figure 5.3A). Neither metformin nor A76 affected the protein expression level or Runx2 (Figure 5.3A). However, treatment with either metformin or A769662 significantly increased Runx2 transcriptional activity in C3H10T1/2 cells (Figure 5.4B), which did not correlate with an increase in AGD-promoted phospho-Runx2 levels, as determined by band-shift (Figure 5.3A).

Figure 5.4 Luciferase reporter assays to determine activity of the transcription factors PPARγ and Runx2. (A) Confluent C3H10T1/2 cells were transfected with a PPARγ and Runx2 luciferase gene reporter constructs, together with control Renilla luciferase vector, and then stimulated for two days with 500 μM metformin or 100 μM A769662, in the presence or absence of 10 μM pioglitazone (PIO), adipogenic medium (IID) or osteogenic medium (AGD). Cell extracts were then prepared and luciferase activities were measured using a dual luciferase reporter assay. PIO and IID significantly induced, whilst metformin and A76 significantly suppressed PPARγ activity Metformin and A76 promoted Runx2 activity in control, adipogenic and osteogenic media (B). Luciferase activities from three separate experiments are shown as means±SEM. Significant increases in luciferase activity are indicated: *, p<0.05, **, p<0.01 and ***, p<0.001, as are significant decreases in activity, #, p<0.05 (n=3). Non significance is indicated (ns).
5.4.2 Molecular mechanisms involved in the action of metformin on adipogenesis

Based on metformin being a known AMPK activator, its ability to activate AMPK was examined by measuring the phosphorylation of a known AMPK substrate, Ser 79 of acetyl coenzyme carboxylase (ACC), using phospho-specific antibodies. A769662 provoked a robust and rapid phosphorylation of ACC, which was maintained for up to 48 hours, but metformin did not induce a significant phosphorylation of ACC, even after 48 hours stimulation (Figure 5.5).

![Figure 5.5](image)

**Figure 5.5** The AMPK activator, A769662, but not metformin activates AMPK in C3H10T1/2 cells. (A) Confluent C3H10T1/2 cells were stimulated for the indicated times with 100 µM A769662 or 500 µM metformin. Cell extracts were then prepared and immunoblotted with antibodies towards the phosphorylated form of the AMPK substrate, ACC, or total ACC. (B) Densitometric values were obtained from immunoblots from three separate experiments and are shown as means±SEM in the line graph. Significant increases in pACC are indicated; **, *p*<0.01 (n=3). Non significance is also indicated (ns).
Treatment of the differentiating cells with rapamycin, a known mTOR/p70S6K inhibitor, dramatically inhibited lipid accumulation associated with adipogenesis, as determined by ORO staining (Figure 5.6A). Rapamycin also suppressed PPARγ activity, as determined by gene reporter assays, indicating that the mTOR/p70S6K pathway is required for adipogenesis of C3H10T1/2 cells (Figure 5.6B).

Figure 5.6 The mTOR inhibitor, rapamycin, suppresses adipogenesis of C3H10T1/2 cells. (A) Confluent CH3H10T1/2 cells were induced to differentiate by addition of 10% foetal calf serum (FCS) supplemented with adipogenic cocktail (IID), in the presence or absence of the mTOR inhibitor, 10 µM rapamycin. After 5 days cells were fixed with formalin and stained with Oil Red O to detect neutral lipid accumulation. Representative micrographs from an experiment carried out on three separate occasions with similar results are shown. (B) Confluent C3H10T1/2 cells were transfected with PPARγ luciferase gene reporter construct, together with control Renilla luciferase vector and then stimulated for two days with 500 µM metformin or 10 µM rapamycin, in the presence or absence of IID. Cell extracts were then prepared and luciferase activity was measured using a dual luciferase reporter assay. Luciferase activities from three separate experiments are shown as means±SEM. Significant increases in PPARγ activity are indicated ***, p<0.001, as are significant decreases in PPARγ activity, #, p<0.05, relative to IID-stimulated cells (n=3).
Rapamycin treatment had no significant effect on phospho-ACC levels (Figure 5.7A and Figure 5.7B), but significantly inhibited phosphorylation of p70S6K on Thr 389 (Figure 5.7C and Figure 5.7D). Both metformin and A769662 also inhibited p70S6K phosphorylation (Figure 5.7C and Figure 5.7D).

**Figure 5.7 The effects of rapamycin on AMPK and p70S6K activities in C3H10T1/2 cells.**

(A) Confluent CH3H10T1/2 cells were stimulated for 5 days with IID, in the presence or absence of 500 µM metformin, 100 µM A769662 or 10 µM rapamycin. Cell extracts were then prepared and immunoblotted with antibodies to phosphorylated ACC (Ser 79). Representative immunoblots from an experiment carried out on three separate occasions with similar results are shown. (B) Densitometric values from 3 separate experiments are shown in the lower panel as means±SEM. Significant increases relative to control are indicated, **, p<0.01 and ***, p<0.001 (n=3). (C) Confluent CH310T1/2 cells were stimulated for 5 days with IID, in the presence or absence of 500 µM metformin, 100 µM A769662, 10 µM compound C or 10 µM rapamycin. Cell extracts were then prepared and immunoblotted with antibodies to phosphorylated p70S6K and total p70S6K. Representative immunoblots from an experiment carried out on three separate occasions with similar results are shown. (D) Densitometric analysis of mean ± SEM p70S6K phosphorylation from 3 separate experiments are shown. Significant increases (*, p<0.05) relative to control, and significant decreases relative to IID-stimulated cells (##, p<0.01 and ###, p<0.001), are indicated (n=3).
Compound C, a known AMPK inhibitor, significantly inhibited both basal p70S6K (Thr 389; Figure 5.7B) and ACC (Ser 79; Figure 5.8A and Figure 5.8B) phosphorylation, suggesting that AMPK is linked to the activation of mTOR/p70S6K signalling in these cells. In addition, 10 µM compound C inhibited adipogenesis of C3H10T1/2 cells treated with IID (Figure 5.8C).

Figure 5.8 Compound C inhibits adipogenesis of C3H10T1/2 cells. (A) Confluent CH3H10T1/2 cells were stimulated for 5 days with IID, in the presence or absence of the AMPK inhibitor, 10 µM compound C. Cell extracts were then prepared and immunoblotted with antibodies to phosphorylated ACC (Ser 79). Representative immunoblots from an experiment carried out on three separate occasions with similar results are shown. (B) Densitometric values from 3 separate experiments are shown as means±SEM. Significant decreases relative to control are indicated, #, p<0.05.(C) Confluent CH3H10T1/2 cells were treated with 10% foetal calf serum (FCS) supplemented with adipogenic cocktail (IID), in the presence or absence of the indicated concentrations of compound C. After 5 days cells were fixed with formalin and stained with Oil Red O to detect neutral lipid accumulation. Representative micrographs from an experiment carried out on three separate occasions with similar results are shown.
5.5 Discussion

In this study, the different effects of antidiabetic drugs on murine C3H10T1/2 mesenchymal stem cell differentiation was established. Pioglitazone, which is a thiazolidinedione, promoted adipogenesis with increased PPARγ protein expression, PPARγ transcriptional activity and increased lipid droplet accumulation. Metformin on the other hand, inhibited adipogenesis with suppression of PPARγ protein expression, PPARγ transcriptional activity and reduced lipid droplet formation. Metformin also promoted osteogenesis with increased Runx2 transcriptional activity, unaided by osteogenic-linked increases in Runx2 phosphorylation. In addition, the novel finding of an AMPK-independent action of metformin on the inhibition of adipogenesis further adds to the current literature in this area.

It has been previously reported that metformin induced osteoblastic differentiation and matrix mineralisation in MC3T3-E1 pre-osteoblasts via activation of the AMPK signalling pathway (Cortizo et al. 2006, Kanazawa et al. 2007, Jang et al. 2011). Metformin also promoted osteogenesis in rat primary osteoblasts (Shah et al. 2010, Zhen et al. 2010), rat bone marrow progenitor cells (Molinuevo et al. 2010) and more recently in human MSCs (Wang et al. 2018). Jang et al found that metformin-induced AMPK activation increased the level of Runx2 mRNA and protein expression (Jang et al. 2011), which increased alkaline phosphatase activity and mineralisation in mesenchymal cells and osteoblast cells in vitro (Ducy et al. 1997, Harada et al. 1999). This contrasted with our finding as metformin promoted Runx2 transcriptional activity with no effect on Runx2 protein expression, which may be due to cell specific differences. Discrepancies between protein expression and enzymatic activity have been previously described highlighting the drawbacks of merely interpreting protein, or even mRNA expressions, to target outcomes (Anderson and Seilhamer 1997, Miyamoto et al. 2001). The examination of enzymatic activity is key considering the complex regulatory mechanisms for gene expression that occur both at post-transcriptional and post-translational levels. A recent breakthrough study provided a plausible explanation, suggesting that because the transcription factors for osteogenesis are already at high basal activity in undifferentiated MSCs, they are therefore only modestly regulated at the mRNA level as opposed to the transcription factors for adipogenesis which are more likely to be transcriptionally regulated being barely active in MSCs (Rauch et al. 2019).

Similar to Molinuevo et al, the current study demonstrated that metformin suppressed adipogenesis with reduced lipid droplet accumulation on Oil red O staining (Molinuevo et
al. 2010), and proceeded to demonstrate the reciprocal effects of metformin on the adipogenic transcription factor PPARγ and osteogenic transcription factor Runx2. Given that increases in PPARγ activity during the early stages of adipogenesis are necessary and sufficient to promote terminal fat cell development (Rosen and Spiegelman 2000), it appears that the ability of metformin and A769662 to inhibit adipogenesis is linked to their ability to suppress PPARγ protein expression. Moreover, given that the PPARγ luciferase reporter assays were carried out after only two days of differentiation, it appears that the adipogenic suppressive actions of metformin and A769662 occur at a very early stage of the differentiation process in favour of Runx2 activity promotion.

Metformin exerted these effects on the transcription factors similarly to A769662, which is a known potent, reversible AMPK activator suggesting the role of AMPK activation in mesenchymal stem cell differentiation. Both metformin and A769662 have been reported to activate AMPK in a variety of cell types (Zhou et al. 2001, Cool et al. 2006). Previous studies have validated the role of AMPK activation in osteogenesis, with the use of metformin and 5-aminoimidazole-4-carboxamide riboside (AICAR), the latter which is another pharmacological activator of AMPK (see section 1.7.2). In human adipose-derived and bone marrow MSCs, AMPK activation was also crucial (Kim et al. 2012, Lee et al. 2014) in osteogenesis with pharmacological induced AMPK inhibition and AMPKα1 knock-out mice demonstrating reduced bone matrix mineralisation, alkaline phosphatase (AP) activity, Runx2 and osteocalcin protein expression (Kim et al. 2012). AMPK knock-out mice have also been shown to have reduced trabecular bone mass and cortical indexes. Paradoxically, studies have also shown that osteoblast differentiation is functionally associated with decreased AMPK activity (Kasai et al. 2009).

The role of AMPK activation in adipogenesis, however, is less well understood. AMPK activation with AICAR has been shown to inhibit the conversion of pre-adipocytes to fat cells (Habinowski and Witters 2001, Lee et al. 2011). Paradoxically, however, the widely used AMPK inhibitor, compound C, has also been reported to inhibit adipogenesis of preadipocyte cell lines (Nam et al. 2008). Previous work has shown that AMPK activation suppresses adipogenesis in favour of osteogenesis in adipocyte-derived human MSCs (hMSCs) and bone marrow-derived MSCs (Kim et al. 2012, Lee et al. 2014). Chava et al. in a recently published study have demonstrated that RUNX2 was in fact a novel substrate for AMPK, with metformin-induced AMPK activation preventing RUNX2 ubiquination in osteogenesis over adipogenesis (Chava et al. 2018). Given the inhibitory effects of these two compounds on early and late markers of adipogenesis in C3H10T1/2 MSCs, it was rather
intriguing to find that metformin did not inhibit adipogenesis through AMPK activation. The use of rapamycin to study the mTOR/p70S6K signalling pathway became apparent following recent work showing the interaction between PPARγ and the mTOR/p70S6K (Sun et al. 2013). Moreover, metformin has been shown to inhibit the activation of the p70S6K pathway independently of AMPK (Vazquez-Martin et al. 2009) in tumour cells and p70S6K has been shown to be required for the growth hormone-dependent adipose conversion of 3T3-F442A preadipocytes (Yarwood et al. 1999). In the present study, rapamycin specifically inhibited phosphorylation of p70S6K on Thr 389, which is the mTOR phosphorylation site critical for kinase function (Saitoh et al. 2002), with no significant effect of phospho-ACC levels highly indicative of an AMPK-independent action.

This, however, does not rule out a role for AMPK in the control of p70S6K activation. In fact, it has been reported that AMPK inhibits mTOR/p70S6K signalling, which is thought to underlie the actions of metformin in a range of cellular contexts (Dowling et al. 2011, Viollet et al. 2012). The suppression of adipogenesis with Compound C, which is an AMPK inhibitor, may suggest that AMPK is linked to the activation of mTOR/p70S6K signalling in these cells, although Compound C itself is a very non-selective AMPK inhibitor (Yu et al. 2008, Vogt et al. 2011). There may be a basal level of AMPK activity that is necessary for supporting adipose conversion of these cells, and this could be examined further with the use of AMPK knock out models. A recent study published has also demonstrated an intriguing dose dependency effect of metformin on adipogenesis (Chen et al. 2018).

The AMPK-independent action of metformin on the inhibition of adipogenesis reported here may be due to cell type-specific effects during the differentiation process. For example, most of the studies demonstrating an AMPK-dependent action of metformin have been conducted in more differentiated cell lines, such as pre-osteoblasts (Cortizo et al. 2006, Kanazawa et al. 2008, Jang et al. 2011), pre-adipocytes (Moreno-Navarrete et al. 2011, Lee et al. 2012), myoblasts (Longnus et al. 2005, Fulco et al. 2008, Kobashigawa et al. 2014) and neuronal mouse cell lines (Bang et al. 2014), instead of the more primitive progenitor cells as investigated here.

In addition, the AMPK-independent action noted may also be due to cell stage-specific effects during the commitment and differentiation process. Pantovic et al. (Pantovic et al. 2013) have demonstrated that there is a coordinated time-dependent activation of different signalling pathways during the osteogenic differentiation of hMSCs, which is AMPK-dependent in the early stages of differentiation followed by late stage activation of the
Akt/mTOR signalling pathway. Given the results presented here, it could be argued that similar mechanisms might regulate adipogenesis in murine MSCs. We found an overall requirement for basal levels of AMPK activity for adipogenesis in C3H10T1/2 cells but the ability of metformin to control the commitment of MSCs to differentiate into either osteoblasts or adipocytes is governed at an early stage through the inhibition of mTOR/p70\textsuperscript{S6K} signalling.

Overall, my work suggests that metformin as an anti-diabetic agent has a promising bone protective function given its positive role in osteogenesis and negative effect on adipogenesis in mesenchymal stem cell differentiation. It is worth noting the supraphysiological dose of metformin used may limit its translation to clinical studies although other biguanides such as phenformin which is much more effective at lower doses is currently being used in clinical trials. It exerts multiple effects to inhibit adipogenesis in different cell types. Therefore, the overriding view that metformin exerts its effects on adipogenesis simply by promoting AMPK activation may therefore need some revision, especially as the role of AMPK itself appears to be complex. In conclusion, further investigation into how metformin suppresses signalling through the mTOR/p70\textsuperscript{S6K} pathway may enable the discovery of new therapeutic intervention strategies to prevent unwanted bone marrow adipogenesis associated with diseases, such as diabetes mellitus, where bone health is impaired.
6 Final Discussion and Future Directions

6.1 Final discussion

Diabetic osteopathy as a complication of diabetes mellitus has undoubtedly gained increasing awareness amongst clinicians and researchers in the last two decades. The programme of research described in this thesis was borne out of an appetite to understand if skeletal fragility affects the paediatric population with Type 1 diabetes (T1D) and the contribution of bone microarchitecture and the intriguing bone-fat balance hypothesis to diabetic osteopathy.

Using novel technique of high-resolution MRI (3T) to image bones in children with T1D, the findings in this thesis add to the current body of evidence in this field, with original results that address the knowledge gap. Findings from Chapter 4 showed that children with T1D demonstrated deficit in bone microarchitecture compared to their healthy age- and gender-matched peers, novel to the current knowledge base. Across all the paediatric age group, children with T1D have lower trabecular bone volume fraction, which was the primary hypothesis of the study. The deleterious effect of T1D on trabecular bone microarchitecture has been shown, not only in mouse models of T1D using microCT (Nyman et al. 2011), but also in adults with T1D from pQCT studies (Shanbhogue et al. 2015). Abdalrahaman et al. performed the first MRI-based assessment of bone microarchitecture in young adults with T1D which concluded similarly of trabecular deficits (Abdalrahaman et al. 2015), but this was contradicted by histomorphometry findings (Armas et al. 2012). Armas et al. showed no abnormalities in trabecular bone on histomorphometry and microCT specimens in a small cohort of adults with T1D, although the study may not be sufficiently powered to detect the differences with a small sample size of 18 cases (Armas et al. 2012). In addition, the study in Chapter 4 found that the trabecular deficits were present early, within 10 years from T1D diagnosis. Comparatively, decrease in trabecular bone volume fraction was noted by 10 weeks of T1D diagnosis in mouse models (Nyman et al. 2011), which is equivalent to 20 human years, supporting the need to intervene early for bone health optimisation.

Intuitively, chronic hyperglycaemia and insulin deficiency have been implicated in the pathogenicity of diabetic osteopathy, along with the complex interplay of GH/IGF-1 pathway inactivity, increased marrow adiposity, chronic inflammation and vasculopathy as detailed in section 1.5.3. The study in Chapter 4 contributed to an improved understanding
of the possible mechanisms for diabetic bone loss. Chronic hyperglycaemia, as reflected by high HbA1c, was significantly associated with reduced bone formation. This is in line with experimental and animal models of type 1 diabetes (T1D) (Rico et al. 1989, Verhaeghe et al. 1990), and consistent with clinical studies reporting a reduction in biochemical markers of bone formation (Gunczler et al. 1998, Pater et al. 2010, Starup-Linde et al. 2014).

The other novel finding in Chapter 4 was the effect of acidosis at initial T1D presentation on lower bone formation marker suggesting the additional programming effect of either sustained hyperglycaemia, or acidosis on skeletal development. Acidosis has been shown to affect bone homeostasis (Topaloglu et al. 2005) and may also have an effect on mesenchymal stem cell differentiation (Guntur et al. 2018). In addition to reduced bone formation, children with T1D also have reduced bone resorption as described in section 1.5.5.1, further confirming the low bone turnover seen in T1D (Campos Pastor et al. 2000, Moyer-Mileur et al. 2008, Starup-Linde et al. 2014, Shanbhogue et al. 2015). This may reflect functional growth hormone resistance as outlined in section 1.5.3.2, although the study found no difference in circulating IGF-1 levels in T1D compared to healthy controls. However, the inverse association between serum ALS and the apparent trabecular bone volume fraction found in this study highlighted a role for the GH/IGF-1 pathway in diabetic osteopathy, which the study was not specifically powered to look for. Bone turnover markers have been known to correlate with bone deficit and predicted fragility fractures (Szulc and Delmas 2008) with IGF-1 reported as a fracture predictor in post-menopausal women with T2D independent of BMD. Further studies with larger sample size are needed to evaluate the validity of IGF-1 as an independent fracture predictor for T1D and to better understand the contribution of the GH/IGF-1 pathway in this condition.

Vasculopathy has been strongly implicated in the pathogenesis of diabetic bone loss as detailed in section 1.5.3.6., with the direct effect of bone microangiopathy and the reduction of blood flow on mesenchymal stem cell differentiation. The independent association of microvascular complications with reduced BMD and increased fracture risk in T1D is well established (Ivers et al. 2001, Miao et al. 2005, Vestergaard 2007, Weber et al. 2015). In addition, clinical studies have demonstrated that the presence of associated microvascular complications, such as retinopathy and nephropathy, are significantly associated with adverse cortical and trabecular bone microarchitecture in individuals with T1D, rather than having T1D on its own (Abdalrahaman et al. 2015, Shanbhogue et al. 2015). The study in Chapter 4 looked at this association and found no difference in the bone microarchitecture
in the T1D children with and without microvascular complications, primarily limited by the very small number of children who have developed retinopathy or nephropathy by this point.

The other possible explanations for bone deficit in T1D include sclerostin-mediated inhibition of bone formation, as detailed in section 1.5.5.1. Sclerostin, produced almost exclusively by osteocytes, is an inhibitor of the canonical Wnt/β-catenin signalling pathway involved in bone formation, thus playing an essential role in osteoblast differentiation and bone turnover (Baron and Kneissel 2013). Many studies have found higher sclerostin levels in T1D individuals (Catalano et al. 2014, Neumann et al. 2014), with overexpression of sclerostin causing osteopenia in mouse models (Baron and Kneissel 2013). Although sclerostin was measured in children with T1D in the study in Chapter 4, the interpretation of this was limited by not measuring the levels in the healthy controls and the paucity of normative data for sclerostin in this age range. An inverse relationship was found between sclerostin and the age of T1D diagnosis. Children who were younger at T1D diagnosis had higher sclerostin levels, as well as higher bone resorption, which may adversely affect their bone health. Manifestation of T1D at an early age has been shown to negatively impact bone development (Bechtold et al. 2006). This may suggest a predisposing variant, the so-called ‘more aggressive’ T1D lending to poorer long-term outcomes, rather than chronic metabolic consequences of the disease, or duration of disease on the bones. The duration of diabetes has, however, been shown to positively correlate with higher sclerostin levels, potentially impairing bone health and increasing fracture risk (Catalano et al. 2014). The study in Chapter 4 did not find any association between sclerostin levels and bone microarchitecture. Interestingly, sclerostin levels have been shown to be inversely associated with fracture in T1D individuals, in that, those with the highest tertile of sclerostin having a markedly reduced (by 81%) risk of fracture compared with those in the lowest tertile (Starup-Linde et al. 2016). This is counterintuitive and remains unexplained. The reproducibility of that study or further mechanistic explanation has yet to be seen.

Ultimately, the most important consequence of diabetic bone loss is fractures with a significant cost to the individual and health economy as a whole, not to mention early onset osteoporosis which also impacts on quality of life. The stimulation for this thesis was the lack of understanding of whether skeletal fragility affects the paediatric population with Type 1 diabetes (T1D), especially given the fact that childhood and adolescents are the peak ages for both the: 1) diagnosis of T1D and the 2) attainment of peak bone mass. Since this work began in 2014, Weber et al. demonstrated in 2015 in a large population-based study that the increased risk of fractures in T1D did not only affect adults, but began in childhood
and extended across the life span (Weber et al. 2015). The clinical study in Chapter 4 confirmed this finding that children with T1D have higher incident fractures compared to their healthy age- and gender-matched peers. Children who fractured had poorer glycaemic control, although HbA1c were not associated with any of the trabecular bone parameters suggesting a complex link between glycaemic control, bone formation and bone microarchitecture. More recent studies, however, have shown an inverse association between trabecular bone microarchitecture and HbA1c in individuals with T2D (de Waard et al. 2018, Karim et al. 2018) although this relationship was not found in young adults with T1D (Abdalrahaman et al. 2015). The absence of a relationship between the bone parameters and HbA1c also beckons the need to explore other markers of glycation such as pentosidine.

Indeed, Granke et al found that the fracture toughness of bone were negatively associated with pentosidinle levels (Granke et al. 2015), a well-known advanced glycation end (AGE) product found in diabetic patients, which may marry better the link between hyperglycaemia, bone microstructure and fractures. Children who fractured also had lower total body BMD. Given that fractures were associated with total body BMD but not lumbar spine BMD, and an association between the deficits in trabecular bone microarchitecture and fractures was not observed, there may be a need to investigate cortical parameters to better understand bone health in T1D as previously done in adults with T1D (Lettgen et al. 1995). A larger cohort study is warranted for more detailed investigations of T1D characteristics and its association with bone microarchitecture in individuals with T1D.

This is the first study to demonstrate a deficit in MRI-based trabecular deficit in children with T1D, contributing not only new findings to the current knowledge base but also evidence of the feasibility of using this non-invasive technique in children in clinical studies. As discussed in section 1.5.6.1.1, although evidence have universally shown reduction in BMD in adults and children with T1D, there is a considerable overlap in BMD values in patients with and without fragility fractures. Specifically, the work in this thesis showed reduction in BMD in T1D children who fractured but did not discover any evidence of an association between bone microarchitecture variables and DXA-derived bone mineral density, for reasons as described in the previous paragraph. MRI-based assessment is free from ionising radiation, as compared to DXA, rendering it superior especially for repeated measurements in prospective clinical trials to further explore the association of clinical markers of T1D, 3D-volumetric density and the different compartments of bone, including trabecular and cortical bones, especially in children.
Additionally, MRI provides the unique imaging modality to study bone marrow adiposity, given that the bone marrow is home to pluripotent mesenchymal stem cells capable of differentiating into osteoblasts and adipocytes. The bone-fat hypothesis suggested that adipogenesis in the marrow occurs at the expense of osteogenesis, which has been shown \textit{in vitro} in mouse and human cells and \textit{in vivo} in mouse models but not in clinical trials in T2D, as detailed in section 1.5.3.4. The study in Chapter 4 supported this mechanism through the negative association of trabecular number with marrow fat fraction, and a corresponding positive association of trabecular separation with marrow fat fraction, when the whole cohort with and without T1D was analysed. This is the first study to demonstrate an association of marrow adiposity and MRI-based bone microarchitecture. Previous studies have shown a similar inverse association between marrow adiposity with pQCT-based trabecular and cortical parameters in obesity (Bredella \textit{et al.} 2012) and DXA-based BMDs in a healthy population (Shen \textit{et al.} 2012a, Shen \textit{et al.} 2012b). However, similar to the only study to date looking at BMA in T1D, this study found a non-significant increase in BMA in individuals with T1D compared to controls. Additionally, there were no association between BMA and glycaemic control, markers of GH/IGF-1 axis, bone turnover markers, or BMD. Poor glycaemic control has been shown to be associated with increased bone marrow adiposity in T2D (Baum \textit{et al.} 2012), where hyperglycaemia diverts the differentiation of mesenchymal stem cells towards adipogenesis (Wang \textit{et al.} 2014). A recent study published, however, demonstrated that adipocytes readily accumulated in the bone marrow of individuals with T2D compared than controls (Ferland-McCollough \textit{et al.} 2018). Unfortunately, our study was limited in its exploration of the bone marrow adiposity by the cross-sectional design and its small sample size, which was not powered to study these associations. With an increasing direct link between BMA with bone strength (Karampinos \textit{et al.} 2015) and BMA with fracture risk (Patsch \textit{et al.} 2013b), it would be critical to explore the role of marrow adiposity in future larger prospective cohort studying diabetic osteopathy.

