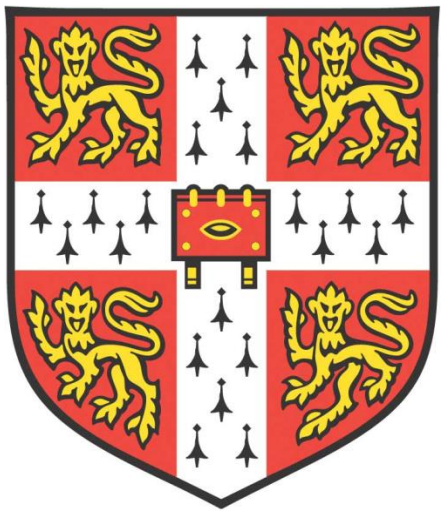
The quantification of pregnancy-induced bone mineral mobilisation in the maternal appendicular skeleton with novel peripheral quantitative computed tomography (pQCT) techniques.



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This dissertation is submitted for the degree of

Doctor of Philosophy

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university or similar institution for any degree, diploma, or other qualification.

In accordance with the degree committee of Biology this thesis does not exceed 60,000 words, excluding bibliography, figure, tables, appendices etc.

Original contribution to knowledge

To my knowledge, this is the first body of work to explore maternal skeletal changes during pregnancy in two discrete populations using novel pQCT imaging techniques. This is the first use of single-slice pQCT in a Sub-Saharan African population during pregnancy (Keneba, The Gambia), and the first application of High Resolution pQCT in pregnancy (Cambridge, UK).

Acknowledgements

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Abstract

The quantification of pregnancy-induced bone mineral mobilisation in the maternal appendicular skeleton with novel peripheral quantitative computed tomography (pQCT) techniques.

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Adaptations in maternal calcium (Ca) economy occur during pregnancy to meet fetal demand and by birth a typical new-born contains 25-30g Ca. At the individual-level if Ca is mobilised it is important to identify where this occurs to identify any potential impact on fracture risk. At present, pregnancy is not considered a risk factor for osteoporosis but data are few. Trabecular bone is more metabolically active than cortical bone in part due to its structure, orientation and much greater surface area. During lactation, studies have consistently reported significant transient bone mineral mobilisation from trabecular-rich axial sites such as the hip and spine, while newer peripheral quantitative computed tomography (pQCT) techniques have found trabecular microarchitectural change in the appendicular skeleton also. My primary objective was to explore whether a similar pregnancy-induced bone mineral mobilisation could be observed in two distinct cohorts of pregnant women using novel pQCT techniques from mid- to late-pregnancy. These techniques provide non-invasive measurements of bone density, mass, geometry, distribution and strength at the radius and tibia.

This thesis aims to determine: whether pregnancy-induced changes occur in a) the trabecular compartment, b) the cortical compartment, c) any potential predictors of a) and b); d) to explore the correlation and agreement of pQCT techniques in-vivo. Single-slice and high-resolution (HR) pQCT data were used to characterise maternal appendicular skeletal change during pregnancy in two studies in contrasting populations: 1) a resource poor subsistence farming Sub-Saharan African community with an habitually low Ca intake and high parity; 2) an affluent Cambridge based population, where Ca intake is higher but parity much lower. In The Gambia, existing pQCT data obtained at 14 and 30 weeks of pregnancy from women (n=811), aged 18-45 and accustomed to low habitual Ca intake, were analysed. In Cambridge,

UK, I designed a study where pregnant women (n=53) and non-pregnant non-lactating (NPNL, n=37) controls aged 30-45 years were scanned with pQCT and HRpQCT at 14 and 36 weeks. These data were modelled to explore the maternal response to pregnancy in these distinct populations.

Contrary to my primary hypothesis there was no evidence of trabecular bone mobilisation in Gambian women, however in contrast in the UK study a -0.5 SD decrease in trabecular vBMD was observed. In The Gambia total vBMD did not change at the radius or tibia, while in Cambridge both techniques detected decreases in total vBMD at the distal tibia. Changes in cortical bone were documented during pregnancy in both populations. In Gambian women small but significant increases in cortical vBMD and BMC were observed at the radius and tibia. In Cambridge at the tibia HRpQCT showed a decrease in cortical vBMD and cortical thickness with an increase in cortical porosity. There were fewer pregnancy-induced changes at the radius but at the cortical- rich diaphysis cortical thickness decreased and endosteal circumference increased. No consistent predictors of these changes were found in either population.

These data from two contrasting populations show a "one size fits all" approach cannot be applied to the maternal skeletal response to pregnancy. In Gambian women with a habitually low Ca intake we have observed the conservation of bone mineral in the appendicular skeleton into the third trimester. In contrast in the Cambridge women evidence of trabecular and cortical mobilisation was observed at the distal tibia. At the radius changes were confined to the cortical-rich proximal radius and supported endosteal resorption during pregnancy. These data suggest that the maternal skeleton is a significant source of fetal Ca in a population accustomed to higher dietary Ca intakes. Further work is needed to determine what this mobilisation means at the individual level and to determine if the mobilised bone mineral is restored postpartum or whether there are lasting consequences for the woman's bone health.