The ability of metformin on a molecular level to manipulate the differentiation of mesenchymal stem cell (MSC) differentiation may be key to the clinical findings of reduced fracture risk in T2D individuals on this medication. The work in this thesis in Chapter 5 studied the effects of metformin on commercially available murine MSCs and found that metformin promoted osteogenesis with reciprocal suppression of adipogenesis, further affirming the bone-fat balance. This is similar to the findings of Molineuvo \textit{et al.} demonstrating a positive osteogenic effect of metformin on rat bone marrow progenitor cells, with partial suppression of adipogenesis (Molinuevo \textit{et al.} 2010). More importantly, a recent study had demonstrated that metformin also promotes osteogenesis in human bone-marrow...
derived MSCs (Wang et al. 2018). Although bone-marrow derived human MSCs can be commercially purchased, these are expensive. Primary human MSCs are less readily available, and can be harvested from bone marrow, peristeum, adipose tissue, amniotic fluid and fetal tissues. Adipose tissue MSCs are seen as most easily available from liposuction procedures or bariatric surgery. However, despite the fact that these can be phenotypically similar, they display heterogeneity in differentiation potential compared to bone-marrow derived MSCs (Pittenger et al. 1999, Anker et al. 2003), hence are not readily interchangeable. Moreover, the challenge remains in identifying factors that promote specific osteogenic and adipogenic commitment of humans MSCs, which is different from that of mouse MSCs (Marie and Fromigue 2006). Primary human MSCs also take longer to grow and achieve confluency for induction of differentiation, taking approximately 4 times longer than the choice of C3H10T1/2 murine MSCs at each subculture (Charbord et al. 2011). Interestingly, metformin appeared to have a negative angiogenic potential on human mesenchymal stem cells, which may potentially negate its positive skeletal effects (Montazersaheb et al. 2018).

The effect of metformin on adipogenesis in MSCs has been less well studied (Molinuevo et al. 2010, Shin et al. 2013). Molinuevo et al found that metformin promoted early markers of adipogenesis with lipid droplet accumulation on ORO but no PPARγ expression (Molinuevo et al. 2010). Data from the experiments in Chapter 5 demonstrated that metformin inhibited adipogenesis through suppression of adipogenic transcription factor PPARγ expression level and transcriptional activity, through AMPK-dependent and independent signalling pathways. The AMPK independent signalling pathway of metformin on adipogenesis, through suppression of mTOR/p70s6K is novel and adds to the current body of literature with new targets for therapeutic potentials. In osteogenesis, however, metformin has been extensively shown to act through AMPK activation. Previous studies in 3T3-L1 pre-adipocytes demonstrated that metformin behaved in a biphasic manner with dose dependency in adipogenic differentiation (Chen et al. 2018). Lower concentrations of metformin (1.25-2.5mM) induced adipogenesis, mediated in an AMPK-independent manner, while higher concentrations of metformin (5-10mM) inhibited adipogenesis via AMPK activation (Chen et al. 2018). The experiments in Chapter 5 were primarily a proof of concept study which demonstrated the positive osteogenic and negative adipogenic effects of metformin at 10mM. This poses translational difficulty due to that being a supraphysiological dose of metformin in human, which is 100 folds the current therapeutic dose of 1g twice daily in the treatment of T2D.
Metformin is not infrequently used in T1D to improve insulin sensitivity and reduce insulin dose requirement with marginal effects on glycaemic control (Vella et al. 2010). A recent randomised controlled trial has shown the additional cardiovascular benefits of metformin in reducing atherosclerosis progression, LDL-cholesterol and weight in adults with T1D (Petrie et al. 2017). With the increasing use of metformin in T1D, it would be conveniently advantageous given that metformin confers a bone protective role on a molecular level. To date, no studies have been published specifically studying the benefits of metformin on bone health. A prospective cohort study is warranted given the therapeutic potentials of metformin, a cheap and safe drug, in addressing the skeletal fragility in T1D.
6.2 Future directions

This thesis has opened up some intriguing avenues for further research, based on some of the limitations of the studies identified as well as new findings from the work. From the limitations of the current work, studies with larger sample sizes are needed to better understand the associations of the clinical characteristics of T1D, such as HbA1c, age at diagnosis, duration of disease and the presence of other microvascular complications, with bone microarchitecture and fractures. It would not be unreasonable to suggest that all intervention studies in diabetes should include some assessment of bone health amongst the secondary outcomes that are assessed.

In my opinion, future interventions to manage and prevent diabetic osteopathy lie in understanding the underlying principles of diabetes induced bone loss. The lack of an overt link between glycaemic control, surrogated by HbA1c, and bone microarchitecture and marrow adiposity highlighted the need to look at other ways chronic hyperglycaemia could detriment bone. As mentioned, it would be worth exploring the relationship of other glycation products such as pentosidine, a well-known advanced glycation end-product in diabetes, particularly as this has been linked to the structural integrity of bone (Granke et al. 2015). In the last decade, glycaemic variability has also become the new subject of contention in interpreting the glycaemic control of individuals with diabetes. Highly variable glucose levels have been implicated as an independent risk factor for developing diabetes-related microvascular complications (Brownlee and Hirsch 2006). It has also been associated with macrovascular complications, with worse cardiovascular outcomes in both T1D (Jun et al. 2019) and T2D (Benalia et al. 2019). With similar HbA1c, individuals with diabetes could have very different glycaemic variability, depending on the extent of their blood glucose excursions. Therefore, understanding the contribution of advanced-glycation end protein and glycaemic variability on the bone microarchitecture may improve the understanding of the effects of hyperglycaemia on skeletal fragility in diabetes, with potential therapeutic implications.

Given that the trabecular bone microarchitecture at the proximal tibia was not significantly different between the fracture and the non-fracture group, this suggests a need to examine the cortical bone architecture in more detail in this condition. Further investigations of cortical bone parameters by using MRI could add mechanistic insights into the pathophysiology of diabetic bone loss in particular looking at its relationship to bone marrow adiposity as well. In addition to exploring different bone compartments, imaging a different
skeletal site such as the hip may be more informative, given that hip fractures are the most common fragility fracture affecting adults with T1D. Future studies should focus on pulse sequences for MR image acquisition from the neck of femur, with thicker cortical bone ideal for better characterization in this condition. Furthermore, the feasibility of 7T-MR imaging at the hip has been demonstrated with promising results (Chang et al. 2014).

With metformin being a promising therapeutic option for bone protection in diabetes mellitus, a prospective longitudinal clinical trial with defined musculoskeletal outcomes should be explored to demonstrate efficacy, determine optimum dose, and possibility a minimum duration of exposure necessary for clinical benefit. This is especially pertinent as bone microarchitecture abnormalities are already observed in the pediatric age group and metformin is not licensed for use in children under 10 years of age. The adult REMOVAL (Reducing with Metformin Vascular Adverse Lesions) cohort (Petrie et al. 2017), which was randomised to 3 years of metformin treatment would provide an ideal starting platform to study the effects of metformin on the bone microarchitecture of individuals with T1D, with the limitation of absent baseline imaging prior to treatment. By incorporating dynamic contrast enhanced (DCE) MRI (Tofts 1997, Radjenovic et al. 2008), the effect of metformin on bone marrow vascularity could also be studied given the potential unwanted anti-angiogenic potential of this drug (Montazersaheb et al. 2018).
List of References


serum IGF-1 are associated with vertebral fractures among postmenopausal women with type-2 diabetes', Bone, 56(2), 355-62.


type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages', *J Cell Biol*, 142(1), 295-305.


Cibula, D., Skrenkova, J., Hill, M., Stepan, J.J. 'Low-dose estrogen combined oral contraceptives may negatively influence physiological bone mineral density acquisition during adolescence', *Eur J Endocrinol*, 166,1003-1011


Day, T. F., Guo, X., Garrett-Beal, L. and Yang, Y. (2005) 'Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis', Dev Cell, 8(5), 739-50.


disease, and bone quality of the distal radius and tibia as measured with high-resolution peripheral quantitative computed tomography - The Maastricht Study', Osteoporos Int.


http://www.revisemri.com/questions/pulse_sequences/se_ge_differences. [online], available: [accessed 28/10].
dihydroxyvitamin D3-induced effect on human osteoblast-like MG-63 cells’, *J Bone Miner Res*, 10(7), 1050-6.


Mastrandrea, L. D., Wactawski-Wende, J., Donahue, R. P., Hovey, K. M., Clark, A. and Quattrin, T. (2008) 'Young women with type 1 diabetes have lower bone mineral density that persists over time', *Diabetes Care*, 31(9), 1729-35.


Okazaki, K., Yamaguchi, T., Tanaka, K., Notsu, M., Ogawa, N., Yano, S. and Sugimoto, T. (2012) 'Advanced glycation end products (AGEs), but not high glucose, inhibit the osteoblastic differentiation of mouse stromal ST2 cells through the suppression of osterix expression, and inhibit cell growth and increasing cell apoptosis', *Calcif Tissue Int*, 91(4), 286-96.


Prockop, D. J. (1997) 'Marrow stromal cells as stem cells for nonhematopoietic tissues', Science, 276(5309), 71-74.


serum markers of bone turnover in healthy children from 2 months to 18 years’, *J Clin Endocrinol Metab*, 92(2), 443-9.


Van Oss, C. J., Good, R. J. and Chaudhury, M. K. (1987) 'Mechanism of DNA (Southern) and protein (Western) blotting on cellulose nitrate and other membranes', *J Chromatogr*, 391(1), 53-65.


Appendices

Clinical Study Documentation

Appendix A  Study Protocol

Appendix B  Case Report Form for Cases

Appendix C  Case Report Form for Controls

Appendix D  Consent/Assent Form for Cases

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Appendix F  Physical Activity Questionnaire (Under 14 years old)

Appendix G  Physical Activity Questionnaire (14-19 years old)

Publications


STUDY PROTOCOL

TITLE The Effects of Type 1 Diabetes Mellitus on the Bone Health of Children and Adolescents

VERSION Version 3.0

DATE 10/12/2014

SPONSOR NHS Greater Glasgow and Clyde (GGC)

FUNDER Glasgow Children’s Hospital Charity

QUALIFICATION MD, University of Glasgow

AIM

This study will be performed according to the Research Governance Framework for Health and Community Care (Second edition, 2006) and World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects 1964
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## Abbreviations

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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>Bic</td>
<td>bicarbonate</td>
</tr>
<tr>
<td>BMC</td>
<td>bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BV/TV</td>
<td>ratio of bone volume over total volume of area scanned</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>CRF</td>
<td>Clinical Research Facility</td>
</tr>
<tr>
<td>CTX</td>
<td>carboxyl-terminal cross-linked telopeptide of Type 1 collagen</td>
</tr>
<tr>
<td>DXA</td>
<td>dual X-ray absorptiometry</td>
</tr>
<tr>
<td>GGC</td>
<td>Greater Glasgow and Clyde</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>H&lt;sup&gt;+&lt;/sup&gt;</td>
<td>hydrogen ions</td>
</tr>
<tr>
<td>HbA1C</td>
<td>glycated haemoglobin</td>
</tr>
<tr>
<td>IGF1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>ALS</td>
<td>acid labile subunit</td>
</tr>
<tr>
<td>IRR</td>
<td>incidence rate ratio</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MRS</td>
<td>magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>phosphate</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RHC</td>
<td>Royal Hospital for Children, Glasgow</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 Diabetes Mellitus</td>
</tr>
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</table>
STUDY SYNOPSIS

<table>
<thead>
<tr>
<th>Title of study</th>
<th>The Effects Of Type 1 Diabetes Mellitus On The Bone Health Of Children And Adolescents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study Centre</td>
<td>Royal Hospital for Children, Glasgow</td>
</tr>
<tr>
<td>Duration of Study</td>
<td>24 months</td>
</tr>
<tr>
<td>Objectives</td>
<td>To use novel methods of microMRI and MRS in assessing bone health of children with T1DM</td>
</tr>
<tr>
<td>Primary Objective</td>
<td>To establish the prevalence of abnormal bone health in children with newly diagnosed and established T1DM, comparing cases and controls</td>
</tr>
<tr>
<td>Secondary Objective</td>
<td>To assess the relationship of abnormal bone health to disease and treatment factors</td>
</tr>
<tr>
<td>Methodology</td>
<td>Prospective study in the newly diagnosed T1DM children over 14 month period from diagnosis</td>
</tr>
<tr>
<td></td>
<td>Cross sectional study in established diagnosed cases of T1DM</td>
</tr>
<tr>
<td>Sample size</td>
<td>Newly Diagnosed Diabetes Study</td>
</tr>
<tr>
<td></td>
<td>Estimated 30 cases, based on annual incidence of T1DM in NHS GGC Paediatric Diabetes service</td>
</tr>
<tr>
<td></td>
<td>Established Diabetes Study</td>
</tr>
<tr>
<td></td>
<td>30 cases, based on power calculation and previous studies</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>Estimated 30 controls, based on power calculation and previous studies but final numbers may vary</td>
</tr>
<tr>
<td>Inclusion criteria</td>
<td>Newly Diagnosed Diabetes Study</td>
</tr>
<tr>
<td></td>
<td>• First presentation of T1DM during study period</td>
</tr>
<tr>
<td></td>
<td>• Age ≥10 and ≤18 yrs</td>
</tr>
<tr>
<td></td>
<td>Established Diabetes Study</td>
</tr>
<tr>
<td></td>
<td>• Children with T1DM of ≥3 yrs duration</td>
</tr>
<tr>
<td></td>
<td>• Age ≥10 and ≤18 yrs</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>• Healthy children age ≥10 and ≤18 yrs</td>
</tr>
<tr>
<td></td>
<td>• No contraindications for MRI</td>
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### Exclusion criteria

<table>
<thead>
<tr>
<th><strong>All potential participants (including controls)</strong></th>
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<tbody>
<tr>
<td>• Age ≥ 19 years</td>
</tr>
<tr>
<td>• Significant other chronic diseases</td>
</tr>
<tr>
<td>• On medication known to affect bone health (e.g glucocorticoids, bone protective therapy)</td>
</tr>
</tbody>
</table>

#### Established Diabetes Study

| • Age < 10 years                                  |
| • Disease duration < 3 years                      |

#### MRI exclusion criteria

| • Contraindications for MRI (e.g cardiac pacemaker, metallic clip in body etc) |

### Study involvement

#### Assessment of bone health by:

| • Dietary, exercise & fracture history            |
| • Biochemical markers of bone metabolism          |
| • Serum GH/IGF1 axis and adipokines               |
| • Dual energy X-ray Absorptiometry (DXA)          |
| • MRI to assess bone microarchitecture            |
| • MRS to assess vertebral bone marrow adiposity   |

#### Assessment of T1DM disease by:

| • Degree of acidosis at presentation              |
| • Glycated haemoglobin (HbA1c) & C-peptide        |
| • Insulin dose (unit/kg)                          |
| • Presence of other microvascular complications   |

### Primary Outcome

| Quantitative measurements of trabecular bone (bone volume/total volume) obtained from microMRI images in cases versus controls |

### Secondary Outcome

1) Measure of bone marrow adiposity by MRS
2) Relationship of markers of bone health (including DXA) to markers of T1DM disease
3) Change in bone and adiposity markers over first year of diagnosis

### Statistical Analysis

Inter-group comparison to be performed by non-parametric analyses of difference and assessment of correlation between continuous variables by univariate and multivariate regression analysis
## STUDY FLOW CHART: Newly Diagnosed Diabetes Study

<table>
<thead>
<tr>
<th></th>
<th>Visit 1 (To be completed within 10 days of discharge)</th>
<th>Visit 2 (6-8 months from diagnosis)</th>
<th>Visit 3 (12-14 months from diagnosis)</th>
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</thead>
<tbody>
<tr>
<td>Obtain informed consent</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Personal and demographic data</td>
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<td>x</td>
<td></td>
</tr>
<tr>
<td>Medical, surgical and medication history</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Review inclusion and exclusion criteria</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Physical examination</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pubertal assessment</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Height (cm) and weight (kg)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>BMI calculation (kg/m²)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Study bloods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- HbA1c* and C-peptide</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>- Laboratory blood glucose*</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Degree of acidosis (H⁺, Bic)*</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Markers of bone metabolism</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>- Serum GH/IGF1 axis and adipokines</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Urinalysis*</td>
<td>x</td>
<td></td>
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</tr>
<tr>
<td>MRI scan</td>
<td>x</td>
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</tbody>
</table>

* Routinely performed for clinical purpose
## STUDY FLOW CHART: Established Diabetes Cases

<table>
<thead>
<tr>
<th>Study Period</th>
<th>Routine clinic visit for initial approach</th>
<th>Visit 1 (Next clinic visit when screening bloods due)</th>
<th>Visit 2 (MRI attendance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obtain informed consent</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Personal and demographic data</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Medical, surgical and medication history</td>
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<tr>
<td>Review inclusion and exclusion criteria</td>
<td></td>
<td>x</td>
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<tr>
<td>Physical examination</td>
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<td>x</td>
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</tr>
<tr>
<td>Pubertal assessment</td>
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<td>x</td>
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<tr>
<td>Height (cm) and weight (kg)*</td>
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<td>x</td>
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<tr>
<td>BMI calculation (kg/m²)*</td>
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<tr>
<td><strong>Study bloods</strong></td>
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<tr>
<td>• HbA1c</td>
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<td>x</td>
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<tr>
<td>• Markers of bone metabolism</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>• Serum GH/IGF1 axis and adipokines</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>DXA scan</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>MRI scan</td>
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<td>x</td>
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</tbody>
</table>

* Routinely performed for clinical purpose
Appendix A

1.0 INTRODUCTION

1.1 Overview
Bone health in diabetes has become an increasing focus of interest as the association between fractures and Type 1 diabetes mellitus (T1DM) becomes increasingly recognised (Nicodemus 2001, Forsen 1999). Patients with Type 1 diabetes have reduced bone mineral density (BMD) as assessed by dual energy X-ray absorptiometry but patients with T2DM have increased BMD (Bouillon 1991, Vestergaard 2006), yet both groups have increased risk of fragility fractures. With a modest degree of reduced BMD, T1DM should have a predicted relative fracture risk of one and a half fold when compared with age-matched non-diabetics, yet a meta-analysis of several large cohort studies (Janghorbani 2007) have demonstrated a disproportionately increased risk of hip fractures by seven-fold.

Such disparities between DXA-derived bone mineral density and actual fracture risk in T1DM suggest that there are other factors contributing to overall bone health. This is acknowledged by the National Institute of Health (NIH) Consortium on Osteoporosis which recognises the importance of bone microarchitecture as a factor determining bone health. Bone microMRI technique is able to distinguish between cortical and trabecular bone hence confer the necessary information of bone microarchitecture to more accurately assess the fracture risk in diabetes patients. Recent evidence has also suggested increasing bone marrow adiposity in osteoporotic bones (Rosen 2006), providing another insight into bone health amenable to attractive treatment options targeting bone marrow adipogenesis.

1.2 Literature background
Earlier studies suggested that the defect in bone mass accretion in T1DM appears quite early on in the course of the disease, with evidence of osteopenia as early as two years after the diagnosis (McNair 1978, Gunczler 1998). More recent studies have shown that children with T1DM have decreased lumbar spine BMD within the first few months of diagnosis (Gunczler 2001). This osteopenia doubles in children with disease duration of more than three months but stabilises out with no further correlation between longer disease duration and BMD (Gunczler 2001).

The mechanisms underlying the abnormalities of bone health may involve:

1) Alteration in bone remodelling, and/or
2) Lack of anabolic action of insulin, and/or
3) Toxic effects of hyperglycaemia or diabetes ketoacidosis

1.2.1 Understanding the changes in biochemical bone markers can provide insight into the pathophysiology of osteopenia in T1DM. Bone formation is a tightly regulated process characterized by a sequence of events starting with commitment of osteoprogenitor cells, and their differentiation into pre-osteoblasts and then into mature osteoblasts, which express high levels of alkaline phosphatase (ALP) and osteocalcin, both of which are markers of bone formation. Experimental and animal models with T1DM have suggested a defect in bone formation with reduction in osteoblastic numbers and function (Rico 1989, Verhaeghe 1990, Hie 2011). Clinical studies in children and young adult with T1DM have shown a reduction in biochemical markers of bone formation.
Appendix A

(Bouillon 1995, Gunczler 1998, Lappin 2009, Pater 2010) thought to be most likely secondary to the maturation defect described above. The degree of bone resorption contributing to the osteopenia remains somewhat more controversial with suggestions of decreased (Pater 2010) but also increased osteoclastic activity (Gunczler 2001) in children with T1DM.

1.2.2) T1DM is a condition of insulinopenia and insulin has an anabolic effect on the bone, both directly and indirectly. In vitro studies have demonstrated insulin as an anabolic agent in osteoblastogenesis as cultured osteoblasts proliferate successfully in response to physiological doses of insulin (Wergedal 1984). In insulin deficient state, immature mesenchymal cells do not differentiate into mature osteoblasts, likely due to altered gene expression of proosteoblastic proteins (Lu 2003). Insulin also exerts indirect but synergistic effects with other anabolic agents in bone, such as parathyroid hormone (PTH) (Hickman 1990) and IGF-1 (Conover 1996). Rats with T1DM rendered insulin-deficient by the chemical streptozotocin (STZ) have non-osmotic hypercalciuria resulting in a negative calcium balance which may indirectly affect bone integrity (Ward 2001).

1.2.3) Animal studies have also shown that the degree of overall glycaemic control correlates with fracture healing (Follak 2004, Follak 2005). Many studies in human have investigated the association of metabolic control in T1DM (as measured by HbA1c) and bone mineral density with some suggesting benefit in good glycaemic control (Valerio 2002, Heap 2004, Pater 2010) whilst others do not (Gunczler 1998, Ersoy 1999). Pater et al showed that HbA1c was inversely correlated to osteocalcin but only at the onset of diagnosis and not later on in the disease process, suggesting that factors other than hyperglycaemia also exert toxic effect on bones in T1DM. One other proposed diabetes-related factor is the effect of acidosis during DKA on bone mineral density. Metabolic acidosis is known to result in negative calcium balance in bone through a reduction in osteoblastic formation, increase in osteoclastic resopotion and bone mineral resolution (Krieger 1992, Bushinsky 1993, Frick 1997). Topaloglu et al have suggested that the decreased osteoblastic activity in DKA is solely due to effect of acidosis without additional influences from other diabetes related-factors such as hyperglycaemia and hypoinsulinaemia (Topaloglu 2005).

1.3 Preliminary data from our group

Preliminary data from a recent study performed on the Scottish national diabetes database has not only confirmed that T1DM patients have a substantially increased relative risk of hip fractures (IRR 3.28 in men, 3.54 in women) compared with age-matched non-diabetics, but also revealed that this increased fracture risk is greatest in younger men and women in their second and third decade of life (Hothersall 2013).

In previous pilot studies, the Developmental Endocrinology Research Group led by Professor Ahmed have developed a method to assess bone microarchitecture using microMRI and demonstrated that this method is sensitive in differentiating between healthy adult volunteers and volunteers with osteogenesis imperfecta as well as growth hormone deficiency (McComb 2013). These studies which employed a 3T-MRI to assess bone microarchitecture and combined this with MR
spectroscopy to assess BMA clearly showed that, in healthy adults, there is an age dependent degradation in bone microarchitecture which is accompanied by increased BMA (McComb 2013). Whilst both groups of cases, OI and GHD, had a reduction in trabecular bone (Fig.1), it was only the GHD cases who showed an increase in BMA. This technique was later applied to young adult women at risk of adverse bone health with childhood onset T1DM. This group has shown that affected women had a lower number of trabeculae and lower BV/TV and these abnormalities were more likely in those patients who were diagnosed at an early age or had a longer duration of disease. These abnormalities were also more profound in those who had retinopathy (Fig.2).

**Fig. 1**

![Control, Osteogenesis Imperfecta, GHD](image)

**Fig. 2**

![Graph showing App BV/TV](image)

### 1.4 Research Strategy

The aim of the current proposal is to explore the use of these novel methods (microMRI and MRS) in children to improve our understanding of the prevalence of early abnormal bone health in conditions such as T1DM.

This research will be dependent on close collaboration between colleagues in endocrinology and diabetes, as well as expertise of the MRI physicists, bone densitometry service and support from laboratory contacts, all of whom have worked together successfully in previous collaborations.

### 2.0 STUDY OBJECTIVES

The objective of this research is to use novel methods of microMRI and MRS in assessing bone health of children with T1DM.
Appendix A

2.1 Primary Objective
To establish the prevalence of abnormal bone health in children with newly diagnosed and established T1DM, comparing cases and controls

2.2 Secondary Objective
To assess the relationship of abnormal bone health to disease (age at diagnosis, severity at disease presentation, duration of disease) and treatment factors (insulin dose, overall glycaemic control)

2.3 Null Hypotheses
2.3.1 Children and adolescent with T1DM have normal bone health
2.3.2 Severity of disease (degree of acidosis and HbA1C) at presentation has no correlation to underlying bone microarchitecture of children with T1DM
2.3.3 Poor diabetes control has no effect on bone health in children with established T1DM

3.0 STUDY DESIGN
This research is divided into:

3.1 Newly Diagnosed Diabetes Cases
A prospective study of bone health in newly diagnosed T1DM children over 14 month period from diagnosis

3.1.1 Setting
Recruitment for the study will be conducted over a period of 18 months between June 2014 – Nov 2015 at Royal Hospital for Children, Glasgow within NHS GGC Paediatric Diabetes Service Network

3.1.2 Subject selection and recruitment
All newly diagnosed T1DM patients between age 10 (inclusive) to 18 years old (inclusive) within these three hospitals will be eligible for recruitment into the study. Recruited subjects will undergo the full study involving blood samples, DXA and MRI scans. The annual T1DM incidence in 2012 within the NHS GGC Health Board is 80, of which 35 are ≥10 years of age.

Patients who are newly diagnosed with T1DM will be identified by the NHS GGC Paediatric Diabetes Service and information leaflet of the study provided. The researcher (also part of diabetes clinical team) will be informed and will make contact and meet with the patients and families in the ward to invite them to take part in the study. There will be opportunities to ask questions, which will be answered comprehensively in the best interest of the patients.

To be enrolled into this study, a child must meet the following inclusion criteria:
- Newly diagnosed case of T1DM
- Age ≥10 and ≤18 yrs old
Appendix A

- Be able and willing to participate in the study as evidence by a parent/guardian signing a valid written informed consent and the patient signing to show their consent/assent (competence appropriate)
- Child and parents must be able to understand English either directly or through interpreters

A child is not eligible for the study if any of the following exclusion criteria apply:
- Age <10 or >19 years old
- Has significant other co-morbidities (e.g. prematurity, congenital heart disease, inflammatory bowel disease etc)
- On medication known to affect bone health (e.g. glucocorticoids, bone protective therapy)

A child will not be eligible for a MRI study if any of the following exclusion criteria apply:
- Age <10 years old
- Has contraindications for MRI scan (e.g. cardiac pacemaker, metallic clip in body etc)

3.1.3 Consent/Assent

Written informed consent will be obtained by the researcher from the family whilst inpatient prior to conducting any study procedures. Informed consent will be obtained in accordance with the Declaration of Helsinki (World Medical Organisation, 2008). The research group is aware that following the new diagnosis of a chronic health problem in children, parents may need more time to assimilate information and this will be respected. In such cases, parents/guardian/young person will be approached again prior to discharge from hospital.

All participation in our study will be consented by the child or young person (ideally with parental consent as well), if deemed competent, or consented by the parents or legal guardian with assent from the child or young person. Consent will be obtained from the study participants, if competent, as assessed by the researcher, according to the guidance from the Scottish Children Research Network (ScotCRN) and the Ethics Working Group of the Confederation of the European Specialists in Paediatrics (CESP). Assent will be obtained from the young person when consent is not appropriate for reasons of competence.

Copies of the consent and/or assent form(s) will be given to the parents/guardian and child respectively, and placed within the child’s medical record. The original(s) will be placed in the site file or subject file, as appropriate.
3.1.4 Schedule of events

Visit 1
Visit 1 is the first contact between the researcher and the patient/family. This will be undertaken whilst patient is still in the ward. After obtaining informed consent, each participant will be screened for eligibility according to the inclusion and exclusion criteria. Other components for the first contact will consist of:

- Medical, surgical and medication information inc. dietary, exercise & fracture history
- Physical examination and pubertal assessment undertaken preferably by examination (or self-reporting if preferred)
- Information on severity of disease at presentation, laboratory glucose, HbA1C and urinalysis result will be collected from medical record
- Study bloods (C-peptide, biochemical markers of bone metabolism, GH/IGF1 axis and adipokines) will be requested on excess blood samples collected during initial routine sampling on admission (if available) or obtained from venepuncture if insufficient and patient agreeable
- Baseline MRI scan will be organised within 10 days of discharge

Visit 2
Visit 2 will be timed with routine diabetes clinic visit at 6 months from diagnosis, where the researcher will meet with participant and family. Components for this contact will consist of:

- Medical, surgical and medication information inc. dietary, exercise & fracture history and severe hypoglycaemia history
- Height and weight
- Pubertal assessment
- Study bloods (not routine) will be obtained for HbA1C, C-peptide, biochemical markers of bone metabolism, GH/IGF1 axis and adipokines

Visit 3
Visit 3 will be timed with routine diabetes clinic visit at 12-14 months from diagnosis where the researcher will meet with participant and family. This visit will involve:

- Medical, surgical and medication information inc. dietary, exercise & fracture history and severe hypoglycaemia history
- Height and weight
- Pubertal assessment
- Study bloods (not routine) will be obtained for HbA1C, C-peptide, biochemical markers of bone metabolism, GH/IGF1 axis and adipokines
- DXA scan at RSC
- An MRI scan will be arranged for the same day at the Clinical Research Facility at RHC, thereafter participant will have completed the study
Appendix A

3.1.5 Controls
In the first instance, age-matched controls from the controls recruited for established children with T1DM will be used. In the event that there are no suitable age-matched controls from the established cohort, more controls will be recruited from healthy age-matched friends or siblings of index cases, children of staff members and from other clinics in RHC. Friends of index cases may be interested to volunteer as controls, and in such instances, parents of the index case may share with the other parents the study information sheet for controls obtained directly from the researchers. If they are keen to know more about the study or proceed to participate, they will respond directly by contacting the researcher (contact details on study information sheet). Children of staff members who are interested to volunteer as controls may also do so. Control participants will only be recruited if they are willing and able to either assent or consent to participation.

All controls will undergo anthropometry, dietary, exercise and fracture history, pubertal assessment and a single MRI scan at the RHC.

3.2 Established Diabetes Cases
A cross sectional case control study of bone health in children with established T1DM

3.2.1 Setting
Recruitment for the study will be conducted over a period of 12 months between June 2014 – May 2015 at the Royal Hospital for Children, Glasgow.

3.2.2 Subject selection and recruitment
A total of 30 patients will be recruited and enrolled (numbers based on power calculation in Section 3.5), along with a group of healthy age-matched controls involving friends or siblings of cases and children of staff members.

All current patients with T1DM of ≥3 years duration will be identified by the researcher from the SCI-Diabetes database and subdivided into three groups based on their glycaemic control (HbA1c ranges: <59mmol/mol, 59-74mmol/mol, >74mmol/mol). The researcher will arrange to meet these potential participants at their next routine clinic appointments to verbally explain the study to them. An information leaflet will be provided by the researcher or another member of the diabetes team to interested participants. There will be opportunities to ask questions, which will be answered comprehensively in the best interest of the patients. This will be considered the initial approach (see Study Flow Chart for Established Diabetes Cases). It is expected that over a period of 3-4 months all interested and eligible patients would have been identified. From this group of willing participants, 30 subjects with 10 subjects from each HbA1c bracket will be recruited into the study based on first come first serve basis.
Appendix A

To be enrolled in the study, a child must meet the following inclusion criteria:
- Diagnosed with T1DM for at least three years
- Age ≥ 10 years and ≤18 years old
- Be able and willing to participate in the study as evidenced by a parent/guardian signing a valid written informed consent and the patient signing to show their consent/assent (competence appropriate)
- Child and parents must be able to understand English either directly or through interpreters

A child is not eligible for the study if any of the following exclusion criteria apply:
- Age <10 or >19 years
- Duration of T1DM less than 3 years
- Has contraindications for MRI scan (e.g. cardiac pacemaker, metallic clip in body etc)
- Has significant other chronic diseases (e.g. prematurity, congenital heart disease, inflammatory bowel disease etc)
- On medication known to affect bone health (e.g. glucocorticoids, bone protective therapy)

Should any participants decide to withdraw from the study before completion, the next eligible and willing participant with a HbA1c within the same range would be recruited.

3.2.3 Consent/Assent
Written informed consent will be obtained by researcher prior to conducting any study procedures at Visit 1. Informed consent will be obtained in accordance with the Declaration of Helsinki (World Medical Organisation, 2008). All participation in our study will be consented by the child or young person (ideally with parental consent as well), if deemed competent, or consented by the parents or legal guardian with assent from the child or young person. Consent will be obtained from the study participants, if competent, as assessed by the researcher, according to the guidance from the Scottish Children Research Network (ScotCRN) and the Ethics Working Group of the Confederation of the European Specialists in Paediatrics (CESP). Assent will be obtained from the young person when consent is not appropriate for reasons of competence.

Copies of the consent and/or assent form(s) will be given to the parents/guardian and child respectively, and placed within the child's medical record. The original(s) will be placed in the site file or subject file, as appropriate.

3.2.4 Schedule of Events

Initial Approach
The researcher will meet with the potential participants at their routine clinic visit at RHC to verbally explain the study to them. An information
leaflet will be provided to participants who satisfy the recruitment criteria. There will be opportunities to ask questions, which will be answered comprehensively in the best interest of the patients. If the patient and family are happy to participate, arrangements will be made to obtain informed consent at Visit 1.

**Visit 1**
This visit will coincide with the eligible patient’s next routine visit to the diabetes clinic at RHC when their routine screening blood is due. The researcher will meet with the participant and undertake the following:
- Informed consent
- Height and weight
- Physical examination and pubertal assessment undertaken preferably by examination (or self-reporting if preferred)
- Study bloods for HbA1C, C-peptide, biochemical markers of bone metabolism, GH/IGF1 axis and adipokines will be performed at the same time as the routine screening bloods
- DXA scan will be performed if possible prior to patient leaving the hospital that day

**Visit 2**
This visit will be within 10 weeks of Visit 1. Participant will be requested to attend for an MRI scan, thereafter participant will have completed the study.

### 3.2.5 Controls
30 healthy age-matched controls will be recruited from friends or siblings of index cases, children of staff members and from other clinics in RHC. Friends of index cases may be interested to volunteer as controls, and in such instances, parents of the index case may share with the other parents the study information sheet for controls obtained directly from the researchers. If they are keen to know more about the study or proceed to participate, they will respond directly by contacting the researcher (contact details on study information sheet). Children of staff members who are interested to volunteer as controls may also do so. Control participants will only be recruited if they are willing and able to either assent or consent to participation.

All controls will undergo anthropometry, dietary, exercise and fracture history, pubertal assessment and a single MRI scan.

### 3.3 Study blood samples
All participants will have blood samples taken at the appropriate intervals according to the schedule of events. Research bloods will involve:
- HbA1c to assess glycaemic control
- C-peptide to assess residual pancreatic beta-cell function
- Biochemical markers of bone metabolism (osteocalcin, BALP, CTX)
- Serum markers of GH/IGF1 axis (IGF-1, IGFBP-3, IGFBP-2, ALS) and adipokines (leptin)
Excluding the HbA1c which is obtained for routine clinical purpose, the total volume of research blood required will be 5mls per collection. At all times possible, study bloods will be timed with routine screening bloods.

All newly diagnosed T1DM patients in Greater Glasgow and Clyde (GGC) have a standard number of blood tests collected at the point of first intravenous access or venous sampling as part of a routine protocol. In order to minimise burden on patient with an extra venepuncture, excess blood samples available from this initial routine collection will be accessed if the patient and parents consent to participate in the study. Once consented, the NHS lab will be contacted by the researcher within a week of discharge, to release the samples for research, all of which will then be stored at the University of Glasgow freezer at RHC. These will be processed in batches at the University of Glasgow lab.

The Research Group has also created its own reference data for markers of bone turnover and GH/IGF-1 axis.

### 3.4 Study imaging

#### 3.4.1 DXA scan

This will be performed at the bone densitometry unit at RHC involving whole body, AP spine and femoral neck. The total maximum dose per scan is 3 microSv, which is equivalent to less than half a day of natural background radiation in the UK.

The Research Group has normative DXA BMC data for 150 school age boys and girls in Glasgow for LS, TB and FN as well as manufacturer’s reference data for LS and TB BMC which are very similar to the Glasgow data. DXA data shall be analysed as per the recommendations of the recent ISCD guidelines as well as following correction for bone area. LS and FN BMC data shall also be mathematically transformed into volumetric data by calculating bone mineral apparent density.

There is a possibility that we might find something unexpected (incidental finding) in the participant’s images. If this happens, the child will be referred to the appropriate specialist for further investigation. All DXA scans will be reviewed by the Bone Densitometry Service as part of the research collaboration. Parents are made aware that they may then have to disclose such findings in future applications for health-related insurance.

#### 3.4.2 MRI/MRS scan

Participants will need to attend dedicated sessions for MRI. The 3T-MRI scanner is based at the RHC Glasgow. Whilst some participants will be able to have the scan on the same day when they are attending the diabetes clinic, it is highly likely they will also need to devote additional time to attend for this. Participants shall be provided with gift vouchers worth £10 per scan visit for this inconvenience and all taxi fares paid for in full from base hospital, or home if no hospital appointment on the day. Base hospital is defined as the local hospital where the patients attends for routine diabetes follow-up.
Participants will be taken through an MRI safety checklist, as per standard MRI practice prior to entering room. The maximum duration of the MRI scan will be one hour, and during this time both microMRI of the tibia and MRS of the lumbar spine will be performed. Participants will be advised to attend for a whole morning or an afternoon so that all the assessments can be performed.

There is a possibility that we might find something unexpected (incidental finding) in the participant’s images. If this happens, the child will be referred to the appropriate specialist for further investigation. All MRI scan images will be clinically reviewed by Dr Ruth Allen, Consultant Paediatric Radiologist, solely for this purpose as part of paid research service. Parents are made aware that they may then have to disclose such findings in future applications for health-related insurance.

3.5 Statistical consideration and power calculation
All data will be presented as medians (ranges) if they are not normally distributed. Group differences will be compared by non-parametric tests and Chi-square test for categorical variable. Repeated measures of ANOVA will be used to test the quantitative measurements of bone health, bone marrow adiposity and metabolic status. Multiple logistic regression analysis will be used to adjust for confounding variables.

Intra-operator repeatability of microMRI and MRS is high with an average CoV of 1% and 2.5%, respectively. The primary hypothesis was that children with T1DM would have a lower appBV/TV compared to healthy children. In our previous work in adult T1DM of 30 cases and control and in adults with OI, which is a more severe osteopathy, the estimated coefficient of variation (CoV) for appBV/TV was less than 5%. In our previous work. Based on this a 7.5% difference at $p<0.05$ with a power of 0.8 would require at least 25 subjects in each arm.

4.0 STUDY OUTCOMES

4.1 Primary Outcome
The primary outcome of the study is the quantitative measurements of bone microarchitecture (apparent BV/TV, apparent trabecular thickness, apparent trabecular number and apparent trabecular separation) obtained from microMRI in cases and controls. Additional measures of bone microarchitecture will also be explored including measures of connectivity (branch density, nodal density)

4.2 Secondary Outcome
The secondary outcome measures include:

- Measure of bone marrow adiposity as assessed by MR spectroscopy
- Relationship of markers of bone health (including DXA) to markers of T1DM disease (duration of disease, age of onset, insulin dose, HbA1C and microvascular complications)
- Change in bone and adiposity markers over first year of diagnosis

5.0 DATA MANAGEMENT
Quantitative data will be collected from a variety of sources – blood and urine samples, questionnaire results, imaging including DXA, MRI and MRS. Data quality will be ensured with repeat measurements taken where appropriate to ensure consistency and regular calibration checks on all equipments used. Only one trained DXA and MRI staff respectively will be used to ensure consistency of results. All personal data will be treated as strictly confidential. Each participant will be given a unique study identifier to preserve anonymity of data stored. All study questionnaires will be filed on site in a security-coded office and results data stored on the researcher’s university computer which is password protected. Personal data will be stored only until completion of assessments and all data anonymised for analysis. All data will be curated responsibly subjected to the University of Glasgow guidance on managing research records. http://www.gla.ac.uk/services/dpfoioffice/guidanceonresearch/

6.0 STUDY TIMELINE
This study will take 24 months to complete from the point of ethics and R&D approval, with timelines as follows:

- Months -6 to 0: Design study protocol
- Months -3 to 0: Obtain ethics and R&D approval
- Months 0 to 1: Identify suitable participants for established T1DM cases
- Months 1-18: Recruit participants, begin assessments and follow-up to completion, with interim analysis at 6 months
- Months 16-20: Perform full data analysis
- Months 20-24: Prepare final thesis

7.0 FUNDING
This study is funded by Glasgow Hospital Children’s Charity, for a one-year full time clinical research fellow with £10,000 for consumables and the Medical Fund of the University of Glasgow for a further £10,000 for consumables. Consumables will include cost of scans, study blood sampling and processing, patient travel and gift vouchers.

The research costs of the proposed study over 2 years are outlined as follows.

**Established diabetes study** (30 cases and 30 controls)
- Biochemical markers (@£25) x 30 samples £ 750
- Minor consumables (purchase of blood bottles, etc) £ 200
- DXA scan (@£50) x 30 scans £ 1,500
- MRI scan (@£250) x 60 scans including controls £15,000
- MRI review for incidental findings (@£12.50 X 60 scans) £ 750
- Patient reimbursement (@20) x 60 visits for MRI £ 1,200

Total £19,400

**Newly diagnosed diabetes study** (estimated 30 patients)
- Biochemical markers (@£25) x 90 sample £ 2,250
- Minor consumables top-up £ 100
- DXA scan (@£50) x 60 scans £ 3,000
- MRI scan (@£250) x 60 scans £15,000
- MRI review for incidental findings (@£12.50 X 60 scans) £ 750
- Patient reimbursement (@20) x 60 visits for MRI £ 1,200
Appendix A

Total

There will also be NHS support costs for obtaining informed consent from NHS patients as per the AcoRD Cost Attribution Guidance 2012 (@£71.08 x90)  

Total consumable costs = £48,097

Additional fundings will be sourced for the full cost of the research and applications have been submitted to NovoNordisk UK Research Foundation and the Chief Scientist Office (CSO).

8.0 PROTOCOL AMENDMENTS

Any change in the study protocol will require an amendment. Any proposed protocol amendment will be submitted to a) the Sponsor for approval, b) REC for favourable opinion and c) R&D for acknowledgement. The sponsor will determine whether an amendment is substantial or non-substantial. The chief investigator will sign and file any amended versions of the protocol before this can be implemented.
9.0 REFERENCES


Frick KK, Jia L, Bushinsky DA. Acute metabolic acidosis inhibits the induction of osteoblastic egr-1 and type 1 collagen. Am J Physiol 1997;272:1450-1456


Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of Type 1 and Type 2 Diabetes Mellitus and Risk of Fracture. Am J Epidemiol 2007;166:495-505


Hickman J, McElduff A. Insulin sensitizes a cultured rat osteogenic sarcoma cell lines to hormones which activate adenylate cyclase. Calcif Tissue Int 1990;46:401-405
Hie M, Iitsuka N, Otsuka T, Tsukamoto I. Insulin-dependent diabetes mellitus decreases osteoblastogenesis associated with the inhibition of Wnt signaling through increased expression of Sost and Dkk1 and inhibition of Akt activation. Int J Mol Med 2011;28:455-462


Lu H, Krant D, Gerstenfeld LC, Graves DT. Diabetes interferes with the bone formation by affecting the expression of transcription factors that regulate osteoblast differentiation. Endocrinology 2003;144:346-352


Nicodemus KK, Folsom AR. Type 1 and Type 2 diabetes and incidence of hip fractures in postmenopausal women. Diabetes Care 2001;24:1192-1197

Pater A, Sypniewska G, Pilecki O. Biochemical Markers of Bone Cell Activity in Children with T1DM. J Pediatr Endocrinol Metab 2010;23:81-86


Appendix A


Appendix B

ESTABLISHED DIABETES STUDY

Case Report Form

Study number:
Information Sheet given: Yes / No
Date of consent:
GP Letter sent: Yes / No

Visit 1

Date of Visit 1:

Demographic and anthropometric data:

<table>
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<tr>
<th>Patient initials</th>
<th>Sex:</th>
<th>Date of birth</th>
<th>Height=</th>
<th>Tel number:</th>
<th>Age in year</th>
<th>Weight=</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Details of diabetes:

Age at diagnosis:
Disease duration:
Severity at presentation:

<table>
<thead>
<tr>
<th>Walking wounded</th>
<th>DKA</th>
</tr>
</thead>
</table>

H+    Bic    Lab glucose    % dehydration    HbA1c

Total insulin dose:
Pump or injection:
Current HbA1c:
Available HbA1C results in the 2 years:

<table>
<thead>
<tr>
<th>Date</th>
<th>HbA1C (mmol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Average HbA1C in the last 2 years:
Average HbA1C in the last year:
Category of HbA1C control:

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<tr>
<th>Good (&lt;59)</th>
<th>Moderate (59-75)</th>
<th>Poor (&gt;75)</th>
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Health behaviour and diet

How often do you eat dairy products? (please circle one)

<table>
<thead>
<tr>
<th>Never</th>
<th>Once a week</th>
<th>2-3 times a week</th>
<th>More than 3 times a week</th>
</tr>
</thead>
</table>

Calcium (mg/day):
Vitamin D (IU/day):
Multivitamin use:
Smoking: (please circle one)

<table>
<thead>
<tr>
<th>Current (number/day)</th>
<th>Past</th>
<th>Never</th>
</tr>
</thead>
</table>

Physical activity (hrs/week): see Physical Activity Questionaire  Completed:
## Medical/Surgical History

### Patient History (inc Birth Hx)
- **Gestation:**
- **Birth weight:**
- **PMHx:**

### Med Hx:

#### Previous surgery:

### Orthopaedic:
- **Joint problems**

### Fracture history:
- **Number**
- **Location**
- **Age**

### Diabetes complications
- **Previous DKAs (details)**
  1.
  2.
  3.

#### Thyroid disorder:
- Last TFT at  
  - **Result**

#### Renal complication (Microalbuminuria):
- Date  
  - **Result**

#### Retinopathy:
- Last follow up at  
  - **Result**

#### Neuropathy:
  
  

### Family History
- **Family history of T1DM:**
- **Family history of osteoporosis:**
- **Family history of fracture:**

## Reproductive history and examination

<table>
<thead>
<tr>
<th>Onset of puberty (years):</th>
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<tbody>
<tr>
<td>Menarche (years):</td>
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<tr>
<td>Puberty status:</td>
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Appendix B

Blood Investigations:

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<tr>
<th>Blood tests</th>
<th>Date done</th>
<th>Date processed</th>
<th>Results</th>
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<tr>
<td>HbA1C</td>
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</tr>
<tr>
<td>C-peptide</td>
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<td></td>
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<tr>
<td>25VitD</td>
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<td></td>
<td></td>
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<td>PTH</td>
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<td>B-ALP</td>
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</tr>
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<td>Osteocalcin</td>
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<td>CTX</td>
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<td>Adiponectin</td>
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<td>Others:</td>
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Storage inform: __________________________________________________________

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<tr>
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<th>Notes</th>
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<tr>
<td>Total body (TB)</td>
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<tr>
<td>Femoral neck (FN)</td>
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<td></td>
</tr>
<tr>
<td>Lumbar spine (LS)</td>
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<td></td>
</tr>
</tbody>
</table>
Appendix B

Visit 2 (Within 6 weeks of Visit 1)

Date of Visit 2:

Taxi organised:

PICU informed: Yes / No

CRF nurses organisation- CM/RW informed : Yes / No

Radiological Investigation:

<table>
<thead>
<tr>
<th>MRI/MRS</th>
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</thead>
<tbody>
<tr>
<td>Proximal tibia</td>
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</tr>
<tr>
<td>Abdominal fat</td>
<td></td>
</tr>
<tr>
<td>Lumbar spine</td>
<td></td>
</tr>
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</table>

Study Completed
Case Report Form (CONTROL)

Study number:

Information Sheet given: Yes / No

Date of consent:

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<th>Visit 1</th>
</tr>
</thead>
<tbody>
<tr>
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Demographic and anthropometric data:

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<th>Date of birth</th>
<th>Height=</th>
</tr>
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<tbody>
<tr>
<td>Tel number:</td>
<td>Male □</td>
<td>Age in year</td>
<td>Weight=</td>
</tr>
</tbody>
</table>

Health behaviour and diet

How often do you eat dairy products? (please circle one)

| Never | Once a week | 2-3times a week | More than 3 times a week |

Calcium (mg/day):

Vitamin D (IU/day):

Multivitamin use:

Smoking: (please circle one)

| Current (number/day) | Past | Never |

Physical activity (hrs/week): see Physical Activity Questionaire
Medical/Surgical History

<table>
<thead>
<tr>
<th>Patient History (inc Birth Hx)</th>
<th>Clinical Examination</th>
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<tr>
<td>Gestation:</td>
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<td>Past Medical History:</td>
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<td>Joint problems</td>
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<td>Fracture history:</td>
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<td></td>
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<tr>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>Age</td>
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</table>

Family History

- Family history of T1DM:
- Family history of osteoporosis:
- Family history of fracture:

Reproductive history and examination

- Onset of puberty (years):
- Menarche (years):
- Pubertal status:

Radiological Investigation:

<table>
<thead>
<tr>
<th>MRI/MRS</th>
<th>Date</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal tibia</td>
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<td></td>
</tr>
<tr>
<td>Abdominal fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumbar spine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Study Completed
For advice on this study  
Dr S Ching Chen  
Royal Hospital for Children  
Glasgow  
Tel 0141 201 0709

For independent advice  
Dr Martina Rodie  
Royal Hospital for Children  
Glasgow  
Tel 0141 201 0236

ESTABLISHED DIABETES STUDY

STUDY OF BONE HEALTH IN CHILDREN
WITH ESTABLISHED TYPE 1 DIABETES MELLITUS (T1DM)

Consent Form – Case

Study Number:.........................................................................................................................

Participant’s Name (Block Capitals):..................................................................................................

Please initial boxes

1. I confirm that I have read and understood the Study Information Sheet for Participant/Parent/Legal Guardian (Case) (Version 3, 16/06/14). I have had the opportunity to consider the information, ask questions and had these questions answered to my satisfaction  

2. I understand that my child’s participation is voluntary and that I am free to withdraw at any time, without giving a reason, without my child’s medical care or legal rights being affected

3. I understand that my child’s GP will be informed that I/he/she am/is participating in this study

4. I allow access for the research team and sponsor representatives to my child’s medical records and patient identifiable information

5. I understand that if anything unexpected is demonstrated on my child’s scan, I/he/she will be referred to the appropriate specialist for further investigation

6. I agree for my child to take part in this study

Name of Participant/Parent/Guardian: ...............................................................

Signature of Participant/Parent/Guardian: .............................................. Date:...........................

Signature of Researcher:.............................................................. Date:...........................

T1DM/Bone/144961/Version 3 160614
ESTABLISHED DIABETES STUDY

STUDY OF BONE HEALTH IN CHILDREN WITH ESTABLISHED TYPE 1 DIABETES MELLITUS (T1DM)

Assent Form – Case

Study Number:.................................................................

Participant's Name (Block Capitals): ............................................

Please initial boxes

<p>| | |</p>
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<thead>
<tr>
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<tbody>
<tr>
<td>1</td>
<td>I confirm that I have read and understood the Study Information Sheet for Participant (Case) (Version 3, 16/06/14)</td>
</tr>
<tr>
<td>2</td>
<td>I have had the time to think about this and ask questions</td>
</tr>
<tr>
<td>3</td>
<td>I understand that I do not have to take part if I do not want to</td>
</tr>
<tr>
<td>4</td>
<td>I agree to take part in this study</td>
</tr>
</tbody>
</table>

Signature of Participant: ........................................... Date: .........................

Signature of Researcher: ............................................. Date: ..........................
| 1. | I confirm that I have read and understood the Information Sheet for Parent/Legal Guardian (Control) dated 10/12/14 (Version 4) for the above study. I have had the opportunity to consider the information, ask questions and had these questions answered to my satisfaction | ☐ |

| 2. | I understand that my child’s participation is voluntary and that I am free to withdraw at any time, without giving a reason, without my child's medical care or legal rights being affected | ☐ |

| 3. | I understand that my child's GP will be informed that he/she is participating in this study | ☐ |

| 4. | I understand that if anything unexpected is demonstrated on my child's scan, he/she will be referred to the appropriate specialist for further investigation | ☐ |

| 5. | I agree for my child to take part in this study | ☐ |

Name of Parent/Guardian: ...................... Date:......... Signature: .............

Name of Researcher:.................................. Date: ............ Signature: .............
### ESTABLISHED DIABETES STUDY

#### STUDY OF BONE HEALTH IN CHILDREN WITH ESTABLISHED TYPE 1 DIABETES MELLITUS (T1DM)

**Consent Form for Young Person – Control**

Study Number: .................................................................

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<td>I confirm that I have read and understood the Information Sheet for Participant (Control) dated 10/12/14 (Version 4) for the above study</td>
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<td>5</td>
<td>I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected</td>
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<td>I understand that sections of my medical notes may be looked at by the research team where it is relevant to my taking part in the research. I give my permission for the research team to have access to my records</td>
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<td>I agree to take part in the above study</td>
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Name of Participant: ........................................ Date: ............ Signature: .................

Name of Parent/Guardian: ............................... Date:............... Signature: .................

Name of Researcher: ........................................ Date: ............ Signature: .................

T1DM/Bone/144961/Version 4 101214
ESTABLISHED DIABETES STUDY

STUDY OF BONE HEALTH IN CHILDREN WITH ESTABLISHED TYPE 1 DIABETES MELLITUS (T1DM)

Assent Form for Young Person – Control

Study Number: ........................................................................................................................................

Please circle your answers:

Have you read (or had read to you) about this project? Yes / No
Has somebody else explained this project to you? Yes / No
Do you understand what this project is about? Yes / No
Have you asked all the questions you want? Yes / No
Have your questions been answered in a way that you understand? Yes / No
Do you understand that it’s OK to stop taking part at any time? Yes / No
Are you happy to take part? Yes / No

If any answers are ‘no’ or you don’t want to take part, don’t sign your name!

If you do want to take part, you can sign your name below

Your name __________________________
Signature __________________________
Date __________________________

The doctor who explained this project to you needs to sign too:

Print Name __________________________
Signature __________________________
Date __________________________

Thank you for your help

(To be completed by the child and their parent/guardian)

T1DM/Bone/144961/Version 4 101214
Physical Activity Questionnaire (<14 years old)

Name: ________________________ Age: ____________

Sex: M_______ F_______ Grade: ____________

We are trying to find out about your level of physical activity from the last 7 days (in the last week). This includes sports or dance that make you sweat or make your legs feel tired, or games that make you breathe hard, like tag, skipping, running, climbing, and others.

Remember:
1. There are no right and wrong answers — this is not a test
2. Please answer all the questions as honestly and accurately as you can — this is very important

1. Physical activity in your spare time: Have you done any of the following activities in the past 7 days (last week)? If yes, how many times? (Check only one box per row)

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<th>Activity</th>
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2. In the last 7 days, during your physical education (PE) classes, how often were you very active (playing hard, running, jumping)? (Check one only)

- I don’t do PE
- Hardly ever
- Sometimes
- Quite often
- Always

3. In the last 7 days, what did you do most of the time at recess? (Check one only)

- Sat down (talking, reading, doing schoolwork)
- Stood around or walked around
- Ran or played a little bit
- Ran around and played quite a bit
- Ran and played hard most of the time
4. In the last 7 days, what did you normally do at lunch (besides eating lunch)? (Check one only)
- Sat down (talking, reading, doing schoolwork)
- Stood around or walked around
- Ran or played a little bit
- Ran around and played quite a bit
- Ran and played hard most of the time

5. In the last 7 days, after school, did you do sports, dance, or play games in which you were very active? (Check one only)
- None
- 1 time last week
- 2 or 3 times last week
- 4 or 5 times last week
- 6 or 7 times last week

6. In the last 7 days, on how many evenings did you do sports, dance, or play games in which you were very active? (Check one only)
- None
- 1 time last week
- 2 or 3 times last week
- 4 or 5 times last week
- 6 or 7 times last week

7. On the last weekend, how many times did you do sports, dance, or play games in which you were very active? (Check one only)
- None
- 1 time
- 2 — 3 times
- 4 — 5 times
- 6 or more times

8. Which one of the following describes you best for the last 7 days? Read all five statements before deciding on the one answer that describes you.
- A. All or most of my free time was spent doing things that involve little physical effort
- B. I sometimes (1 — 2 times last week) did physical things in my free time (e.g. played sports, went running, swimming, bike riding, did aerobics)
- C. I often (3 — 4 times last week) did physical things in my free time
- D. I quite often (5 — 6 times last week) did physical things in my free time
- E. I very often (7 or more times last week) did physical things in my free time

9. Mark how often you did physical activity (like playing sports, games, doing dance, or any other physical activity) for each day last week.

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10. Were you sick last week, or did anything prevent you from doing your normal physical activities? (Check one)
- Yes
- No
- If Yes, what prevented you? ___________________________
Physical Activity Questionnaire (14-19 years old)

Name: __________________________ Age: ____________
Sex: M ______ F ______ Grade: ____________

We are trying to find out about your level of physical activity from the last 7 days (in the last week). This includes sports or dance that make you sweat or make your legs feel tired, or games that make you breathe hard, like tag, skipping, running, climbing, and others.

**Remember:**
1. There are no right and wrong answers — this is not a test
2. Please answer all the questions as honestly and accurately as you can — this is very important

1. Physical activity in your spare time: Have you done any of the following activities in the past 7 days (last week)? If yes, how many times? (Check only one box per row)

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(Check one only)

- I don’t do PE
- Hardly ever
- Sometimes
- Quite often
- Always
### Appendix G

3. In the last 7 days, what did you normally do at lunch (besides eating lunch)?
   (Check one only)
   - Sat down (talking, reading, doing schoolwork)
   - Stood around or walked around
   - Ran or played a little bit
   - Ran around and played quite a bit
   - Ran and played hard most of the time

4. In the last 7 days, on how many days right after school, did you do sports, dance, or play games in which you were very active?
   (Check one only)
   - None
   - 1 time last week
   - 2 or 3 times last week
   - 4 or 5 last week
   - 6 or 7 times last week

5. In the last 7 days, on how many evenings did you do sports, dance, or play games in which you were very active? (Check one only)
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9. Were you sick last week, or did anything prevent you from doing your normal physical activities? (Check one.)

   - Yes
   - No
   - If Yes, what prevented you? __________________________
There are several mechanisms by which diabetes could affect bone mass and strength. These mechanisms include insulin deficiency; hyperglycemia; the accumulation of advanced glycation end products that may influence collagen characteristics; marrow adiposity and bone inflammation. Furthermore, associated diabetic complications and treatment with thiazolidinediones may also increase risk of fracturing. The following article provides its readers with an update on the latest information pertaining to diabetes related bone skeletal fragility. In the authors’ opinion, future studies are needed in order to clarify the impact of different aspects of diabetes metabolism, glycemic control, and specific treatments for diabetes on bone. Given that dual energy x-ray absorptiometry is a poor predictor of bone morbidity in this group of patients, there is a need to explore novel approaches for assessing bone quality. It is important that we develop a better understanding of how diabetes affects bone in order to improve our ability to protect bone health and prevent fractures in the growing population of adults with diabetes.

**KEYWORDS:** diabetes • fractures • glucose • insulin • skeleton

Diabetes mellitus is a common metabolic disorder with an increasing prevalence throughout the world. In addition to the many target organs that are typically associated with dysfunction, the skeleton has been recognized as another organ that is adversely affected in diabetes mellitus with fragility fractures been increasingly recognized in people with diabetes mellitus [1–3]. An extensive volume of clinical data supports altered bone structure and biochemical markers of bone turnover in people with both Type 1 and Type 2 diabetes (T1D and T2D) [4–6]. In addition, a series of experimental studies have reported several possible mechanisms that could explain the link between diabetes and adverse bone health. In the present article, we review the pathophysiological mechanisms of diabetic bone loss and the evidence that exists for abnormalities of bone health in people with diabetes.

**Pathogenesis of altered bone metabolism in diabetes**

**Hyperglycemia**

Several *in vivo* and *in vitro* studies indicate that hyperglycemia contributes to bone loss through a variety of mechanisms: direct glucose toxicity to osteoblast through polyol-pathway activity affecting osteoblast numbers and function [7,8]; osmotic and nonosmotic pathways which independently suppress osteoblast maturation and mineralization [9,10]; down regulation of vitamin D receptor thus indirectly impairing osteoblast maturation in response to 1,25(OH)D3 [8]; oxidative stress [11]; and formation of glycation products [12].

Hyperglycemia leads to increased nonenzymatic protein glycation and excessive formation of these modified proteins called advanced glycosylation end-products (AGEs). AGEs appear to be the common biochemical entity accumulating in individuals with long-term diabetic complications. They are seen in various sites such as vascular tissues, kidneys and bones [13,14]. The accumulation of AGEs increases formation of collagen cross-links which results in increased stiffness of the collagen network in the cortical bone, but this has no overall effect on the stiffness of the mineralized bone [15]. Accumulation of pentosidine, a well-characterized AGE, is negatively correlated to trabecular bone volume and the structural strength of the trabecular bone, which may explain how AGEs contribute to bone fragility [16–18]. In support of this hypothesis, *in vivo* studies in both T1D and T2D rats have confirmed that an increase in AGE production is negatively correlated with bone...
mineral density (BMD) and bone strength [19,20]. Moreover, AGE was found at higher concentration in cortical and cancellous bones of people with femoral neck fracture compared to post-mortem controls [17,19]. In addition to affecting bone physical properties, AGES also have direct effects on bone cells. In vitro and ex vivo findings suggest that elevated AGE can inhibit proliferation and differentiation of osteoblast [21,22] in mouse and human mesenchymal stem cells (MSCs). It is also associated with reduced osteocalcin mRNA expression as seen in rodents with diabetes [20,23]. Furthermore, Okazaki et al. demonstrated that the inhibition of osteoblastic differentiation by AGES can occur independently of hyperglycemia [24]. AGES can also cause osteoblast death by provoking inflammation and generation of reactive oxygen species (ROS) which in turn lead to cell apoptosis as a result of oxidative stress [11,23,25,26]. However, reports regarding the influence of AGES on osteoclasts and bone resorption show inconsistent effects with some evidence of increased [27], decreased [28] as well as no effect of AGE on bone resorption [21]. Higher pentosidine levels and AGE products have been found to reduce bone strength and elevated vertebral fracture risks in T2D [17,29].

MSCs are pluripotent stem cells capable of differentiating into bone, muscle, cartilage and adipose tissues. Emerging evidence suggests that hyperglycemia may also alter the differentiation of the MSCs promoting adipogenesis instead of osteogenesis [30]. Mice with spontaneously and pharmacologically induced diabetes with bone loss have shown increased marrow adiposity, determined by histology and by mRNA markers of adipogenesis (PPARγ and aP2) [31]. Hyperglycemia diverts the MSC differentiation signaling pathways toward adipogenesis at the expense of osteogenesis. This effect is achieved via ERK-1/-2-activated PI3K/Akt-regulated pathway in mouse MSCs [32], and via suppression of HO-1 expression [33] and Wnt/protein kinase C noncanonical pathway in human MSCs [34], evidenced by increased PPARγ expression in high glucose stimulated and reduction of osteocalcin levels. Wang et al. demonstrated that hyperglycemia can also promote adipogenesis through synthesis of hyaluronan matrix which promotes an inflammatory response culminating in demineralization of trabecular bones [35].

**Insulin, IGF-1 & other growth factors**

Insulin is increasingly recognized to have an anabolic effect on bone that is distinct from the effect of its analogue IGF-1. This effect is mediated either indirectly through its control of blood glucose concentration while maintaining the levels of parathyroid hormone (PTH), IGF-1 vitamin D [36,37], or directly on bone cells through specific insulin receptors (IRs) and IGF-1 receptors in osteoblast. IRs are present on osteoblasts and its expression varies with differentiation status of the osteoblast both in vivo and in vitro [38,39]. In vitro studies on primary osteoblasts and clonal osteoblast cell lines show insulin promotes glucose uptake [40], alkaline phosphatase activity, collagen synthesis [41,42] and osteoblast proliferation [43] of these cells. Maor et al. demonstrated reduced IRs in the skeletal growth centers of mice with streptozocin (STZ)-induced diabetics and these were reversed with insulin therapy [44]. Evidence for a direct link between insulin action and bone formation in vivo can be observed in IR knock out mice which show altered bone formation [45], abnormal trabecular microarchitecture [46] and reduced bone strength [47]. Insulin positively regulates bone formation by stimulating osteocalcin production. Observational studies in people with T1D and T2D, with insulin deficiency and resistance respectively, demonstrate reduced osteocalcin levels compared with controls [48,49]. Furthermore, there is a positive correlation between insulin dose and BMD [50,51]. The dichotomy of lower BMD in T1D with insulinopenia and higher BMD in T2D with clinical hyperinsulinaemia further implicates a causal anabolic effect of insulin on bone.

The direct effect on bone may also be mediated by the IGF-1 pathway. IGF-binding protein (IGFBP) serves as a carrier protein for IGF1. Insulin inhibits IGFBP-1, 4 expression in osteoblasts and therefore lack of insulin leads to an increase in IGFBP-1 and 4 levels, accordingly decreasing the availability of unbound IGF-1 for anabolic effects on bone [52]. In addition to insulin deficiency, T1D individuals and animal models demonstrate dysregulation of a variety of endocrine factors including reduced amylin [2] and IGF-1 [53]. Amylin is another osteotropic factor that is cosecreted by pancreatic beta-cells and absent in T1D [54]. Amylin-deficient mice displayed low bone mass with increased number of osteoclast [55] and that treatment with amylin in diabetic rat result in increased BMD and bone strength [54]. Similarly, diabetic animals with low blood IGF-1 and knock-out mice for its receptor displayed diminished bone formation [56,57]. Also, serum IGF-I level was another predictor of prevalent VF’s in postmenopausal women with T2D [58].

**Calcium, vitamin D & calcitropic hormones**

It is well known that calcium homeostasis plays a major role in regulating bone metabolism. Therefore, imbalance in systemic factors which are capable of regulating calcium balance have been found to influence diabetic bone loss [59]. Similar to diabetic patients, several but not all studies demonstrate that diabetic animal models have reduced levels of PTH, vitamin D, calcium, magnesium and phosphate [60]. The effect of DM on calcium metabolism is complex, but essentially it is associated with a negative calcium balance hallmark by both bone and renal loss [61]. There is a growing body of evidence which demonstrate exacerbation of osteopenia and osteoporosis in animals and humans with vitamin D deficiency and T1D or T2D [62-64]. Verhaeghe et al. found that diabetic rats have higher urinary calcium excretion and significantly lower serum concentrations of both 1,25-dihydroxyvitamin D3 and vitamin D-binding protein [63]. Frazer et al. demonstrated that alteration in vitamin D metabolism in young insulin-dependent diabetics aged 7–18 years old, who have markedly reduced 1,25-dihydroxyvitamin–D but normal serum calcium, phosphate and PTH concentrations, could be related to their
observed decrease in cortical bone mass [64]. More recently, Zhang et al. aimed to explain the mechanism involved by demonstrating that male STZ-induced diabetic mice have high urinary calcium excretion and decreased BMD [59]. Quantified PCR results showed alteration of vitamin D metabolic enzyme expression and down regulation of mRNA expression levels for renal calcium transporter receptors, plasma membrane Ca-ATPase, and vitamin D receptor. In support of this, treatment with calcitriol in STZ-induced rats demonstrated recovery of BMD [65].

In terms of PTH, several in vivo and in vitro studies indicate that an imbalance in PTH was associated with bone loss and increase fracture risk [66,67]. PTH encourages bone formation and turnover by increasing the expression of osteocalcin, IGF-1, IGFBP-3, bFGF, MMP-1 and MMP-13 in rat osteoblasts in vitro (7) [68], as well as MMP-13 in mice in vivo [69]. Moreover, treatment with PTH to T1D and T2D mice demonstrated reversal of trabecular bone parameters through its anabolic effects on osteoblasts differentiation and maturation [67,70]. PTH-treatment was also associated with reduced TUNEL-staining of osteoblast suggesting its antiapoptotic effect on osteoblasts. It has been suggested that PTH promotes repair of DNA damage by increasing PCNA and Foxo3a [71], hence prolonging osteoblast survival. Intermittent PTH-treatment reversed bone loss to baseline in mice compared to only partial reversal in continuous PTH-treatment, although this effect may be due to underlying differences between the T1D and T2D mouse models [67,70]. Similarly, treatment with PTH-related protein, which is produced at high levels by differentiating osteoblasts, also reverses trabecular bone loss in STZ-induced diabetic mice suggesting its modulatory effect on osteoblast function and role in diabetic osteopenia [72].

**Marrow adiposity & adipokines**

There is a growing body of evidence that suggests that the fat-bone connection plays an important role in the pathophysiology of bone loss. Osteoblasts and adipocytes share a common precursor, both derived from the pluripotent MSCs located in the bone marrow suggesting a mutually exclusive and reciprocal lineage selection of one or the other. The main lineage-specific transcription factors that direct the differentiation of MSC are the runt-related transcription factor 2 (Runx2) for osteoblastogenesis and the PPARγ for adipogenesis [73]. It has been suggested that lineage selection could regulate bone density and result in bone loss when MSCs commit to the adipocytes at the expense of osteoblasts [33,74,75]. Botolin et al., 2005 were the first to report an increase in the expression of PPARγ and an increase in visible adipocytes in tibia of T1D mouse models compared with control [76]. Subsequently, several experimental animal studies have demonstrated an increase in bone marrow adiposity in both spontaneously and STZ-induced T1D mice compared to controls [31,72,77]. As described earlier, hyperglycemia is capable of promoting adipogenesis by altering the lineage commitment of MSCs to adipocytes through various signaling pathways contributing to the diabetic osteopenia [32,34,78]. Rzonca et al. demonstrated in vivo that rosiglitazone (PPARγ agonist) administration results in significant bone loss whilst Cock et al. 2004 demonstrated that congenitally PPARγ deficient mice had increased BMD and bone trabecular microarchitecture parameter including BV/TV and trabecular thickness [79]. However, Botolin and McCabe found that inhibition of PPARγ by administration of an antagonist to insulin deficient T1D mice prevented bone marrow adiposity but not bone loss [31]. This inability in preventing bone loss in T1D mice suggested that bone marrow adiposity may only be partially responsible for diabetic bone loss.

Bone marrow adiposity can also have direct effect on osteoblasts as demonstrated by several coculture studies in mouse and human cells [80–82]. The presence of adipocytes inhibit osteoblast proliferation through the lipotoxic effect of free fatty acid in the bone marrow microenvironment [81]. Coe et al. demonstrated that the diabetic bone marrow itself is a mediator for osteoblast death with an increase in caspase-3 activity, a marker for extrinsic apoptotic pathway which are activated by extracellular ligands such as TNFα, in bone marrows of both spontaneous and pharmacologically-induced diabetic mice. By treating the cocultures with TNFα antibodies, they prevented osteoblast death further supporting the negative correlation of diabetic bone marrow adiposity and bone mass.

In addition to releasing large amounts of free fatty acid, adipocytes in the bone marrow also secretes cytokines including leptin, resistin and TNFα, the last of which will be discussed in the next section. Leptin is an adipokine which has a complex regulatory role on bone metabolism, with an indirect inhibitory effect on osteoclastogenesis and a direct stimulatory effect on osteoblastogenesis through a central (hypothalamic) and peripheral pathway [83–85]. Despite being an adipokine, in vitro studies indicate that leptin promotes an osteoblast rather than adipocyte lineage [84,85]. In contrast to T1D patients who demonstrate increased [86] or slightly decreased leptin level [87], leptin levels were found to be significantly suppressed in T1D mice, its absence results in reducing bone mass with increasing marrow adiposity [83]. T2D is typically associated with obesity, which has been associated with higher leptin level and higher BMD. Vasilkova et al., however, demonstrated that leptin has an independent positive correlation to BMD, irrespective of BMI [88]. In addition, interventional studies in mice have demonstrated that leptin administration reduces bone marrow adiposity and increase bone mass [89,90]. However, similar to their work on PPARγ antagonist, Motyl and McCabe concluded that leptin administration to T1D mice modify and prevent marrow adiposity but did not prevent diabetic bone loss [91]. Adiponectin (APN), the most abundant adipocyte-secreted adipokine, regulates energy homeostasis and exerts well-characterized insulin-sensitizing properties. The peripheral and central effects of APN on bone metabolism are beginning to be explored but are still not clearly understood. APN-knockout (APN-KO) mice fed a normal diet exhibit decreased trabecular structure and mineralization and increased bone marrow adiposity and central administration of APN decreased osteoclast numbers, whereas osteoblast osteogenic marker expression and trabecular bone mass increased,
both, in APN-KO and WT mice [92]. The insulin-sensitising effect of OC is known be due to the upregulation of the expression of the insulin-sensitizing APN gene in adipocytes [93] but there is some suggestion that in humans the link between OC and APN may be gender specific [94].

**Chronic inflammation**

The pathogenesis of both T1D and T2D are associated with activation of the immune system, especially so in T1D which involves an autoimmune destruction of pancreatic β-cells whilst T2D involves a more chronic low-grade inflammatory process [95,96]. Several experimental studies using T1D mice model indicate that systemic and local inflammatory cytokines are increased at the onset of diabetes with rapid suppression of osteoblast markers and increase in adipocyte markers, indicating that bone inflammation may be another contributing factor to the diabetic bone pathology [82,97,98]. Serum cytokine levels (TNFα, IFN-γ, IL-1Rα and LT-β) and corresponding bone cytokine mRNA expression were increased from as early as 5 days after induction of diabetes in mouse models [31,97] with decrease in osteocalcin mRNA expression in bone RNA extracts, and it remains suppressed at 40 days postinduction. TNFα can either directly suppress osteoblast maturation as well as promote osteoblast death in vitro [98] or act indirectly by contributing to elevation of ROS causing osteoblast apoptosis from oxidative stress [11,26]. Coe et al. also found that TNFα in the bone marrow microenvironment directly mediate osteoblast death with increase in expression of pro- apoptotic factors and osteoblast TUNEL staining, further contributing to T1D bone loss [82]. Inhibition of the cytokines with TNFα neutralizing antibodies prevented osteoblast apoptosis [82] but transgenic mice with IFN-γ KO proceeded to have diabetic bone pathology [97], supporting the idea that diabetic inflammatory bone loss involves an interplay of more than one cytokine and/or a combinations of other factors. Apart from proinflammatory cytokines, abnormal hyaluronan production in bones of diabetic rodents also induced monocyte apoptotic factors and osteoblast TUNEL staining, further concerning the cytokine mRNA expression were increased from as early as 5 days after induction of diabetes in mouse models [31,97] with decrease in osteocalcin mRNA expression in bone RNA extracts, and it remains suppressed at 40 days postinduction. TNFα can either directly suppress osteoblast maturation as well as promote osteoblast death in vitro [98] or act indirectly by contributing to elevation of ROS causing osteoblast apoptosis from oxidative stress [11,26]. Coe et al. also found that TNFα in the bone marrow microenvironment directly mediate osteoblast death with increase in expression of pro-apoptotic factors and osteoblast TUNEL staining, further contributing to T1D bone loss [82]. Inhibition of the cytokines with TNFα neutralizing antibodies prevented osteoblast apoptosis [82] but transgenic mice with IFN-γ KO proceeded to have diabetic bone pathology [97], supporting the idea that diabetic inflammatory bone loss involves an interplay of more than one cytokine and/or a combinations of other factors. Apart from proinflammatory cytokines, abnormal hyaluronan production in bones of diabetic rodents also induced monocyte and macrophage infiltration into the bone collagen matrix, promoting adipogenesis at the expense of osteogenesis [35].

The concept of T2D as an inflammatory disease is relatively new with increased fat depots in T2D implicated as the source for more proinflammatory cytokines and adipokines [95]. However, a large prospective population study (EPIC) in Germany found that systemic inflammatory markers are independent predictors for the development of T2D, independent of degree of insulin resistance and obesity [99].

**Vasculopathy**

Similar to other diabetic microvascular complications of retinopathy, nephropathy and neuropathy, bone microangiopathy has been insinuated as another possible mechanism for diabetic bone loss. In hypoxic condition (2% oxygen), the bone marrow shifts toward adipogenic lineage by enhancing expression of genes associated with adipogenic/lipogenic phenotype (C/EBPβ, PPARγ2, and αP2) and by suppressing expression of genes associated with osteoblast differentiation (alkaline phosphatase, AP) [100]. Oikawa et al. found that the cumulative vascular density was reduced by threefold in bone marrow of STZ-induced diabetic mice compared to control, along with reduction in blood flow [101]. In addition, there is also evidence of reduction in bone marrow volume and bone marrow remodeling with cell depletion mainly affecting the osteoblastic niche secondary to hypoperfusion and oxidative stress [101]. Correspondingly, boosting the antioxidative pentose phosphate pathway with benfotiamine supplementation prevented microangiopathy and hypoperfusion in the bone marrow with reduction in cell apoptosis, providing further compelling evidence for vasculopathy in diabetic bone pathology. Regenerating mouse tibia has reduction in blood vessels with lower expression of VEGF, a signaling protein which regulate angiogenesis, and its receptor [72] with increased risk of falls from hypoglycemic attacks resulting in fractures [103]. Total daily insulin requirement has a positive predictive value for low BMD, although the need for higher insulin dose may reflect the presence of more severe disease [104]. Different classes of anti-diabetic medication, such as thiazolidinediones (TZDs), metformin and glycogen-like peptide 1 inhibitors have been reported to have both varying effects on bone. TZDs such as pioglitazone and rosiglitazone has been shown to increase adipocyte differentiation and decreased osteoblast differentiation through activation of PPARγ activity [79,105,106]. Consistent with these preclinical observations, a meta-analyses clearly indicate that TZD use is associated with a higher risk of fracture, particularly in women [107]. In contrast, metformin and sulfonylurea have been reported to be bone protective with a reduction in risk of fractures in patients treated with these agents [108,109]. Both compounds exert a direct osteogenic effect in vitro by stimulating proliferation and differentiation of osteoblast [110-114] through preventing AGE-induced deleterious effects in osteoblastic cells [115] and various signaling pathways including the PI3K/Akt pathway [113], ERK-1/2 [110] and AMPK activation [112,114]. Zhen et al. also demonstrated an indirect effect of metformin on osteoblast survival through reduction of intracellular ROS [116]. In addition to its osteoblastogenic potency in vitro, metformin can further prevent bone loss by inhibiting osteoclastic differentiation [117]. However, ovariectomized rodents treated with metformin demonstrated inconsistent radiological findings of no change in bone microarchitectural increased BMD [118]. A newer class of oral anti-diabetic agent, the dipeptidyl peptidase-4 (DPP-4) inhibitors, such as sitagliptin, has been observed to have anabolic effects on the bone [119] but the long-term skeletal consequences of these
drugs are yet unclear. Lastly, dapagliflozin, a highly selective inhibitor of sodium-glucose cotransporter 2 which reduces hyperglycemia and weight in patients with T2D by increasing urinary glucose excretion has also been studied for its effects on bone metabolism but no clear effects have been described [120,121].

Clinical evidence of fractures

Type 1 diabetes

Although fracture data for T1D is scarce, the existing evidence indicates that people with T1D have a higher fracture risk compared to the general population and these fractures occur more frequently in the lower limbs [1,122-124]. More recently, vertebral fractures have been also reported [125,126]. The risk of hip fractures in those with T1D is reported to be 7–12 times greater [122,127] and this increased risk is also evident in young adults [3]. The increased fracture risk was much higher than expected based on BMD assessed by dual energy x-ray absorptiometry (DXA), which was only 10% lower than normal [2]. In a meta-analysis that highlighted the fracture rates and the discrepant relationship with BMD, the relative risk for hip fracture amongst T1D studies was almost 7 [1]. Poor glycemic control and disease duration have also been reported to play a contributory role in this raised

Table 1. Studies that evaluated fracture risk in people with Type 1 diabetes mellitus.

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>Study design</th>
<th>Sex</th>
<th>Study population</th>
<th>Age (mean ± SD or range)</th>
<th>Fracture site and assessed outcome</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicodemus and Folsom (2001)</td>
<td>Prospective cohort</td>
<td>F</td>
<td>30,377</td>
<td>55–69</td>
<td>Hip</td>
<td>Women were 12.25 times more likely to report an incident hip fracture than women without diabetes</td>
<td>[122]</td>
</tr>
<tr>
<td>Miao et al. (2005)</td>
<td>Prospective cohort</td>
<td>F/M</td>
<td>24,605</td>
<td>20.7 ± 10.9</td>
<td>Hip</td>
<td>Elevated risks were observed in both sexes (standardized hospitalization ratios = 7.6 [95% CI: 5.9–9.6] and 9.8 [7.3–12.9], respectively), increasing with follow-up time. Ophthalmic, nephropathic, neurological, and cardiovascular complications were indicators of particularly high risks</td>
<td>[129]</td>
</tr>
<tr>
<td>Ahmed et al. (2006)</td>
<td>Prospective cohort</td>
<td>F/M</td>
<td>12,639</td>
<td>25–98</td>
<td>All nonvertebral and hip</td>
<td>Men had an increased risk of all non-vertebral [RR, 3.1 (95% CI: 1.3–7.4)] and hip fractures [RR 17.8 (95% CI: 5.6–56.8)]. Men and women using insulin had increased hip fracture risk. Duration of disease did not alter hip fracture risk</td>
<td>[124]</td>
</tr>
<tr>
<td>Janghorbani et al. (2007)</td>
<td>Prospective cohort</td>
<td>F</td>
<td>1,01,343</td>
<td>54.7 ± 9.3</td>
<td>Hip</td>
<td>RR of hip fracture was 7.1 (95% CI: 4.4–11.4), after adjustment for BMI, smoking, physical activity, menopausal status, intake of calcium, vitamin D and protein, and postmenopausal hormone use, RR for T1D was 6.4 (3.9–10.3)</td>
<td>[4]</td>
</tr>
<tr>
<td>Zhukouskaya et al. (2013)</td>
<td>Cross-sectional</td>
<td>F/M</td>
<td>82/82</td>
<td>31.1 ± 8.6</td>
<td>VFs</td>
<td>No association between VF and lumbar spine BMD in the T1D group. No effect of age of diagnosis, disease duration, HBA1c and related complication on the prevalence of VF</td>
<td>[125]</td>
</tr>
<tr>
<td>Neumann et al. (2014)</td>
<td>Cross-sectional</td>
<td>F/M</td>
<td>128/77</td>
<td>43.4 ± 8.8</td>
<td>Fractures of ribs, fingers and toes were excluded</td>
<td>Those with prevalent fractures had higher HbA1c, pentosidene level and more diabetes-related complications. BMD was lower in those with prevalent fractures</td>
<td>[126]</td>
</tr>
</tbody>
</table>

BMD: Bone mineral density; RR: Relative risk; T1D: Type 1 diabetes; VF: Vertebral fractures.
fracture risk irrespective of BMD [108,127,128]. The fracture risk could also be attributed to the microvascular complications of diabetes such as neuropathy, retinopathy and nephropathy [122,129]. Recently, elevated AGEs and pentosidine levels has been found to impair bone strength and cause fragility fractures in T1D [126]. Studies that have evaluated fracture risk in patients with T1D are summarized in Table 1.

**Type 2 diabetes**

There is growing supportive evidence for an increased risk of fractures in patients with T2D despite them having higher or normal BMD [1,130,131]. A meta-analysis of eight prospective studies in adults with T2D concluded that the relative risk of hip and wrist fractures was 1.4 and 1.2, respectively, while there was no increase in vertebral fractures [1] and another meta-analysis [4] of 16 epidemiologic studies, of which 4 investigated vertebral fractures, found that the relative risk of hip fracture was 1.8 but the risk was not elevated at other regions of the body. However, other studies have reported an increased risk of vertebral fractures in T2D patients, and these presented conflicting results [29,106,108,112,130,132–134]. Duration of T2D, glycemic control, anti-diabetic medications, propensity to fall and T2D-related complications were observed as possible predicting factors for the increased fracture risk in these studies. Cross-sectional studies have indicated that those with increased fractures are older [4,29,122] and have a longer duration of T2D [29,133]. The incidence was also higher in those treated with insulin [4,134]. In T2D, falls are common [106,135] and the risk of falling may be higher in those with poor balance, arthritis, cardiovascular disease, depression, poor vision and use of medications for sleeplessness or anxiety [135]. It is unclear whether this increased risk of fall is also associated with more fractures. Studies that have evaluated BMD in patients with T2D are summarized in Table 2.

**Bone turnover studies**

**Type 1 diabetes**

In people with T1D, an assessment of markers of bone turnover often shows a state of reduced bone formation which may be associated to factors such as deficiency of insulin and chronic hyperglycemia but this needs further investigation [6]. Abnormalities in the growth hormone–IGF-1 axis may also play a contributory role but this needs further investigation.

Among the markers of bone turnover, serum osteocalcin level has often been reported to be low whereas bone alkaline phosphatase has been found to be increased in some studies [136,137]. This imbalance of bone formation markers (increased ALP, decreased osteocalcin) may reflect an impairment of osteoblast differentiation and maturation as bone ALP is expressed early during osteoblast development and osteocalcin is released from the mature osteoblast. Reports on markers of bone resorption are scarce and conflicting, with studies reporting either normal or reduced circulating levels bone resorption markers [128,138,139]. Serum PTH has been found to be increased or normal [128]. Though levels of the Wnt signaling antagonist, sclerostin, are reported to be higher, its link to other markers of bone metabolism has not been confirmed yet [126]. Only one longitudinal study showed that bone turnover returned to normal after improved glycemic control [140].

**Type 2 diabetes**

Bone formation is consistently lower in people with T2D, as evidenced by lower serum osteocalcin and procollagen type 1 N-terminal propeptide (P1NP) levels, [112,141], as well as reduced histologic measures of mineralizing surface and bone formation rate [142]. Another study has reported no difference in P1NP in adults with T2D compared to normoglycemic adults, but this lack of difference may be due to small sample size [143]. CTX level has been extensively investigated in adults with T2D and is consistently lower compared to age-matched healthy controls [141,144]. Reports of the effects of T2D on other markers of bone resorption are less consistent, with studies reporting either no difference or reduced bone resorption markers in people with T2D [141]. Serum PTH tends to be lower [126] while levels of sclerostin, are higher in patients with T2D [145]. The majority of bone turnover data that show a net bone loss effect of T2D were from adults with longer-standing T2D. The strongest predisposing factors for this effect included persistent hyperglycemia [146,147] reduced insulin sensitivity [148] and lower circulating levels of IGF-1 and IGF-binding protein-3 [149]. The ratio of OC to bone ALP ratio has also been recently reported as a clinically useful marker for assessing the risk of vertebral fractures independent of BMD in men with diabetes [150]. Elevated sclerostin levels were associated with an increased risk of VFs in T2D patients independently of BMD and bone turnover in both genders [151].

**Bone imaging studies in DM**

**Type 1 diabetes**

Since then the association between diabetes mellitus and bone changes became a subject of extensive research, but results so far are inconclusive. There is still an open question of whether the effect of diabetes on bone health is primarily due to changes in bone density or changes in the inherent material properties of bone tissue. The majority of studies in children have reported either a slightly low BMD or a normal BMD (Table 3) [152–155]. In adults with T1D, the most consistent observation is that BMD is reported to be significantly decreased at lumbar spine and femoral neck (Table 4) [104,139,152,153,156–163]. In one meta-analysis, a significantly lower BMD Z-score at the hip (-0.37 ± 0.16) and spine (-0.22 ± 0.01) [1] was observed. These findings have been confirmed by several subsequent studies [160,161] but there are also a small number of studies that have reported no abnormalities in BMD [156,159,164]. The effect of T1D on cortical and trabecular bone structure in humans has not been as well characterized as it has in rodent models [165]. Studies that have evaluated bone mass and structure by using pQCT showed reduced total and cortical cross sectional area and lower muscle mass in children and adolescence with T1D compared to healthy control [165–169].
### Table 2. Studies that evaluated fracture risk in people with Type 2 Diabetes (T2D).

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>Study design</th>
<th>Sex</th>
<th>Study population</th>
<th>Age (years) (mean ± SD or range)</th>
<th>Fracture site and assessed outcome</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strotmeyer et al. (2005)</td>
<td>Prospective</td>
<td>Both</td>
<td>323 men and 243 women T2D, 106 men and 71 women with impaired fasting glucose. Control group: 1209 women and 1207 men</td>
<td>70–97</td>
<td>All fractures</td>
<td>Adjustment for hip BMD, lean mass, fat mass and abdominal visceral fat indicated that older adults with DM are still at higher risk</td>
<td>[132]</td>
</tr>
<tr>
<td>De Liefde et al. (2005)</td>
<td>Prospective</td>
<td>Both</td>
<td>F, 483/3,481; M, 309/2,382</td>
<td>73.8–68.8</td>
<td>Any non-VF</td>
<td>IGT was associated with decreased fracture risk. Established and treated DM had an increased fracture risk. Adjusted for age, BMI, creatinine, visual acuity, falling frequency, lower limbisability, smoking, and BMD</td>
<td>[182]</td>
</tr>
<tr>
<td>Bonds et al. (2006)</td>
<td>Prospective</td>
<td>F</td>
<td>Case: 5,285; Ctrl: 88,120</td>
<td>64.9 ± 7.0</td>
<td>Determined whether risk varies by fracture site, ethnicity, and baseline bone density.</td>
<td>After adjustment for multiple risk factors including falls and BMD, the fracture risk was elevated among black women</td>
<td>[183]</td>
</tr>
<tr>
<td>Schwartz et al. (2009)</td>
<td>Observational</td>
<td>Both</td>
<td>Case: 501; Ctrl 427</td>
<td>73.6 ± 2.9</td>
<td>VF and pentosidine level</td>
<td>Higher pentosidine levels are a risk factor for fracture in older adults with diabetes and may account in part for reduced bone strength in T2D</td>
<td>[17]</td>
</tr>
<tr>
<td>Yamamoto et al. (2009)</td>
<td>Cross-sectional</td>
<td>Both</td>
<td>F, 137/622; M, 161/76</td>
<td>F, 67.5 ± 9.8 M, 66.0 ± 8.1</td>
<td>VF and its association with BMD</td>
<td>After adjustment for age, body mass index, LS-BMD and diabetes complications, T2D patients have an increased risk of vertebral fractures</td>
<td>[130]</td>
</tr>
<tr>
<td>Kanazawa et al. (2011)</td>
<td>Cross-sectional</td>
<td>Both</td>
<td>F, 334; M, 479</td>
<td>F, 67.6 ± 9.4 M, 60.3 ± 12.8</td>
<td>The relationship between serum IGF-I and vertebral fractures</td>
<td>Serum IGF-I level was inversely associated with vertebral fracture prevalence in postmenopausal women T2DM. Association persisted despite adjustment for age, duration of diabetes, body mass index, serum creatinine, and HbA1c and BMD at the lumbar spine</td>
<td>[58]</td>
</tr>
<tr>
<td>Vélegas et al. (2011)</td>
<td>Cross-sectional</td>
<td>F</td>
<td>148</td>
<td>61.87 ± 7.85</td>
<td>Vertebral fracture</td>
<td>High prevalence of osteoporosis and vertebral fractures (23%) even after adjustment for disease parameters; more frequent in long-standing disease and in those with retinopathy and impaired renal function</td>
<td>[133]</td>
</tr>
<tr>
<td>Yamamoto et al. (2013)</td>
<td>Cross-sectional</td>
<td>both</td>
<td>F,146; M,175</td>
<td>F 64.9 ± 9.3 M 65.7 ± 8.7</td>
<td>Vertebral fracture and its association with sclerostin level</td>
<td>Sclerostin was higher in men than women and associated with an increased risk of vertebral fractures independent of BMD and bone turnover in both genders</td>
<td>[151]</td>
</tr>
</tbody>
</table>

BMD: Bone mineral density; BMI: Body mass index; IGF-I: Insulin-like growth factor-I; IGT: Impaired glucose tolerance; LS: Lumbar spine; VF: Vertebral fracture.
Another pQCT study by [170] indicated that as T1D adolescents reached 14 and 15 years of age, their cortical CSA normalized, becoming equivalent to the cortical CSA of nondiabetics at the same age. However, T1D was associated with a decreased bone CSA at the radius at the end of pubertal growth comparing to controls [171]. In light of the emerging importance of the interactions between marrow fat and bone and the evidence for the negative effects of marrow fat on bone density, marrow fat as an independent surrogate for bone quality has also been recently assessed by MRI [172]. The factors that may be associated with BMD in T1D are not well-known yet. Some studies have documented a decreased BMD in those with a recent onset of T1D [158,166,173] indicating the existence of mechanisms before the appearance of clinical symptoms.

### Table 3. Bone densitometry studies in children with Type 1 diabetes mellitus T1D.

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>N(f/m)</th>
<th>Age (years) (range or means ± SD)</th>
<th>Dis duration (years) (range, means ± SD)</th>
<th>Modality</th>
<th>Site</th>
<th>Major finding in cases versus controls</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gunczler et al. (1998)</td>
<td>26(11/15)</td>
<td>12.1 ± 3.1</td>
<td>4 years</td>
<td>DXA</td>
<td>LS, FN</td>
<td>↓ LS BMD</td>
<td>Adjusted for age, sex and pubertal status. No association with disease duration, glycemic control, 24 h urinary calcium excretion or bone turnover markers</td>
<td>[177]</td>
</tr>
<tr>
<td>Léger et al. (2006)</td>
<td>127 (54/73)</td>
<td>6–20</td>
<td>4.1–8.6</td>
<td>DXA</td>
<td>LS, TB</td>
<td>↓ Total BMC and BMD</td>
<td>Only present in girls. Adjusted for age, sex, pubertal stage, and BMI SDS</td>
<td>[179]</td>
</tr>
<tr>
<td>Brandao et al. (2007)</td>
<td>44 (22/22)</td>
<td>8.8 ± 4.4</td>
<td>6.6 ± 3.9</td>
<td>DXA</td>
<td>LS</td>
<td>No abnormalities</td>
<td>After adjustment for weight, height and pubertal development, the BMD was ±2.0 S.D. in only two diabetic patients (4.5%). Longer duration and poor metabolic control may have a negative impact on bone mass</td>
<td>[155]</td>
</tr>
<tr>
<td>Moyer-Mileur et al. (2008)</td>
<td>11(11/0)</td>
<td>12.9 ± 1.0</td>
<td>5.9 ± 3.7</td>
<td>PQCT DXA</td>
<td>Tibia LS, FN</td>
<td>↓ Tibial and FN BMD</td>
<td>Altered markers of GH/IGF-1 axis associated with low BMD. The results were adjusted for height and puberty</td>
<td>[167]</td>
</tr>
<tr>
<td>Heilman et al. (2009)</td>
<td>30 (11/19)</td>
<td>4.7–18.6</td>
<td>5.4 ± 3.4</td>
<td>DXA</td>
<td>LS, TB</td>
<td>↓ Total BMC and LS BMD</td>
<td>Only present in boys. Inverse association to urinary markers of oxidative stress, plasma ICAM-1 levels and HbA1c. The result was adjusted for age, sex and BMI</td>
<td>[174]</td>
</tr>
<tr>
<td>Haerati et al. (2009)</td>
<td>56 (23/33)</td>
<td>17.2–24.8</td>
<td>10.6 ± 3.9</td>
<td>pQCT</td>
<td>DR</td>
<td>Similar trabecular BMD, ↓ CSA</td>
<td>Adjusted for age, gender, height score and BMI. In girls, the CSA SDS correlated negatively with the BMI SDS and positively with the height SDS</td>
<td>[171]</td>
</tr>
<tr>
<td>Loureiro et al. (2014)</td>
<td>75(39/36)</td>
<td>6-20</td>
<td>-</td>
<td>DXA</td>
<td>LS (L1-L4)</td>
<td>↓ LS BMD</td>
<td>Children and adolescents with early onset T1D presented with low BMD associated with poorer glycemic control. BMD was not adjusted for any variables</td>
<td>[154]</td>
</tr>
</tbody>
</table>

BMC: Bone mineral content; BMD: Bone mineral density; BMI: Body mass index; CSA: Cross sectional area; DR: Distal radius; DXA: Dual x-ray absorptiometry; FA: Forearm; FN: Femoral neck; GH/IGF-1: Growth hormone/IGF-I; ICAM-1: intercellular adhesion molecule-1 (ICAM-1); LS: Lumbar spine; pQCT: Peripheral quantitative computed tomography; T1D: Type 1 diabetes.
such as autoimmune and inflammatory mechanism [154]. However, others studies have not detected an abnormality of BMD in recently diagnosed children indicating that the metabolic consequence of the disease over time may play an important role in a predisposing covariant. Some studies have suggested that metabolic control, including its effect on the growth hormone/IGF-1 axis, plays a strong role in the genesis of osteopenia [104,162,166,173–175]. These findings are supported by a study of intensive insulin therapy that stabilized BMD [138]. However, others have failed to show this association [1,157,176–180].

In summary, the available data on the structural quality of bone in T1D are still scarce and controversial. There are limitations such as small sample sizes, different techniques to measure and interpret data and inadequate adjustment of confounders such as age, disease duration and metabolic status.

**Type 2 diabetes**

Reports of the effects of T2D on BMD as measured by DXA are still contradictory, with majority of studies reporting increased BMD [1,123,181–187]. A small number of studies have shown no difference [188–191] comparing to nondiabetics. A meta-analysis of 15 cross-sectional, cohort and case-control studies demonstrated that men and women with type 2 diabetes had elevated BMD compared to controls [192]. In adults with type 2 diabetes, BMD was greater by a difference of +0.06 g/cm² at the lumbar spine, +0.04 g/cm² at the femoral neck, and +0.06 at the total hip [192]. Gender was a significant predictor for BMD, with a higher BMD in women than in men even after adjusting for confounding factors such as body weight and age [181,192–194]. BMI and glycemic control have been reported as strong predictors of elevated BMD [1,187,192] but despite adjusting for BMI and HbA1c some studies found that BMD remains higher in adults with T2D [182,185,189,191,193–195]. Another possibility is that the lumbar spine BMD is over-estimated in adults with T2D due the changes that are associated with osteoarthritis [196].

Although generally limited to small sample sizes, cross-sectional studies have been used to examine cortical and trabecular bone density at the hip and spine, as well as peripheral skeletal sites, in adults with T2D [146,197–201]. Trabecular bone density in T2D is generally similar to or significantly greater than that of nondiabetic controls [146,197–201]. The effects of T2D on cortical bone are more variable, with some studies reporting no differences, [146,199], whereas others finding deficits in bone size and cortical bone structure in T2D [198,199,201,202]. Although two recent studies found that cortical porosity is increased only in postmenopausal diabetics with a prior fragility fracture, and that diabetics without prior fracture had similar cortical bone density, porosity and microarchitecture as nondiabetic controls [199,201] a more recent study has reported raised radial cortical porosity despite adjustment for confounding variables [202]. In addition, it was the first study that demonstrated compromised bone material strength in patients with T2D, and was negatively correlated with the average glycated hemoglobin level over the previous 10 years. More recently, with the help of specific software, a group of investigators have calculated the trabecular bone score based on pixel variations on DXA images in young women with T2D and showed that it was lower and associated with worse control [5]. Recently, in vivo assessment of bone quality by microindentation methods has also revealed that the bone material strength may also be reduced in people with T2D [202]. Collectively, current evidence indicate that apart from BMD measured by DXA, an assessment of cortical bone quality may be important to consider for assessing risk of fragility fractures. However, future research is needed to characterize bone microstructure in T2D, taking in account the confounder factors as BMI, age and associated diabetic complication. Studies that have evaluated BMD in adult patients with T2D were summarized in Table 4.

**Effect of anti-diabetic therapies**

The effect of anti-diabetic therapies on bone has recently been reviewed in detail [203]. TZDs such as rosiglitazone and pioglitazone increase insulin sensitivity via activation of PPARγ and although they possess beneficial antihyperglycemic profiles, their use is associated with adverse effects on bone as highlighted very clearly in the ADOPT studies [204]. Metformin is the most commonly used to increase insulin sensitivity in people with diabetes. Population based studies suggest that metformin decreases fracture risk in T2D [134]. Although the ADOPT studies did not demonstrate beneficial effects of metformin on fracture risk, they showed reduced bone turnover [199]. Head to head studies comparing a combination of rosiglitazone and metformin to metformin alone in T2D show that although the combination is superior over monotherapy with metformin for glycemic control, the former was associated with significantly reduced BMD in lumbar spine and hip, while metformin monotherapy therapy did not have an effect on bone [205]. The link between insulin therapy and fractures is less clear. Whilst treatment of severe insulin deficiency and improved glycemic control is associated with positive changes in markers of bone turnover [140], the use of insulin in people with T2D has been reported to be associated with an increased incidence of fractures [134]. Finally, incretin analogues and DPP-4 inhibitors that enhance the effect of enteric hormones such as glucagon-like peptides in stimulating insulin release and inhibiting glucagon production may also have a beneficial effect on bone health. A meta-analysis of DPP4 inhibitors has revealed a lower risk of fractures in those treated with these drugs [206]. There is a need for further prospective studies to understand these effects in greater detail.

**Conclusion**

The evidence from experimental studies, with supporting evidence from bone marker and bone density data, suggest that adults with diabetes are at increased risk for fractures. Patients with T1D are at a higher risk for osteoporotic fractures compared to T2D patients or general population. This may be explained by the initial period of insulin deficiency which results in impaired bone formation and prevents accrual of an
Table 4. Bone densitometry studies in adults with Type 1 diabetes.

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>Subject N(f/m)</th>
<th>Age, years (range or means ± SD)</th>
<th>Disease duration, years (mean ± SD)</th>
<th>Modalities Site</th>
<th>Major findings in cases</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kayath et al. (1994)</td>
<td>90 (18–54)</td>
<td>2–20 (mean ± SD) DXA LS, FN</td>
<td></td>
<td>BMD in 34% of IDDM patients</td>
<td>Positive correlation with glucose levels duration of disease and insulin dosage</td>
<td><img src="#" alt="152" /></td>
<td></td>
</tr>
<tr>
<td>Lunt et al. (1998)</td>
<td>99 (99/0)</td>
<td>42 median 27 median DXA LS FN</td>
<td></td>
<td>No difference in BMD from control</td>
<td>Positive correlation with BMI and height</td>
<td><img src="#" alt="156" /></td>
<td></td>
</tr>
<tr>
<td>Kemink et al. (2000)</td>
<td>35 (14/21)</td>
<td>37.6 ± 9.9 8.5 ± 3.5 DXA LS FN</td>
<td></td>
<td>FN BMD, LS BMD, Osteopenia in 67% of men 57% of women</td>
<td></td>
<td><img src="#" alt="157" /></td>
<td></td>
</tr>
<tr>
<td>Lopez-Iberra et al. (2001)</td>
<td>32 (10/22)</td>
<td>20–39 At diagnosis DXA LS FN</td>
<td></td>
<td>LS BMD, FN BMD</td>
<td></td>
<td><img src="#" alt="158" /></td>
<td></td>
</tr>
<tr>
<td>Liu et al. (2003)</td>
<td>33 (33/0)</td>
<td>20–37 14.5 ± 5.7 DXA FN, LS, wrist, TB</td>
<td></td>
<td>LS BMD, FN BMD</td>
<td></td>
<td><img src="#" alt="153" /></td>
<td></td>
</tr>
<tr>
<td>Bridges et al. (2005)</td>
<td>35 (0/35)</td>
<td>49.3</td>
<td>20 DXA DR</td>
<td>No difference in BMD from control</td>
<td></td>
<td><img src="#" alt="159" /></td>
<td></td>
</tr>
<tr>
<td>Strotmeyer et al. (2006)</td>
<td>67 (67/0)</td>
<td>35–55 32.2 ± 5.3 DXA QUS Total hip FN, TB LS</td>
<td></td>
<td>total hip BMD p &lt; 0.001 FN BMD p &lt; 0.001 TB BMD p &lt; 0.01 Calcaneal BUA p &lt; 0.001</td>
<td></td>
<td><img src="#" alt="160" /></td>
<td></td>
</tr>
<tr>
<td>Hamilton et al. (2009)</td>
<td>102 (52/50)</td>
<td>20–71 5.5–23.6 DXA FA, FN, LS</td>
<td></td>
<td>BMD at LS, FN and FA</td>
<td>Only present in women, Lower BMD was associated with impaired bone formation</td>
<td><img src="#" alt="161" /></td>
<td></td>
</tr>
<tr>
<td>Danielson et al. (2009)</td>
<td>75 (75/0)</td>
<td>28 16 DXA Calcaneus FA, FN, LS</td>
<td></td>
<td>BMD at FA and FN</td>
<td>Inverse correlation with HbA1c and markers of bone formation in patients with BMD</td>
<td><img src="#" alt="162" /></td>
<td></td>
</tr>
<tr>
<td>Eller-Vainicher et al. (2011)</td>
<td>175 (104/71)</td>
<td>32 ± 8.4 13 ± 8.4 DXA LS FN</td>
<td></td>
<td>BMD at LS and FN</td>
<td>Low BMD is associated with low BMI; chronic complications have a negative association with BMD</td>
<td><img src="#" alt="104" /></td>
<td></td>
</tr>
<tr>
<td>Botushanov et al. (2014)</td>
<td>162 (97/65)</td>
<td>29.17 /</td>
<td></td>
<td>BMD at FN and LS</td>
<td>LS BMD negatively correlated with marker of bone formation</td>
<td><img src="#" alt="163" /></td>
<td></td>
</tr>
<tr>
<td>2 year prospective Mastrandrea et al. (2008)</td>
<td>63 (63/0)</td>
<td>15–39</td>
<td></td>
<td>BMD at FN and TB</td>
<td>Statistically significant in women ≥20 years of age</td>
<td><img src="#" alt="139" /></td>
<td></td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Comment</th>
<th>Study (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>† Decrease in BMD; ‡ Increase in BMD; BMD: Bone mineral density; BMI: Body mass index; BUA: Broadband ultrasound attenuation; CSA: Cross sectional area; DR: Distal radius; DXA: Dual X-ray absorptiometry; FA: Forearm; FN: Femoral neck; LS: Lumbar spine; QUS: Quantitative ultrasound; T1D: Type 1 diabetes; TB: Total body.</td>
<td></td>
</tr>
<tr>
<td>Study (year)</td>
<td>N (f/m)</td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
</tr>
<tr>
<td>Strotmeyer et al. (2004)</td>
<td>566 (243/323)</td>
</tr>
<tr>
<td>Register et al. (2006)</td>
<td>775 (410/365)</td>
</tr>
<tr>
<td>Tao et al. (2008)</td>
<td>76 (76/0)</td>
</tr>
<tr>
<td>Melton et al. (2008)</td>
<td>49 (28/21)</td>
</tr>
<tr>
<td>Anaforoglu et al. (2007)</td>
<td>206 (206/0)</td>
</tr>
<tr>
<td>Shan et al. (2009)</td>
<td>1042 (1042/0)</td>
</tr>
<tr>
<td>Petit et al. (2010)</td>
<td>190 (90/100)</td>
</tr>
<tr>
<td>Burghardt et al. (2010)</td>
<td>19 (9/10)</td>
</tr>
<tr>
<td>Shu et al. (2012)</td>
<td>25 (25/0)</td>
</tr>
<tr>
<td>Farr et al. (2014)</td>
<td>30 (30/0)</td>
</tr>
<tr>
<td>Pritchard et al. (2012)</td>
<td>30 (30/0)</td>
</tr>
</tbody>
</table>

↓: Decrease in BMD; ↑: Increase in BMD; 1T-MRI: 1 Tesla magnetic resonance imaging; BMC: Bone mineral content; BMD: Bone mineral density; BMI: Body mass index; CSA: Cross sectional area; DR: Distal radius; DXA: Dual x-ray absorptiometry; FN: Femoral neck; HR-pQCT: High resolution-peripheral quantitative computed tomography; LS: Lumbar spine; pQCT: Peripheral quantitative computed tomography; QCT: Quantitative computed tomography; QUS: Quantitative ultrasound; vBMD: Volumetric bone mineral density.
adequate peak bone mass. This maybe complicated further by subsequent poor glycemic control. T1D is typically associated with reduced BMD, whereas those with T2D usually have normal or elevated BMD. This inconsistency indicates that impaired bone quality may further explain the higher fracture risk in addition to frequent falls and associated comorbidities. There are several mechanisms by which diabetes could affect bone mass and strength, including insulin level, hyperglycemia, AGE accumulation which influences collagen characteristics, increased marrow adiposity and increased bone inflammation. In addition, associated diabetic complications and treatment with TZDs may increase fracture risk. Future studies are needed to clarify the impact of different aspects of diabetes metabolism, glycemic control and specific treatments for diabetes on bone. Given that DXA related BMD is a poor predictor of bone morbidity in this group of patients, there is a need to explore novel approaches to assessing bone quality. A better understanding of how diabetes affects bone will improve our ability to protect bone health and prevent fractures in the growing population of adults with diabetes.

**Five-year view**

With limited information from current studies to explain the increased risk of fragility fracture, the focus on understanding the effects of diabetes, in particular T1D, on bone health needs to explore methods of assessing bone health that involve more advanced imaging modalities as well as other molecular and biochemical markers of bone health and diabetes control. Increased availability of these tools in the near future will be particularly useful in the pediatric setting given that the peak age of diagnosis of T1D is between 10 and 14 years old, which is also the critical physiological period of bone development. Dissecting the conundrum of diabetic bone loss in T2D may be complicated by many confounding factors, but the suggestion of diabetic osteopathy as the obesity of bone opens up exciting avenues for exploration. Finally, the suggestion that bone loss may be another manifestation of diabetes-related vascular disease, raises the possibility that assessment of bone health may become a routine component of screening for health complications in diabetes. Routine screening of bone health by tools that are specific for the bone pathology in this condition is likely to lead to improved targeted prevention of fractures in T1D and T2D.

**Financial & competing interests disclosure**

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No writing assistance was utilized in the production of this manuscript.

**Key issues**

- The awareness of bone as another target organ of diabetes complications remains low clinically out with the research arena.
- Small samples sizes and problems with addressing confounding factors limit the evidence on the effect of glycemic control on bone health in diabetes.
- Abnormalities of bone health may be present early after diagnosis.
- Diabetic bone loss is most likely due to a complex interplay between the effects of hyperglycemia, insulin deficiency, IGF-1 deficiency, increased bone marrow adiposity, inflammation and vasculopathy on bone formation.
- *In-vitro* studies support the osteogenic potential of metformin, which offers a rational prevention strategy.
- The disparity between dual x-ray absorptiometry-derived bone mineral density and actual fracture risk highlights the limitation of dual x-ray absorptiometry as a predictor of fracture risk in conditions secondary to systemic disease.

**References**


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98. Rous TD. Inflammation as death or life signal in diabetic fracture healing. Inflamm Res 2011;60:3-10


122. Nicodemus KK, Folsom AR. Type 1 and type 2 diabetes and incident hip fractures in postmenopausal women. Diabetes Care 2001;24:1192-7


130. Schwartz AV. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA 2011;305:2184-92


151. Brandao FR, Vicente EJ, Dalter CH, et al. Bone metabolism is linked to disease...


179. Léger J, Marinovic D, Alberi C, et al. Lower bone mineral content in children with type 1 diabetes mellitus is linked to female sex, low insulin-like growth factor type 1 levels, and high insulin requirement. J Clin Endocrinol Metab 2006;91:3947-53


194. Register TC, Lenchik L, Hsu F-C, et al. Type 2 diabetes is not independently associated with spinal trabecular volumetric bone mineral density measured by QCT in the Diabetes Heart Study. Bone 2006;39:628-33


Metformin suppresses adipogenesis through both AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms

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ABSTRACT

People with Type 2 diabetes mellitus (T2DM) have reduced bone mineral density and an increased risk of fractures due to altered mesenchymal stem cell (MSC) differentiation in the bone marrow. This leads to a shift in the balance of differentiation away from bone formation (osteogenesis) in favour of fat cell development (adipogenesis). The commonly used anti-diabetic drug, metformin, activates the osteogenic transcription factor Runt-related transcription factor 2 (Runx2), which may suppress adipogenesis, leading to improved bone health. Here we investigate the involvement of the metabolic enzyme, AMP-activated protein kinase (AMPK), in these protective actions of metformin. The anti-adipogenic actions of metformin were observed in multipotent C3H10T1/2 MSCs, in which metformin exerted reciprocal control over the activities of Runx2 and the adipogenic transcription factor, PPARγ, leading to suppression of adipogenesis. These effects appeared to be independent of AMPK activation but rather through the suppression of the mTOR/p70S6K signalling pathway. Basal AMPK and mTOR/p70S6K activity did appear to be required for adipogenesis, as demonstrated by the use of the AMPK inhibitor, compound C. This observation was further supported by using AMPK knock out mouse embryonic fibroblasts (MEFs) where adipogenesis, as assessed by reduced lipid accumulation and expression of the adipogeneic transcription factor, C/EBPβ, was found to display an absolute requirement for AMPK. Further activation of AMPK in wild type MEFs, with either metformin or the AMPK-specific activator, A769662, was also associated with suppression of adipogenesis. It appears, therefore, that basal AMPK activity is required for adipogenesis and that metformin can inhibit adipogenesis through AMPK-dependent or -independent mechanisms, depending on the cellular context.

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1. Introduction

Type 2 diabetes mellitus (T2DM) is characterized by chronic elevation of blood glucose levels because of systemic insulin resistance. In addition to the reduction of insulin sensitivity in muscle, adipose tissue and the liver, it has been noted recently that people with diabetes have increased risk of bone fractures (Janghorbani et al., 2007,Kilpadi et al., 2014,Hothersall et al., 2013). Furthermore, the use of the thiazolidinedione (TZD) antidiabetic drug class, which includes pioglitazone and rosiglitazone, has been shown to increase the risk of bone fractures and secondary osteoporosis (Lecka-Czernik, 2009,Grey et al., 2007,Schwartz et al., 2006).

TZDs act as agonists for the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) which is considered to be the master regulator of fat cell development (adipogenesis) (Tontonoz et al., 1994a). It is therefore thought that the detrimental
effects of TZDs on bone health is through the activation of PPARγ in mesenchymal stem cells (MSCs) causing adipogenesis and, consequently, suppressing bone development (osteogenesis) (Lecka-Czernik et al., 2007, Shockley et al., 2009). Adipogenesis is regulated by a temporally induced cascade involving PPARγ and members of the CCAAT/enhancer binding protein (C/EBP) transcription factor family. In the initial stages of adipogenesis there is a transient accumulation of C/EBPβ and C/EBPδ proteins, leading to a later accumulation of C/EBPa and PPARγ (Cao et al., 1991, Yeh et al., 1995). C/EBPa and PPARγ then promote gene expression characteristic of an adipocyte phenotype and their expression remains elevated for the life of the differentiated cell (Tontonoz et al., 1994b).

Metformin has been used clinically for the treatment of T2DM since the 1960s and it is thought to function primarily through the inhibition of hepatic gluconeogenesis (Cusi et al., 1996). Metformin interferes with oxidative phosphorylation in the mitochondria by inhibiting complex I in the electron transport chain, although the exact mechanism of inhibition is not yet known (El-Mir et al., 2000, Lantier et al., 2014). Metformin has also been shown to stimulate osteogenic differentiation of MSCs towards osteoblasts in vitro through the trans-activation of Runt-related transcription factor 2 (Runx2), the key regulatory transcription factor for osteogenic differentiation (Jang et al., 2011) and, unlike TZDs, has been shown to be associated with a reduced risk of fractures. Osteoblast differentiation has been proposed to be dependent on the cellular energy sensor AMP-activated protein kinase (AMPK), as the expression of various osteogenic genes has been shown to be inhibited by compound C, a chemical inhibitor of AMPK, and a dominant negative form of AMPK (Banerjee et al., 1997). Furthermore, metformin stimulates AMPK activation through the inhibition of oxidative phosphorylation in hepatocytes (Zhou et al., 2001).

AMPK is a heterotrimeric serine/threonine protein kinase that acts as a cellular energy sensor due to its ability to be activated by an increase in the AMP-ATP ratio, which leads to phosphorylation of Thr172 on AMPKα by liver kinase B1 (LKB1) (Hardie, 2015, Woods et al., 2003). AMPK can also be phosphorylated and activated at Thr172 by calcium/calmodulin-dependent protein kinase kinase (CaMKK) in a Ca2+-dependent, AMP-independent manner (Hawley et al., 2005). AMPK functions to inhibit ATP consuming pathways and at the same time activate catabolic pathways to re-establish cellular energy homeostasis. It has also been shown that AMPK has an array of non-metabolic functions including promotion of nitric oxide synthesis and numerous anti-inflammatory actions (Jones et al., 2005, Reihill et al., 2007, Salminen et al., 2011, Morrow et al., 2003, Salt and Palmer, 2012). Recently, it has been shown that AMPK functions in cell differentiation by promoting osteogenic differentiation while suppressing adipogenic differentiation (Kanazawa et al., 2008, Vila-Bedmar et al., 2010), however, the role of AMPK in cell commitment to differentiation remains unclear. Therefore, the main aim of the current study is to determine the effect of metformin on adipogenesis and, in particular, to understand the role of the AMPK signalling pathway in these processes.

2. Materials and methods

2.1. Cell culture and induction of differentiation

AMPK α1/α2 knockout mouse embryonic fibroblasts (MEFs), C3H10T1/2 mouse mesenchymal stem cells (Clone 9; ATCC CCL-
Fig. 2. Effects of Metformin and the AMPK-activator, A769662, on early markers of differentiation in C3H10T1/2 cells.

A) Confluent C3H10T1/2 cells were stimulated for 5 days with 10 μM pioglitazone (PIO) or an adipogenic (IID) or osteogenic (AGD) cocktail, in the presence or absence of 500 μM metformin or 100 μM A769662. Cell extracts were then prepared and immunoblotted with antibodies to PPARγ, Runx2 and AMPK. The phosphorylation-dependent electrophoretic mobility shift of Runx2 induced by AGD treatment is indicated (pRunx2). Representative immunoblots from an experiment carried out on three separate occasions with similar results are shown.

B) Densitometric analysis of PPARγ1 (upper panel) and PPARγ2 (lower panel) levels relative to Runx2 are shown as means ± SEM. Significant increases (*, p < 0.05) relative to control, and significant decreases relative to PIO-stimulated cells (#, p < 0.05), are indicated (n = 3). Non-significant changes are also indicated (ns).

C) Confluent C3H10T1/2 cells were transfected with a PPARγ (upper panel) and Runx2 (lower panel) luciferase gene reporter constructs, together with control Renilla luciferase vector, and then stimulated for two days with 500 μM metformin or 100 μM A769662, in the presence or absence of 10 μM pioglitazone (PIO), adipogenic medium (IID) or osteogenic medium (AGD). Cell extracts were then prepared and luciferase activities were measured using a dual luciferase reporter assay. Luciferase activities from three separate experiments are shown as means ± SEM. Significant increases in luciferase activity are indicated; *, p < 0.05, **, p < 0.01 and ***, p < 0.001, as are significant decreases in activity, #, p < 0.05 (n = 3). Non significance is also indicated (ns).
C3H10T1/2 cells were harvested and nuclear extracts prepared using the Nuclear Extraction kit from Active Motif, Belgium. Briefly, the media was aspirated and cells were harvested in ice-cold PBS containing phosphatase inhibitors and centrifuged (200 x g, for 5 min at 4 °C) to obtain a cell pellet. The cell pellet was re-suspended in complete lysis buffer containing 10 mM DTT and phosphatase inhibitor cocktail and then centrifuged (14 000 x g, for 10 min at 4 °C) to obtain nuclear and supernatant fractions.

2.3. Western blotting

Protein samples were separated by SDS-PAGE and then transferred electrophoretically onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) Marvel milk powder in TBS (20 mM Tris–HCl pH 7.5, 150 mM NaCl) for 1 h. Membranes were then incubated with primary antibodies overnight at 4 °C. Following washes with TBST (TBS supplemented with 0.5% (v/v) Tween-20), membranes were incubated with secondary antibodies for 1 h at room temperature. Antibodies were diluted to the required concentration in 5% (v/v) Sea Block (Thermo Scientific) and 5% (v/v) TBST. The primary antibodies included those purchased from Cell Signalling Technologies (CST), Danvers, MA and included perilipin, peroxisome proliferator-activated receptor gamma (PPARγ; marker for adipogenesis), Runx-related transcription factor 2 (Runx2; marker for osteogenesis), phosphorylated-ACC (P-ACC (Ser79); marker for AMPK activity), phosphorylated AMPKα (P-AMPKα (Thr172)); detects active AMPK) and phosphorylated-p70S6K (P-p70S6K (Thr389); upstream regulator of mTOR signalling). The rabbit polyclonal adiponectin antibody was generated in house and the tubulin antibody was bought from Abcam, Cambridge, UK. Antibodies were detected using a LI-COR Odyssey Infrared Imaging systems and densitometric analysis was carried out using ImageJ software (National Institute of Health, UK) software Version 1.47.

2.4. Oil Red O staining

Cells were incubated with adipogenic IID media in the presence or absence of either 10 μM pioglitazone, 500 μM metformin, 100 μM of the AMPK-activator, A769662, or 10 μM of the p70S6K inhibitor, rapamycin. Following differentiation, media was aspirated and cells were fixed to cell culture plates with 10% (v/v) neutral buffered formalin for 30 min. The formalin was then aspirated and staining was carried out with the addition of 0.3% (w/v) Oil Red O in isopropanol:water (60:40) for 5 min in room temperature. The Oil Red O was then aspirated and wells washed with distilled water four times. Imaging was carried out using a Zeiss Axiovert 25 microscope with QImaging camera and supporting software.

2.5. AMPK activity assays

AMPK activity was determined in AMPK α1 plus AMPK α2 immuno-complexes through phosphorylation of the peptide HMRSAMSGGLHVLKRR [SAMS], as previously described (Morrow et al., 2003). Briefly, the AMPK immunoprecipitates were re-suspended in 20 μl of HEPES Brij-35 buffer. Reaction mixtures (20 μl) containing 5 μl of HEPES Brij-35 buffer, 5 μl of 1 mM SAMS peptide in HEPES Brij-35 buffer, 5 μl of 1 mM AMP in HEPES Brij-35 buffer and 5 μl of immunoprecipitate re-suspended in HEPES Brij-35 buffer, were prepared in 1.5 ml microcentrifuge tubes on ice and the reaction initiated by the addition of 5 μl of MgATP solution (1 mM [γ-32P] ATP, 250–500 c.p.m./pmol; 25 mM MgCl2 in HEPES Brij-35 buffer). Reaction mixtures were then incubated on a vibrating platform in an air incubator at 30 °C for 10 min. Assay mixtures (15 μl) were spotted onto P81 phosphocellulose paper, and rinsed, with gentle stirring to remove free ATP, for 5 min in 1% (v/v)
phosphoric acid. A further 2/C2 5 min water washes were performed on the phosphocellulose paper, before a final 5 min wash with 1% (v/v) phosphoric acid. A Beckman Multi-Purpose scintillation counter LS 6500 was used to measure [32P]-labelled substrate. 3 ml of scintillation fluid was used per sample. Results were corrected for radioactivity recovered in blank reactions lacking the SAMS peptide. One unit of AMPK activity is that required to incorporate 1 nmol of 32P into the SAMS substrate peptide/min/mg protein.

### 2.6. Transient transfection and luciferase assay

C3H10T1/2 cells were transfected with the indicated plasmids in 6-well plates, with 1.125 μg/well PPARE (PPARγ reporter, purchased from Adgene) and 6xOSE reporter constructs (Runx2 reporter, supplied by Jian Huang, Rush Medical Centre, Chicago, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and then treated with metformin, A769662 or rapamycin with and without adipogenic differentiation media. Cells were then harvested 48 h after transfection and assayed using the Luciferase reporter assay system (Promega, Madison, WI) according to manufacturer’s instructions. As a transfection control, the Renilla plasmid 0.125 μg/well was co-transfected in each transfection experiment, and the luciferase activity was normalised to the Renilla activity.

#### 2.7. Statistical analysis

All experiments were performed in triplicate and statistical analysis was performed using Student’s t-test or one-way ANOVA. Results are expressed as mean ± standard error (SEM) and differences with p < 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Metformin suppresses adipogenesis in C3H10T1/2 MSCs

It has been previously reported that treatment of preadipocyte cell lines with AMPK activators inhibits their conversion to fat cells (Lee et al., 2011, Habinowski and Witters, 2001). Paradoxically,
however, the widely used AMPK inhibitor, compound C, has also been reported to inhibit adipogenesis of preadipocyte cell lines (Nam et al., 2008). To try and address this apparent contradiction and to further investigate the role of AMPK in the control of adipogenesis of multipotent mesenchymal stem cells (MSCs), we stimulated murine C3H10T1/2 MSCs with two known activators of AMPK, metformin (500 μM) and A769662 (100 μM). Confluent cultures of C3H10T1/2 MSCs were treated for 5 days with medium containing 10% foetal calf serum (FCS) supplemented with either an insulin-containing, adipogenic medium (IID) and/or the anti-diabetic drug, pioglitazone (PIO), which is a known agonist of the adipogenic transcription factor, PPARγ (Day and Bailey, 2007). Cells were also incubated with 10% FCS alone, as negative control for differentiation. After 5 days of treatment, cells were fixed and then stained with the neutral lipid stain, Oil Red O, to monitor lipid accumulation, which is a widely used late marker of adipogenesis (Fig. 1a). We found that treatment of cells with IID-containing medium stimulated lipid accumulation in C3H10T1/2 MSCs, an effect that was further enhanced by co-treatment of cells with PIO (Fig. 1a). We also found that treatment of cells with either metformin or A769662 suppressed adipogenesis promoted by IID alone or by a combination of IID plus PIO (Fig. 1A), with metformin being a more effective inhibitor of lipid accumulation (Fig. 1A). The effects of metformin on the suppression of adipogenesis was confirmed by western blotting for two late markers of fat cell conversion, adiponectin and perilipin, the expression of which were strongly induced following IID treatment, but were suppressed in the presence of metformin (Fig. 1B). These results suggest that AMPK activators inhibit adipogenesis of C3H10T1/2 MSCs. To further elucidate the mechanisms of action of metformin and A769662 in these cells, we next treated cells with 10% FCS, supplemented with either IID or PIO, and measured the protein expression levels of the transcription factor, PPARγ (Day and Bailey, 2007). Cells were also incubated with 10% FCS alone, as negative control for differentiation. After 5 days of treatment, cells were fixed and then stained with the neutral lipid stain, Oil Red O, to monitor lipid accumulation, which is a widely used late marker of adipogenesis (Fig. 1a). We found that treatment of cells with IID-containing medium stimulated lipid accumulation in C3H10T1/2 MSCs, an effect that was further enhanced by co-treatment of cells with PIO (Fig. 1a). We also found that treatment of cells with either metformin or A769662 suppressed adipogenesis promoted by IID alone or by a combination of IID plus PIO (Fig. 1A), with metformin being a more effective inhibitor of lipid accumulation (Fig. 1A). The effects of metformin on the suppression of adipogenesis was confirmed by western blotting for two late markers of fat cell conversion, adiponectin and perilipin, the expression of which were strongly induced following IID treatment, but were suppressed in the presence of metformin (Fig. 1B). These results suggest that AMPK activators inhibit adipogenesis of C3H10T1/2 MSCs. To further elucidate the mechanisms of action of metformin and A769662 in these cells, we next treated cells with 10% FCS, supplemented with either IID or PIO, and measured the protein expression levels of the transcription factor, PPARγ (Day and Bailey, 2007). Cells were also incubated with 10% FCS alone, as negative control for differentiation. After 5 days of treatment, cells were fixed and then stained with the neutral lipid stain, Oil Red O, to monitor lipid accumulation, which is a widely used late marker of adipogenesis (Fig. 1a). We found that treatment of cells with IID-containing medium stimulated lipid accumulation in C3H10T1/2 MSCs, an effect that was further enhanced by co-treatment of cells with PIO (Fig. 1a). We also found that treatment of cells with either metformin or A769662 suppressed adipogenesis promoted by IID alone or by a combination of IID plus PIO (Fig. 1A), with metformin being a more effective inhibitor of lipid accumulation (Fig. 1A). The effects of metformin on the suppression of adipogenesis was confirmed by western blotting for two late markers of fat cell conversion, adiponectin and perilipin, the expression of which were strongly induced following IID treatment, but were suppressed in the presence of metformin (Fig. 1B).
Red O staining experiments in Fig. 1, we found that treatment of cells with either metformin or A769662 effectively suppressed IID- and PIO-stimulated increases in PPARγ protein levels (Fig. 2A and B), as well as IID- and PIO-stimulated PPARγ activity (Fig. 2C), as determined by gene reporter assays. Given that increases in PPARγ activity during the early stages of adipogenesis are necessary and sufficient to promote terminal fat cell development (Rosen and Spiegelman, 2000), it appears that the ability of metformin and A769662 to inhibit adipogenesis of C3H10T1/2 MSCs is linked to their ability to suppresses increases in PPARγ protein levels promoted by treatment of cells with either IID or PIO. Moreover, given that the PPARγ luciferase reporter assays were carried out after only two days of differentiation, it appears that the suppressive actions of metformin and A769662 occur at a very early stage of the adipogenic process.

The control of differentiation of MSCs into fat and bone is thought to be controlled through reciprocal regulation of PPARγ and the osteogenic transcription factor, Runx2 (Jeon et al., 2003,Muruganandan et al., 2009), during the commitment stage of differentiation. We therefore also examined Runx2 protein levels (Fig. 2A and B) and activity (Fig. 2C). As a positive control for these experiments, cells were incubated with 10% FCS supplemented

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**Fig. 6. Compound C inhibits adipogenesis of C3H10T1/2 cells.**

A) Confluent C3H10T1/2 cells were stimulated for 5 days with IID, in the presence or absence of the AMPK inhibitor, 10 μM compound C. Cell extracts were then prepared and immunoblotted with antibodies to phosphorylated ACC (Ser 79). Representative immunoblots from an experiment carried out on three separate occasions with similar results are shown (upper panel). Densitometric values from 3 separate experiments are shown in the lower panel as means ± SEM. Significant decreases relative to control are indicated, #, p < 0.05.

B) Confluent C3H10T1/2 cells were treated with 10% FCS supplemented with adipogenic cocktail (IID), in the presence or absence of the indicated concentrations of compound C. After 5 days cells were fixed with formalin and stained with Oil Red O to detect neutral lipid accumulation. Representative micrographs from an experiment carried out on three separate occasions with similar results are shown.

C) Confluent C3H10T1/2 MSCs were induced to differentiate by addition 10% FCS in the presence or absence of IID medium and/or 10 μM Compound C or 10 μM rapamycin. Cell extracts were then prepared after 5 days and immunoblotted with antibodies to perilipin and tubulin. Representative immunoblots from an experiment carried out on three separate occasions with similar results are shown. Densitometric analysis of three immunoblots are shown as means ± SEM in the lower panel. Significant increases relative to control are indicated, ***p < 0.001 and significant decreases with respect to IID-treated cells are indicated, ###, p < 0.001.
with a widely used osteogenic medium (AGD) (Shea et al., 2003) to induce Runx2 activity. We found that treatment of cells with 10% FCS plus AGD for 5 days did not significantly affect PPARγ protein levels (Fig. 2A and B) or activity (Fig. 2C). However, AGD treatment alone did promote a noticeable phosphorylation band-shift of Runx2 protein in treated cells (Fig. 2A), which correlated with an increase in Runx2 activity, as determined by a Runx2 gene reporter assay (Fig. 2C). Treatment with either metformin or A769662 significantly increased Runx2 activity in C3H10T1/2 cells (Fig. 2C), which did not correlate with an increase in AGD-promoted phosho-Runx2 levels, as determined by band-shift (Fig. 2A). Since osteogenesis and adipogenesis of MSCs are thought to be reciprocally regulated by the PPARγ:Runx2 activation ratio (Chen et al., 2016), we can conclude that the inhibitory actions of metformin and A769662 on the adipogenesis of C3H10T1/2 MSCs can partly be explained by reciprocal control of PPARγ and Runx2 activity, thereby favouring an osteogenic lineage. In addition, whereby the actions of metformin and A769662 appear to be through the suppression of adipogenic-dependent increases in PPARγ expression, the actions on Runx2 activity remain to be determined, but appear to be independent of osteogenic-linked increases in Runx2 phosphorylation (Fig. 2A).

3.2. Metformin suppresses adipogenesis in C3H10T1/2 MSCs through the inhibition of the p70S6K signalling pathway and not through the activation of AMPK

Both metformin and A769662 are reported to activate AMPK in a variety of cell types (Zhou et al., 2001, Cool et al., 2006). Given the inhibitory effects of these two compounds on early and late markers of adipogenesis of C3H10T1/2 MSCs (Figs. 1 and 2), we next tested their ability to activate AMPK in these cells. We did this by measuring the phosphorylation of a known AMPK substrate, Ser 79 of acetyl coenzyme carboxylase (ACC), using phospho-specific antibodies. Intriguingly, although A769662 provoked a robust and rapid phosphorylation of ACC, which was maintained for up to 48 h, metformin did not induce a significant phosphorylation of ACC, even after 48 h stimulation (Fig. 3). It is likely, therefore, that while metformin is an effective inhibitor of the adipogenic differentiation of C3H10T1/2 MSCs, in response to insulin-containing IMD medium, or activation of PPARγ by PIO, this occurs through mechanisms that are independent of AMPK activation. Recent work, however, has shown that the control of osteogenesis is regulated through interactions between PPARγ and the mTOR/p70S6K signalling pathway (Sun et al., 2013). Moreover, metformin has been shown to inhibit the activation of the p70S6K pathway independently of AMPK (Vazquez-Martín et al., 2009) in tumour cells and p70S6K has been shown to be required for the growth hormone-dependent adipose conversion of 3T3-F442A preadipocytes (Yarwood et al., 1999). We therefore examined the role of the p70S6K pathway on IMD-induced adipogenesis of C3H10T1/2 cells by incubating cells with the mTOR/p70S6K inhibitor, rapamycin. We found that incubation of differentiating cells with rapamycin dramatically inhibited lipid accumulation associated with adipogenesis, as determined by Oil Red O staining (Fig. 4A). Furthermore, rapamycin also suppressed PPARγ activity, as determined by gene reporter assays, indicating that the mTOR/p70S6K pathway is required for adipogenesis of C3H10T1/2 cells (Fig. 4B). We found that the effects of rapamycin were specific to inhibition of mTOR/p70S6K, since rapamycin treatment had no significant effect of phospho-ACC levels (Fig. 5A), indicating no effect on AMPK activity, but, rather, significantly inhibited phosphorylation of p70S6K on Thr 389 (Fig. 5B), which is the mTOR phosphorylation site critical for kinase function (Saitho et al., 2002). Importantly, both metformin and A769662 also inhibited p70S6K phosphorylation (Fig. 5B), indicating that suppression of adipogenesis of C3H10T1/2 MSCs by these compounds may involve suppression of mTOR/p70S6K signalling at early stages of commitment to differentiation. Although we found that metformin inhibits mTOR/p70S6K signalling apparently independently of AMPK activation (Figs. 3 and 5A), this does not rule out a role for AMPK in the control of p70S6K activation. In fact, it has been reported that AMPK inhibits mTOR/p70S6K signalling, which is thought to underlie the actions of metformin in a range of cellular contexts (Violett et al., 2012, Dowling et al., 2011). To determine whether the same relationship exists in C3H10T1/2 MSCs, we incubated cells with the AMPK inhibitor, compound C, and determined its action on ACC (Ser 79) and p70S6K (Thr 389) phosphorylation (Fig. 5A and B, respectively). We found that compound C significantly inhibited both basal p70S6K (Thr 389; Fig. 5B) and ACC (Ser 79; Fig. 6A) phosphorylation, suggesting that AMPK is linked to the activation of mTOR/p70S6K signalling in these cells. We also found that 10 μM compound C was able to inhibit adipogenesis of C3H10T1/2 cells treated with IMD, as determined by lipid accumulation (Fig. 6B) and expression of the adipogenic marker, perilipin (Fig. 6C), as well as suppressing AMPK activation in the presence or absence of IMD (Fig. 6A). This suggests that basal levels of AMPK activity, perhaps acting through the mTOR/p70S6K pathway (Fig. 5B), are important for supporting adipose conversion of these cells.

3.3. AMPK plays a dual role in regulating the adipogenesis of mouse embryonal fibroblasts (MEFs)

Our findings in C3H10T1/2 cells may provide the explanation for the apparently conflicting, previous reports that both activation AMPK and inhibition of AMPK block adipogenesis of 3T3-L1 pre-adipocytes (Lee et al., 2011, Habinowski and Witters, 2001, Nam et al., 2008). This has been suggested to be a result of AMPK exerting differential control during the process of differentiation; due to AMPK exerting different control at early time points versus late time points. Instead, we hypothesise there is a threshold level of AMPK activity required for adipogenesis, above which further activation leads to a break on the process. Indeed, it is worth noting that AMPK activity levels do not change significantly during

**Fig. 7. Metformin suppresses adipogenesis in MEFs**

A) Wild type and AMPK knockout (−/−) mouse embryonic fibroblasts (MEFs) were treated with IMD plus 10 μM pioglitazone (PIO) for 7 or 9 days as indicated. Wild type MEFs were also stimulated in the presence or absence of 1 mM metformin or 100 μM A769662. Cells were then stained with Oil Red O. Representative micrographs from an experiment carried out on three separate occasions with similar results are shown.

B) Confluent wild-type (upper panel) and AMPK−/− (lower panel) MEFs were stimulated for the indicated times with 10 μM pioglitazone (PIO) plus (BD) and, for wild-type MEFs, in the presence or absence of 1 mM metformin or 100 μM A769662. Cell extracts were then prepared and immunoblotted with antibodies to C/EBPβ and/or C/EBPδ, as indicated. Representative immunoblots from experiments carried out on three separate occasions with similar results are shown.

C) Densitometric values taken at day 5 from the experiment carried out in Fig. 7B are shown in the lower panel as means ± SEM. Significant increases in expression are indicated, **, p < 0.01 and ***, p < 0.001. Significant decreases relative to control are also indicated, #, p < 0.05. Non-significance is also indicated (ns).

D) Wild-type MEFs were incubated with 1 mM metformin for the times indicated or with 100 μM A769662 for 30 min. Cells were then lysed and the lysates were immunoblotted with antibodies specific to the indicated proteins (upper panel). Results from densitometric analysis of three separate immunoblots are shown in the lower panel as means ± SEM. Significant increases relative to t = 0 are indicated, * p < 0.05.
adipogenesis of 3T3-L1 preadipocytes (Supplementary Fig. 1) and C3H10T1/2 MSCs (unpublished observations) indicating that AMPK activity must be kept under stringent control to allow the differentiation of these cells. To investigate this relationship further, we next examined the effects of metformin and A769662 and adipogenesis in wild type and AMPKα knockout (−/−) mouse embryonal fibroblasts (MEFs). We first incubated wild type or AMPKα (−/−) MEFs with 10% FCS, in the presence or absence of a combination of adipogenic medium (IID) plus PIO, (Fig. 7A). Cells were then fixed and stained with Oil Red O at days 7 and 9, to monitor late-stage triglyceride accumulation (Fig. 7A), and cell extracts were prepared at days 1, 2 and 5, to detect levels of the early marker of differentiation, the transcription factor C/EBPβ, by western blotting (Fig. 7B). We found that a combination of IID and PIO promoted a large increase in lipid accumulation in wild type MEFs but not AMPKα (−/−) MEFs, at days 7 and 9 (Fig. 7A). We also found that after an initial increase in the expression of C/EBPβ and C/EBPα at day 1 in wild type MEFs, following IID and PIO treatment, levels fell by day 5 but remained significantly above basal (Fig. 7B). This was not the case in AMPKα (−/−) MEFs, where C/EBPβ levels returned to basal by day 5 and remained at that level for up to 9 days of treatment with differentiation medium (Fig. 7B). These results suggest that a basal level of AMPK activity is required for adipogenesis of MEFs and exerts actions on both early and late markers of adipose conversion. We next examine the effects of metformin and A769662 on adipogenesis of MEFs in response to IID and PIO treatment. We found that treatment of wild type MEFs with either metformin or A769662 led to a reduction in lipid accumulation (Fig. 7A) and C/EBPβ protein levels (Fig. 7B), indicating that both compounds are anti-adipogenic in these cells. We also found that both metformin (1 mM) and A769662 (100 μM) significantly increased phosphorylation of ACC at (Ser 79; Fig. 7C), indicating that they both exert their anti-adipogenic actions through the activation of AMPK. Together with the results obtained from C3H10T1/2 MEFs this suggests that the role of AMPK in the control of adipogenesis is complex, suggesting both positive and negative regulation that may depend on the cellular context. Moreover, the anti-adipogenic actions of the anti-diabetic drug, metformin, may be both AMPK-dependent and AMPK-independent, again depending on the cellular context.

4. Discussion

Previous work has shown that AMPK activation reduces adipogenesis in favour of osteogenesis in adipocyte-derived human MSCs (hMSCs) and bone marrow-derived MSCs (Kim et al., 2012;Lee et al., 2014). In the present work, we found that the AMPK activators, metformin and A769662, inhibited adipogenesis in murine C3H10T1/2 MSCs and in wild type MEFs. Both AMPK activators promoted a significant activation of AMPK in wild type MEFs, although, interestingly, we found that metformin, did not promote AMPK activation in C3H10T1/2 cells (Fig. 3). Metformin has been shown to activate AMPK in many different cell types; however a requirement of AMPK for the therapeutic actions of metformin has been questioned following genetic loss of function experiments that demonstrated AMPK-independent mechanisms of action of metformin during the inhibition of hepatic gluconeogenesis (Foerst et al., 2010). The AMPK-independent action of metformin on the inhibition of adipogenesis reported here might be due to cell type specific effects or stage-specific effects during the differentiation process. For example, most of the studies demonstrating an AMPK-dependent action of metformin were conducted in more differentiated cell lines, such as pre-osteoblasts (Jang et al., 2011; Kanazawa et al., 2008; Cortizo et al., 2006), pre-adipocytes (Moreno-Navarrete et al., 2011;Lee et al., 2012), myoblasts (Longnus et al., 2005;Kobashigawa et al., 2014;Fulco et al., 2008) and neuronal mouse cell lines (Bang et al., 2014), instead of the more primitive cell progenitors investigated here.

With regards to stage-specific effects, Pantovic et al. (Pantovic et al., 2013) demonstrated that there is a coordinated time-dependent activation of different signalling pathways during the osteogenic differentiation of hMSCs, which is AMPK-dependent in the early stages of differentiation followed by late stage activation of the Akt/mTOR signalling pathway. Given the results presented here, it could be argued that similar mechanisms might regulate adipogenesis in murine MSCs. For example, we find an overall requirement for basal levels of AMPK activity for adipogenesis of C3H10T1/2 cells, as demonstrated by the use of the AMPK inhibitor compound C (Fig. 6B) and verified by the use of AMPK knockout (−/−) MEFs (Fig. 7A). It should be noted, however, that AMPK activity levels remain constant throughout the process of adipogenesis, as determined by AMPK activation assays (Supplementary Fig. 1) and phosphorylation of ACC on Ser 79 (results not shown). Despite this, the ability of metformin to inhibit adipogenesis was found to be due to a reduction in the PPARγ-Runx2 activation ratio (Fig. 2C) and this was linked to the inhibition of mTOR/p70S6K signalling (Fig. 4). This suggests that the ability of metformin to control the commitment of MSCs to differentiate into either osteoblasts or adipocytes is governed at an early stage through the inhibition of mTOR/p70S6K signalling. Moreover, wild type MEFs were observed to accumulate lipid and increase expression of C/EBPβ in response to an adipogenic cocktail of IID plus PIO (Fig. 7). These effects were blocked in AMPK (−/−) MEFs, which may indicate that AMPK is required for efficient, late stage lipid accumulation or, since AMPK is involved in mitochondrial biogenesis, altered mitochondrial function (Bergeron et al., 2001).

Overall, our work suggests that metformin exerts multiple effects to inhibit adipogenesis in different cell types. Therefore, the overriding view that metformin exerts its effects on adipogenesis simply by promoting AMPK activation may therefore need some revision. In particular, the role of AMPK itself appears to be complex, in that it appears to exert both positive and negative effects during the adipogenic conversion of MEFs and C3H10T1/2 MSCs. In conclusion, further investigation into how metformin suppresses signalling through the mTOR/p70S6K pathway may lead to new therapeutic intervention strategies to prevent unwanted bone marrow adipogenesis associated with diseases, such as T2DM, where bone health is impaired.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.11.011.

References

Bergeron, R., Ren, J.M., Cadman, K.S., Moore, LK, Perret, P., Pypaert, M., Young, L.H.,


S.C. Chen a, d, R. Brooks a, J. Houskeeper b, S.K. Bremner b, J. Dunlop b, B. Viollet c, d, e, P.J. Logan f, I.P. Salt f, S.F. Ahmed a, S.J. Yarwood g, *

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The authors regret that the crop lines in the western blot in Figure 6c were put in the wrong place when the figure was prepared. The corrected version of the figure is:

![Western Blot Diagram]

Revising the figure does not alter the densitometry measurements we made or change the conclusions we draw from the data. The authors would like to apologise for any inconvenience caused.

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Skeletal Fragility & Its Clinical Determinants In Children With Type 1 Diabetes

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Skeletal fragility in T1D

**Skeletal Fragility & Its Clinical Determinants In Children With Type 1 Diabetes**

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**Context** Type 1 diabetes (T1D) is associated with an increased fracture risk at all ages.

**Objective** To understand the determinants of bone health and fractures in children with T1D.

**Design** Case-control study of children with T1D on bone turnover markers, DXA and 3T-MRI of the proximal tibia to assess bone microarchitecture and vertebral marrow adiposity, compared to age and sex-matched healthy children.

**Results** 32 children with T1D at a median (range) age of 13.7 years (10.4,16.7) and 26 controls aged 13.8 years (10.2,17.8) were recruited. In children with T1D, serum BAP SDS, CTX SDS, and total body and lumbar spine BMD SDS were lower (all p<0.05). Children with T1D also had lower trabecular volume [0.55 (0.47,0.63) vs 0.59 (0.47,0.63); p=0.024], lower trabecular number [1.67 (1.56,1.93) vs 1.82 (1.56,1.99); p=0.004] and higher trabecular separation [0.27 (0.21,0.32) vs 0.24 (0.20,0.33); p=0.001] than controls. Marrow adiposity was similar in both groups (p=0.25). Bone formation as assessed by BAP was lower in children with poorer glycemic control (p=0.009) and who were acidic at initial presentation (p=0.017) but higher in children on continuous subcutaneous insulin infusion (p=0.025). Fractures were more likely to be encountered in children with T1D compared to controls [31% vs 19%; p<0.001]. Compared to those without fractures, the T1D children with a fracture history had poorer glycemic control (p=0.007) and lower total body BMD (p<0.001) but no differences in bone microarchitecture.

**Conclusion** Children with T1D display a low bone turnover state with reduced bone mineralisation and poorer bone microarchitecture.

Micro-MRI based assessment of bone microarchitecture demonstrated poorer trabecular bone in children with T1D compared to healthy control.
Introduction

Adults with T1D have a significantly increased risk of fracture compared to the general population (1, 2). New presentation of T1D peaks between the ages of 9 and 14 years (3, 4) and given that childhood and adolescence are important physiological periods for optimal bone development (5), it is possible that these young people with T1D may be especially susceptible to abnormalities of bone health. Indeed, recent studies also confirm that children and young people with T1D may also have a higher risk of fractures (6, 7). Earlier studies suggested have that the deficit in bone mass in T1D may be present at an early stage after diagnosis (8) but it is unclear whether the duration of the condition or any other clinical parameters of diabetes or its control are associated with adverse skeletal morbidity. As the significantly increased fracture risk is disproportionate to the marginal reduction in bone mineral density in adults with T1D (1), there is an increasing realization that a comprehensive assessment of bone requires an assessment of bone microarchitecture as well as bone density (9).

Metabolic conditions that may affect bone health, such as diabetes, are also associated with marked alterations in body composition, adiposity and bone marrow adiposity (10-12). Glycemic control in diabetes has been shown to be positively related to lumbar spine marrow adiposity (10, 13). MRI with its ability to study microarchitecture as well as bone marrow adiposity promises to be a useful imaging modality. Indeed, recent MRI-based studies in young women with childhood-onset T1D revealed a deficit in trabecular bone microarchitecture (14). The current study was performed to determine whether these MRI-based findings in trabecular bone microarchitecture were present at an earlier age in children with T1D. The detailed multimodality analysis of bone health that was performed in these children also provided a mechanistic insight into the effect of diabetes on the developing skeleton.

Methods

Subjects

Children and adolescents aged between 10-18 years old with T1D for at least three years were recruited from the Paediatric Diabetes Service at the Royal Hospital for Children, Glasgow during the period August 2014 and January 2016. The diagnosis of T1D was based on clinical presentation and ongoing insulin requirement with autoantibody confirmation in uncertain cases. For the MRI-based studies, age and sex-matched children and adolescents without were recruited to provide a reference group. Exclusion criteria included other chronic diseases or medications known to affect bone health. Information on personal health, medication history and lifestyle habits, including dairy intake, use of vitamins or calcium supplementation, cigarette smoking and a family history of early osteoporosis was collected. Age-appropriate questionnaires were used to collect information on level of physical activity (15, 16). Information was also collected on age at diagnosis, severity of initial presentation, disease duration, glycemic control, as measured by glycosylated haemoglobin (HbA1c) averaged over the last 12 months, and presence of microvascular complications in the children with T1D. Glycemic control was categorised as good (HbA1c <7.5% or <58 mmol/mol), moderate (HbA1c 7.5-9.0% or 58-75mmol/mol) or poor (HbA1c >9.0% or >75mmol/mol) (17). A history of radiologically-confirmed fractures was also collected, and in children with T1D only fractures sustained after the diagnosis of T1D were included. The study protocol was approved by the West of Scotland Research Ethics Committee and informed consent was obtained from the study participants.

Anthropometry and puberty staging
Age- and sex-adjusted Z scores for height, weight and BMI were calculated using the LMS method based on UK population reference data (18). Puberty assessment was undertaken by a single researcher (SCC) and categorised according to Tanner stages for girls and boys, with the additional option of self-assessment in those who did not want to be examined (19). For analysis, pubertal status was defined by genital status in male and breast development in female.

**Biochemical Markers of Bone Metabolism & GH/IGF-1 Axis**

In all 32 cases, blood samples were collected in the morning to coincide with the clinic visit, centrifuged and the supernatant stored at -80°C. Osteocalcin (OC), bone-specific alkaline phosphatase (BAP), C terminal telopeptide of Type I collagen (CTX) were measured by ELISA (Immunodiagnostic systems, Boldon, UK). The intra-assay variation for OC, BAP and CTX was 1.4%, 9.6% and 3.1%, respectively. Plasma IGF-I and its binding protein-3 (IGFBP3) and acid labile subunit (ALS) were also determined, using ELISA (Mediagnost GmbH, Reutlingen, Germany). Intra-assay variabilities were 16.0%, 2.9%, and 10.9%, respectively. Sclerostin (SOST) was also measured by ELISA (TECO, Pathway Diagnostics, Surrey, UK) with intra-assay variation of 14.1%. Parathyroid hormone (PTH) and alkaline phosphatase (ALP) were measured using chemiluminescent microparticle immunoassay (Abbott Diagnostics, IL, USA) with intra-assay variations <6.3% and <8.0% respectively. 25-hydroxy-Vitamin D was measured by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) based on an in-house method with an intra-assay variation of <10% (20).

**Dual energy X-ray absorptiometry**

Lumbar spine (LS, L2-L4) and total body (TB) measurements were acquired by a Lunar Prodigy densitometer (GE Medical Systems, Waukesha, Wisconsin, USA) and analyzed using Encore software (Version 13.0). As outlined in previous studies, reference data were used to calculate a predicted and a percentage predicted bone area (BA) for age and sex (21, 22). The reference data allowed for a comparison of the actual bone mineral content (BMC) of the individual with the predicted BMC of a subject of the same sex and bone area from which the percentage predicted BMC (ppBMC), expressed as an SDS (BMC SDS) could be calculated. The coefficient of variation (%CV) calculated on repeated DXA measurement of a phantom was <1% of BMC and the in vivo %CV was <2.5%. DXA was also used to perform thoracolumbar vertebral fracture assessment, as previously described (23).

**MRI Assessment Of Microarchitecture**

MRI images of the proximal tibia with a resolution of 0.2 mm x 0.2 mm x 0.4 mm (slice thickness) were acquired with a 3T-MRI scanner (Siemens MAGNETOM Prisma, Erlangen, Germany) using a transmit/receive extremity coil for the knee as previously described (9, 14). A fully balanced true fast imaging with steady-state free precession (TrueFISP) pulse sequence with the following parameters was used to provide a 3D volume of isotropic resolution: TE=4.69ms, TR=10.83ms, flip angle=60°, no. of averages=15, field of view=100mm x 100mm, matrix=448 x 448, number of slices=20, bandwidth (Hz)=189 and total scan time=10 mins. A low pass filter was applied to the images to correct for any bone marrow inhomogeneity. The images were then coded and analyzed blind using MATLAB (Mathworks Inc, San Mateo, CA, USA) to obtain measures for apparent bone volume to total volume ratio (appBV/TV), apparent trabecular number (appTbN), apparent trabecular thickness (appTbTh) and apparent trabecular separation (appTbSp). Standardized analysis was performed to obtain a mean value from five image slices (Slice 9, 10, 11, 12 and 13 out of the 20 slices) per participant. The images were of sufficiently good quality for analysis in 30 of the 32 cases and in all 26 controls.

**MR spectroscopy (MRS) Assessment Of Bone Marrow Adiposity**
1H-MRS was performed using an 18-channel body array (anterior) and a 32-channel spine coil (posterior). Spectra were obtained from a 20mm × 20mm × 20mm volume within the vertebral body of L3, using a method described previously (9, 14). A Point Resolved Spectroscopy Sequence (PRESS) with no water suppression was used with the following parameters: TE=30ms, TR=2000ms, flip angle=90°, no. of averages=80, bandwidth (Hz)=1200 and total scan time = 2.5 mins. Analysis was performed following fitting of the spectrum in the time domain using a nonlinear least-squares algorithm, AMARES (24) in the java-based MR user interface (jMRUI) software package (25). The areas under the water peak and lipid peak were obtained and used to calculate the lipid-to-water ratio (LWR) and percentage fat fraction (%FF) as a measure of bone marrow adiposity (26). MRS analysis was performed in 29 cases and in 26 control participants.

Sample size calculation and statistical analyses
The primary hypothesis was that children with T1D would have a lower appBV/TV compared to healthy children. The estimated coefficient of variation (CoV) for appBV/TV was less than 5% in our previous work (9, 14) and based on this a 7.5% difference at p<0.05 with a power of 0.8 would require at least 25 subjects in each arm. Data were analyzed using IBM SPSS software (Version 22, SPSS, Chicago, IL, USA) and described as medians (ranges). Group differences between cases and controls were initially compared by Mann-Whitney test for continuous variables and Chi-square test for categorical variables, and then adjusted for multiple testing using false discovery rates (FDR) (27). Specifically, biochemical markers of bone turnover and DXA parameters were compared against 0, using the above tests depending on the variables. Spearman’s rank correlation co-efficient was used to assess the association between variables. Further analysis was performed to compare the characteristics of those children with T1D with a history of fractures with those without fractures. Factors significant on univariate analysis (HbA1c, TB BMC and physical activity score) were included in a multivariate logistic regression for comparison between the fracture and non-fracture groups. All continuous data were presented as median (ranges) with p<0.05 considered as significant, unless otherwise specified.

Results
Clinical characteristics
Thirty-two children with T1D, median (range) age 13.7 years (10.4, 16.7), were recruited (Table 1). The median age at diagnosis was 5.9 years (1.3, 10.8) and median duration of disease was 7.2 years (3.1, 12.4). The median average HbA1c in the preceding 12 months was 8.1% (4.6, 11.3) or 65mmol/mol (27, 100); glycemic control was good in seven, moderate in 19 and poor in six. Of the 32 children, 3 were pre-pubertal, 17 in early puberty (Tanner stages 2 and 3) and 12 in late puberty (Tanner stages 4 and 5). Of the 32, 10 presented with diabetic ketoacidosis (DKA) at the time of diagnosis. The median insulin dose was 1.0unit/kg/day (0.6, 1.8) with 10 out of 32 on continuous subcutaneous insulin infusion (CSII) via pump. Of the 24 children with T1D, 16 had undergone retinopathy screening and 19 had assessment of microalbuminuria. Mild background retinopathy was present in 3 (19%) and microalbuminuria was absent in all.

Bone Metabolism & GH/IGF-1 Axis
Children with T1D had reduced BAP SDS -0.57 (-2.50, 2.10), with median absolute value 59.2mcg/L (14.4, 118.5). Those with poor control had a lower BAP SDS of -0.79 (-2.5, -0.54), compared to 0.50 (-0.64, 2.10) for children with good glycemic control (p=0.009) (Figure 1A). Children with T1D who were in DKA at initial presentation had significantly lower BAP SDS than those who were not acidic (p=0.017) (Figure 1B). Further hospital admissions with DKA subsequent to the initial presentation, however, did not appear to have
an effect on BAP ($p=0.734$) (Figure 1C). A higher BAP SDS was noted in T1D children on CSII compared to those on MDI of insulin (Figure 1D). The children on pump therapy had a lower HbA1c [58mmol/mol (49, 71) vs 68mmol/mol (58,100) in controls; $p=0.005$] and lower daily insulin requirement for weight [0.93 unit/kg/day (0.60, 1.16) vs 1.2 unit/kg/day (0.73,1.81) in controls; $p=0.04$]. The median serum OC and sclerostin in cases were 56.5ng/ml (13.9, 262.8) and 0.22 ng/ml (0.01,0.7) respectively. Serum OC did not show an association to glycemic control, age or duration of diagnosis. Although median CTX SDS was also lower compared to controls ($p<0.001$) (Table 2), it did not show a significant association to glycemic control. However, median CTX SDS ($r=-0.44, p=0.012$) and sclerostin ($r=-0.38, p=0.038$) were inversely associated to age at diagnosis (Figures 1E and 1F). Although serum IGF-1 was not significantly lower in those with T1D [-0.24 (-3.64, 1.48); $p=0.364$], the children had lower ALS SDS [-0.70 (-1.67,0.76; $p<0.001$] and higher IGFFBP3 SDS [1.66 (0.13,3.81); $p<0.001$]. The median 25-OH vitamin D level was 48nmol/L (18, 75) and PTH 3.6mmol/L (1.5, 6.6) in the children with T1D. There were no intergroup differences in 25-OH vitamin D levels in the children with [47nmol/L (25,60)] and without [49nmol/L (18,75)] DKA at initial presentation ($p=0.555$) or the children on continuous subcutaneous insulin infusion (CSII; pump therapy) [49nmol/L (37,60)] compared to those on multiple daily injections (MDI) [47nmol/L (18,75)] of insulin ($p=0.602$).

**Bone Densitometry by DXA**

Median BMC SDS for TB and LS was marginally but significantly lower in the cases than 0 at -0.1 (-1.1, 0.9) and -0.3 (-1.0, 1.8), respectively. TB and LS BMC SDS did not show any correlation to glycemic control, age at diagnosis, disease duration and acidosis at presentation (all $p>0.05$). TB BMC SDS, however, was inversely associated to CTX SDS ($r=-0.5, p=0.003$).

**Bone microarchitecture by MRI**

Comparison of bone microarchitecture variables revealed that appBV/TV and appTbN were significantly lower and appTbSp was higher in cases compared with controls (Table 2). The appBV/TV difference between cases and controls were evident across all ages. In the cases, serum ALS showed an inverse association to appBV/TV ($r=-0.54, p=0.002$), appTbTh ($r=-0.42, p=0.02$) and appTbSp ($r=0.46, p=0.01$); however, removal of two cases with high values of ALS SDS resulted in an absence of this association except for appBV/TV where an inverse association was still present at $r=-0.44$ ($p=0.02$). In the children with T1D, there was no association of bone microarchitecture parameters with HbA1c, age at diagnosis, disease duration, acidosis at presentation or presence of retinopathy. However, daily insulin dose for weight was positively associated to appTbN ($r=0.41, p=0.036$). There was no evidence of an association between any of the bone microarchitecture variables and BMC SDS for TB and LS.

**Bone Marrow Adiposity by MRI**

Median vertebral bone marrow adiposity, expressed as percentage fat fraction (%FF), was higher in cases than controls, but this did not reach statistical significance (Table 2). In the cases, there was no association between %FF and markers of GH/IGF-1 axis, markers of bone turnover, BMC SDS for TB and LS. Combined analysis of the cases and controls showed an inverse association between %FF and appTbN ($r=-0.40, p=0.004$) and a positive association with appTbSp ($r=0.33, p=0.021$) (Figures 2A and 2B).

**Fractures**

Children with T1D were more likely to fracture compared to healthy controls ($p<0.001$) (Table 1). Of the 32 children with T1D, 10 (31%) had a fracture following the diagnosis of T1D (Table 3). None of the children had any abnormalities identified on vertebral fracture assessment by DXA. The clinical characteristics of the fracture and non-fracture group were...
comparable for age, age at diagnosis, gender, puberty, anthropometric measurements, insulin doses and delivery systems (all \( p > 0.05 \)). The median age of diagnosis was 4.4 years (1.3, 10.8) in the fracture group and 6.5 years (3.8, 10.8) in the non-fracture group \( (p = 0.077) \) (Figure 3A). The median disease duration was 8.6 years (3.1,12.4) in the fracture group and 7.0 years (3.1,10.9) in the non-fracture group \( (p=0.204) \). The median HbA1c was higher in the fracture group at 8.7\% (6.6, 11.3) or 72 mmol/mol (49, 100) compared to the non-fracture group of 7.8\% (4.6, 10.1) or 62 mmol/mol (27, 87) \( (p=0.005 \text{ and } p=0.007 \) respectively) (Figure 3B). The children with a history of fracture had a median TB BMC SDS of -0.5 (-1.1, 0.0) and significantly lower than the non-fracture group \( (p<0.001) \) (Figure 3C). Both groups had similar BAP SDS, IGF-1 SDS and CTX SDS. The median physical activity score (1=least, 5=most active) for the fracture and non-fracture groups were not significantly different, with 2.8 (1.7, 4.1) and 2.2 (1.3, 3.7), respectively, after correction for multiple testing (Figure 3D). There was no significant difference in bone microarchitecture or bone marrow adiposity between these fracture groups. Children with fractures had lower TB BMC SDS (adjusted OR 0.002; 95\% CI 0.0 – 0.769, \( p=0.041 \)) after adjusting for HbA1c and physical activity score in multiple logistic regression.

Discussion
This is the first study to look in detail and specifically at the association between T1D disease characteristics, bone microarchitecture and marrow adiposity by MRI and fracture incidence in children. The group of children studied had an overall diabetes control which was better than expected for adolescents \( (28) \) but similar to that described recently in a population based study of skeletal morbidity in young people with T1D \( (6) \). In addition, contrary to other studies of bone health in children and adolescents with T1D \( (29) \), the current cohort were well nourished and were also vitamin D sufficient.

Previous studies on bone turnover status of children and adolescents with T1D have produced variable results \( (30-32) \). Given that these markers vary markedly in growing children, the values for the two commonly studied markers of bone formation and bone resorption, BAP and CTX, were adjusted for age and sex in the current study. The existence of a low bone turnover state, characterised by low circulating levels of both BAP and CTX, is important to highlight in a group of well-controlled children with T1D. This biochemical picture has also been described in young adults with T1D \( (14, 33) \). Whilst, it is possible that this low bone turnover state may also be a reflection of functional growth hormone resistance which may exist in people with diabetes \( (34) \), circulating IGF1 was not particularly low in the current cohort. Given that there was a clear inverse association between bone formation and glycemia and between bone resorption and the age at diagnosis of diabetes, it is possible that the mechanisms that influence bone turnover in T1D are mediated through several pathways including those that may be dependent on chronic hyperglycemia or insulinopenia \( (35) \). For instance, the inverse association between circulating sclerostin and age at diagnosis further supports the notion of a sclerostin-mediated signalling pathway inhibition of bone turnover \( (36) \).

Marked reduction in diabetes complications have been reported in T1D adolescents with a shift towards a more physiologic insulin replacement using continuous subcutaneous insulin infusion (CSII) via pump \( (37) \). The higher levels of BAP in T1D children on CSII compared to those on multiple daily injections (MDI) suggests that this form of replacement may indeed be advantageous for bone health preservation. However, given that this group also had a lower HbA1c could suggest a degree of selection bias. The lower levels of BAP in children who presented in DKA at initial diagnosis is very interesting and suggests the possibility of an additional programming effect of acidosis or sustained hyperglycemia prior to initial presentation on skeletal development. Given that the effects of acidosis on bone homeostasis
may be independent of calcitropic hormones (38) and it is possible that acidosis may have an effect on mesenchymal stem cell differentiation (39), a long lasting effect on bone health that may result from the initial acidic insult deserves further exploration.

In the current study, children with T1D had marginally lower bone mineral density when corrected for size and this is consistent with previous studies demonstrating that children and adolescents with T1D have lower bone mineral density status (40-42). Although bone microarchitecture studies have been performed in T1D and some have reported deficits (43,44) the current study is the first study in children with T1D that has shown a deficit in MRI-based assessment of trabecular bone microarchitecture, a finding which is similar to what has been described before in young women with T1D (14). It is interesting to note that that the two studies (43,44) that have shown a deficit and the one (45) which did not show a deficit examined the tibia and radius, respectively and the possibility of a selective effect on a weight bearing site needs further exploration in adequately powered studies.

The inverse association of bone microarchitecture parameters with bone marrow adiposity in children confirms our previous reports of this finding in young women (46) and not only does this further reinforce the hypothesis that the observed skeletal deficit in T1D may have its origins in a shift of mesenchymal stem cell differentiation towards adipogenesis rather than osteogenesis but it also highlights the unique benefit of MRI based assessment for providing mechanistic insight when studying bone health. Furthermore, the positive association between insulin dose and trabecular number substantiates the anabolic effect of insulin on bone (47, 48). The lack of an association between bone microarchitecture parameters and DXA-based measures of bone density has also been previously noted in young women with T1D (46) and highlights the point that bone microarchitecture assessment provides additional information on bone health that cannot be obtained from bone densitometry. Recent histomorphometric and microCT based studies of trabecular bone microarchitecture have shown an inverse association with HbA1c (49, 50) but this was not observed in the current study or our previous study in young women (9) and perhaps these studies were not sufficiently powered to investigate this association. An association between the deficits in trabecular features and fractures was also not observed and this may also require a larger cohort as well as a study of cortical parameters, as described recently in adults (44). In the previous study performed in young women with T1D, we had reported that bone microarchitectural deficits were more likely in those who had retinopathy (14). However, given the low prevalence of microvascular complications in children (51), the current study was not sufficiently powered to investigate this finding in this age group.

Approximately a third of the current cohort of children with T1D had suffered a radiologically confirmed fracture before the median age of 13.8 years. Fracture rates in children with T1D have rarely been reported but the current rate of fractures was higher than previously reported in children and young people with T1D (28, 29, 51) and the control group. In the UK, approximately one third of children are reported to suffer a fracture before the age of 17 years, with a fracture incidence of 3% in boys and 1.5% in girls at their peak (52). Therefore, it is possible that the control group had a relatively lower incidence of fractures. However, there were some notable differences between the groups of children with T1D who suffered a fracture compared to those who had not suffered a fracture. Children who fractured had lower total body bone mineral density and poorer glycemic control compared to the non-fracture group. Abnormalities in cortical bone mineral density with reduced muscle cross-sectional area have been previously reported in T1D and are consistent with an anabolic bone deficit (53). This is further supported by our finding of significantly lower total body, but not lumbar, bone mineral density in T1D children with fracture. Given that bone microarchitecture at the proximal tibia was not significantly different between the fracture and the non-fracture group, this reinforces the need to study cortical bone
architecture in more detail in this condition. Although the combination of lower bone mineral density, poorer glycemic control and an increased risk of fractures could have been explained by reduced physical activity (54), this confounder was discarded as both groups had comparable levels of physical activity.

In summary, children and adolescents with relatively well controlled T1D are more likely to have a history of fractures and show deficits in trabecular bone microarchitecture, BMD and bone formation. Although, the association of these markers of bone health to clinical markers of T1D and its management require further exploration through prospective clinical trials, we would recommend that assessment of bone health should become routine in all therapeutic trials in the field of diabetes.

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Contribution statement
SCC recruited the cases, performed the study, analyzed the data, wrote the initial manuscript and subsequent revisions. SS performed the DXA scans and revised the manuscript, MM and JM performed the assays and revised the manuscript, JF designed the MRI acquisition parameters and revised the manuscript, JW assisted with analysis and revised the manuscript, KJR assisted with recruitment and revised the manuscript; SFA conceived and designed the study, revised the manuscript and acts as the guarantor.

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Disclosure Summary –
All authors have no conflicts of interest to declare.

References


Figure 1. The relationship between T1D disease characteristics and markers of bone turnover (as assessed by bone formation marker BAP, bone resorption marker CTX and sclerostin). (A) BAP in children with good, moderate and poor glycemic control. [HbA1c categorised as good (HbA1c <7.5% or <58 mmol/mol), moderate (HbA1c 7.5-9.0% or 58-75mmol/mol) or poor (HbA1c >9.0% or >75mmol/mol). Children with poor glycemic control had significantly lower bone formation marker. (B) Children who presented in DKA at the time of T1D diagnosis also had reduced bone formation marker although subsequent number of DKAs (C) did not affect BAP SDS. (D) T1D children on CSII (insulin via pump) had higher BAP SDS than those on MDI (insulin via injection). The relationships between CTX (E) and sclerostin (F) with age at diagnosis of T1D. Children diagnosed younger with T1D had higher bone resorption and sclerostin levels. [BAP bone alkaline phosphatase, CTX c-terminal telopeptide of type 1 collagen, DKA diabetic ketoacidosis, CSII continuous subcutaneous insulin infusion, MDI multiple daily injections, ‡ p not significant post multiple testing]

Figure 2. Relationship between bone marrow adiposity and bone microarchitecture in the whole cohort. Marrow fat fraction (%FF) was inversely associated with appTbN (A) and positively associated with appTbSp (B) [appTbN apparent trabecular number, appTbSp apparent trabecular separation].

Figure 3. Subanalysis of children with T1D depending on fracture status. T1D children with fracture, had a lower age at diagnosis (A), worse glycemic control (as assessed by HbA1c) (B), lower total body bone mineral content for bone area (TB BMC-for-BA) SDS (as assessed by DXA) (C) and tended to have a higher physical activity score (D) which was not significantly different on post multiple testing.

Table 1. Clinical characteristics of the whole cohort, with sub-categories of children with T1D based on their glycemic control as per HbA1c

<table>
<thead>
<tr>
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<th>Whole Cohort</th>
<th>T1D Cases Based on Glycemic control (HbA1c)</th>
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<tbody>
<tr>
<td></td>
<td>T1D (n=32)</td>
<td>Control (n=26)</td>
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<tr>
<td></td>
<td></td>
<td>Good (n=5) Moderate (n=20) Poor (n=6)</td>
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<tr>
<td>Age (years)</td>
<td>13.7 (10.4,16.7)</td>
<td>13.8 (10.2,17.8)</td>
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<td>Gender (M/F)</td>
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<td>13/13</td>
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<tr>
<td>Height SDS</td>
<td>0.3 (1.5,2.5)</td>
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<tr>
<td>Weight SDS</td>
<td>0.8 (1.3,3.2)</td>
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<td>BMI SDS</td>
<td>0.5 (0.6,2.9)</td>
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<td>Age at diagnosis (yr)</td>
<td>5.9 (3.1,10.8)</td>
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<td>Disease duration (yr)</td>
<td>7.2 (3.1,12.4)</td>
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<td>HbA1c average in last 12m</td>
<td>8.1 (4.6,11.3)</td>
<td>7.0 (6.6,7.3)</td>
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<td>HbA1c average in last 12m (mmol/mol)</td>
<td>65 (27.100)</td>
<td>53 (49.56)</td>
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<td>HbA1c at diagnosis (%)</td>
<td>10.7 (7.3,17.2)</td>
<td>9.5 (7.3,9.9)</td>
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<td>HbA1c at diagnosis (mmol/mol)</td>
<td>93 (56,164)</td>
<td>80 (56,85)</td>
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<td>DKA at initial presentation (Y/N) (n=31)</td>
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<td>Insulin dose (unit/kg/day)</td>
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<tr>
<td>25-OH Vitamin D (mmol/L)</td>
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<td>53 (46,60)</td>
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<td>Physical activity score</td>
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<td>Previous fracture(s)(Y/N)</td>
<td>10/22</td>
<td>5/21</td>
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Values are presented as median (range). a n=25, b n=27, c n=24, d n=19
Table 2. MRI-based Measures of Bone Microarchitecture and Vertebral Bone Marrow Adiposity in the whole cohort and subgroup analysis of these parameters in children with T1D with and without fractures post-T1D diagnosis

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<th>Parameters</th>
<th>Whole cohort</th>
<th>T1D Children</th>
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<td>T1D (n=32)</td>
<td>Controls (n=26)</td>
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<td></td>
<td>p</td>
<td>Fracture</td>
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<td>appBV/TV</td>
<td>0.55 (0.47,0.63)</td>
<td>0.59 (0.47,0.63)</td>
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<td></td>
<td>a</td>
<td>c</td>
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<tr>
<td>appTbN (mm⁻¹)</td>
<td>1.67 (1.56,1.93)</td>
<td>1.82 (1.56,1.99)</td>
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<td></td>
<td>b</td>
<td>c</td>
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<tr>
<td>appTbSp (mm)</td>
<td>0.27 (0.21,0.32)</td>
<td>0.24 (0.20,0.33)</td>
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<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>appTbTh (mm)</td>
<td>0.32 (0.27,0.39)</td>
<td>0.32 (0.25,0.38)</td>
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<td>Marrow FF (%)</td>
<td>23.1 (11.0,66.0)</td>
<td>20.0 (8.0,61.1)</td>
</tr>
</tbody>
</table>

Values are presented as median (range). MRI = magnetic resonance imaging, appBV/TV = apparent bone volume to total volume, appTbN = apparent trabecular number, appTbSp = apparent trabecular separation, appTbTh = apparent trabecular thickness. FF = fat fraction. n=30, n=29, n=9, n=21, n=20

Table 3. Age, distribution, sites of fracture and the events preceding in the fracture cohort

<table>
<thead>
<tr>
<th>Age at diagnosis (year)</th>
<th>Sex</th>
<th>Age at fracture (year)</th>
<th>Duration of disease to fracture time (year)</th>
<th>Fracture distribution (UL/LL/Other)</th>
<th>Site of Fracture</th>
<th>Event preceding fracture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3 M</td>
<td>12.3</td>
<td>11.0</td>
<td>LL</td>
<td>Left tibia + metatarsals</td>
<td>Hyperextension injury during football</td>
<td></td>
</tr>
<tr>
<td>1.3.1</td>
<td>11.8</td>
<td>LL</td>
<td>Left calcaneum</td>
<td>Fell off skateboard at low speed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.2</td>
<td>11.9</td>
<td>UL</td>
<td>Left 5° proximal phalange</td>
<td>Hyperextension injury during football</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2 F</td>
<td>4.9</td>
<td>2.7</td>
<td>LL</td>
<td>Right fibula</td>
<td>Fall in park from standing height</td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>4.5</td>
<td>UL</td>
<td>Left suprACLyde</td>
<td>Fell off motorcycle with protective gear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.8 M</td>
<td>5.9</td>
<td>3.1</td>
<td>Other</td>
<td>Right clavicle</td>
<td>Fall in park from standing height</td>
<td></td>
</tr>
<tr>
<td>7.2 M</td>
<td>6.4</td>
<td>2.7</td>
<td>UL</td>
<td>Left radius and ulnar</td>
<td>Fall on outstretched hand</td>
<td></td>
</tr>
<tr>
<td>8.3 M</td>
<td>10.8</td>
<td>6.6</td>
<td>UL</td>
<td>Right radius and ulnar</td>
<td>Fall in school from standing height</td>
<td></td>
</tr>
<tr>
<td>4.5 F</td>
<td>7.2</td>
<td>2.9</td>
<td>UL</td>
<td>Right radius</td>
<td>Trip over schoolbag</td>
<td></td>
</tr>
<tr>
<td>5.0 M</td>
<td>8.3</td>
<td>3.1</td>
<td>UL</td>
<td>Left suprACLyde</td>
<td>Fell onto left side from standing height</td>
<td></td>
</tr>
<tr>
<td>7.2 M</td>
<td>10.3</td>
<td>3.1</td>
<td>UL</td>
<td>Left radius</td>
<td>Slipped and fell during football</td>
<td></td>
</tr>
<tr>
<td>10.3 F</td>
<td>11.5</td>
<td>1.2</td>
<td>UL</td>
<td>Left radius</td>
<td>Hyperextension injury during football</td>
<td></td>
</tr>
<tr>
<td>10.8 M</td>
<td>11.2</td>
<td>0.4</td>
<td>UL</td>
<td>Left scaphoid</td>
<td>Fall on outstretched hand</td>
<td></td>
</tr>
<tr>
<td>11.8</td>
<td>1.0</td>
<td>UL</td>
<td>Left humeral suprACLyde</td>
<td>Fall off bicycle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

UL = upper limb, LL = lower limb
Figure 1

A. BAP SDS vs. HbA1c by category
- Good
- Moderate
- Poor

B. BAP SDS vs. DKA at initial presentation
- Yes
- No

C. BAP SDS vs. DKA since presentation
- No
- Yes

D. BAP SDS vs. Insulin delivery
- MDI
- CSII

E. CTK SDS vs. Age at diagnosis (year)

F. Selenotin (ng/ml) vs. Age at diagnosis (year)

Correlations:
- $r = -0.44, p = 0.012$
- $r = -0.38, p = 0.038$
- $p = 0.009$
- $p = 0.017$
- $p = 0.03$
- $p = 0.009$
- $p = 0.017$
- $p = 0.03$

Note:‡ indicates a significant result.
Figure 2

A

$r, -0.40, p=0.004$

% FF

appTbN (mm$^{-1}$)

B

$r, 0.33, p=0.021$

% FF

appTbSp (mm)
Figure 3

A

Age at diagnosis (year)

Fracture since diagnosis

B

Average HbA1c in last 12 months (mmol/mol)

Fracture since diagnosis

C

T3 BMC for BA SDS

Fracture since diagnosis

D

Physical activity score

Fracture since diagnosis

p = 0.077

Fracture since diagnosis

p = 0.007

Fracture since diagnosis

p = 0.043

Fracture since diagnosis

p = 0.043