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Abbreviations

aBMD	areal Bone Mineral Density
AD-SOS	Amplitude Dependant Speed Of Sound
AIC	Akaike Information Criterion
ANOVA	Analysis Of Variance
BA	Bone Area
BAP	Bone-Specific Alkaline Phosphate
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
BMI	Body Mass Index
BMP	Bone Matrix Proteins
BMU	Basic Multicellular Unit
BTM	Bone Turnover Marker
BTT	Bone Transmission Time
BUA	Bone Ultrasound Attenuation
BV/TV	Bone Volume/Total Volume
CSA	Cross Sectional Area
CSMI	Cross Sectional Moment Of Inertia
CT	Computer Tomography
CTx	C-Terminal Telopeptide Of Type 1 Collagen
DPA	Dual-Photon X-Ray Absorptiometry
DPD	Deoxypyridinoline
DXA	Dual-Energy X-Ray Absorptiometry
ECM	Extra-Cellular Matrix
ENID	The Early Nutrition And Immune Development Trial
FEFOL	Iron Folate
FFM	Fat Free Mass
FFQ	Food Frequency Questionnaire
FFQ	Food Frequency Questionnaire
FGF23	Fibroblast Growth Factor 23
FM	Fat Mass
FRAX®	Fracture Risk Assessment Tool
HA	Hydroxyapatite
HIC	High Income Countries
HRpQCT	High Resolution Pqct
HRT	Hormone Replacement Therapy
HSA	Hip Structural Analysis
IBD	Irritable Bowel Disease
ICTP	Carboxyterminal Telopeptide Of Type I Collagen
IGF1	Insulin-Related Growth Factor 1
IOF	International Osteoporosis Foundation
IQR	Inter Quartile Range
ISCD	International Society for Clinical Densitometry
IVF	In-Vitro Fertilisation
LMIC	Low And Middle Income Countries
MLM	Multi-Level Model
MMN	Multiple Micronutrients
MRC	Medical Research Council
MSF	Macrophage Colony-Stimulating Factor
MUAC	Mid-Upper Arm Circumference
NOF	National Osteoporosis Foundation
NOS	National Osteoporosis Society
NP	Non-Pregnant
NPNL	Non-Pregnant Non-Lactating
NTx	N-Terminal Telopeptide Of Type 1 Collagen
OC	Osteocalcin
OCP	Osteoclast Precursor Cells
ONS	Office Of National Statistics
OPG	Osteoprotegerin
P1CP	C-Propeptides Of Type I Collagen
P1NP	N-Propeptides Of Type I Collagen
PA	Physical Activity
PABS	Pregnancy And Bone Study
PBM	Peak Bone Mass
PE	Protein Energy
PEMMN	Protein Energy & Multiple Micronutrients
PHV	Peak Height Velocity
pQCT	Peripheral Quantitative Computer Tomography
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone Related Protein
PVE	Partial Volume Effect
PYD	Hydroxyproline
QCT	Quantitative Computer Tomography
QUS	Qualitative Ultrasound
RANK	Receptor Activator Of NF-Kb
RANK-L	Receptor Activator Of NF-Kb Ligand
RNI	Refernce Nutrient Intake
ROI	Region Of Interest
SA-BMC	Size Adjusted BMC
SOS	Speed Of Sound
SXA	Single-Photon X-Ray Absorptiometry
SPA	Single Photon Absorptiometry
SSI	Stress-Strain Index
SSI	Stress-Strain Index
SXA	Single-Energy X-Ray Absorptiometry
TAP	Total Alkaline Phosphate
TBS	Trabecular Bone Score
vBMD	Volumetric Bone Mineral Density
VOI	Volume Of Interest
µCT	Micro Computed Tomography

1 Background and Introduction

1.1 Introduction

Through the lifecourse the skeleton experiences significant change and many factors determine whether healthy bones are maintained at each life-stage and into advanced age. Bone strength in later life depends on optimal skeletal growth, development, and maintenance during childhood, adolescence and adulthood (Ward, 2012). At birth the skeleton contains 25-30 g of calcium, the primary bone forming mineral. Rapid mineral accrual occurs during childhood growth and into puberty, where 30-40% of total bone mass is accrued (Bonjour et al., 1991, Slemenda et al., 1994, Holroyd et al., 2012). Peak bone mass (PBM) is achieved at end of longitudinal growth between the late-second to early-third decade of life (Teegarden et al., 1995). Steady bone remodelling follows, with little net change in mineral content, until midlife when remodelling shifts in favour of resorption beginning a slow gradual decline from PBM (Nordin et al., 1998, Uusi-Rasi et al., 2007). However significant bone mineral loss does not occur in women until the onset of menopause, where large quantities are irreversibly lost. Half of women aged over 50 years will be affected by osteoporosis, a disease of the bones, which occurs when you lose too much bone, make too little bone, or both. Bones become weak and are at risk of fragility fractures i.e. non-traumatic fractures from minor falls (at the hip and distal radius) or in the absence of any insult (vertebral fractures) (NOS, 2014, NOF, 2015). Fractures result in pain, loss of independence, increased morbidity and mortality.

Reproduction can occur across a significant proportion of the lifecourse both before PBM is achieved and until shortly before the mineral losses of menopause. Pregnancy and lactation are not considered risk factors for osteoporosis (Kalkwarf and Specker, 1995). There are few, conflicting, studies concerning maternal skeletal adaptation to pregnancy, while we have well documented evidence of transient mineral mobilisation during lactation. It is known that adaptations in maternal Ca economy occur during gestation to facilitate fetal Ca demand including elevated gastrointestinal

absorption and decreased urinary excretion (Prentice, 2000a). While there is a lack of consensus from maternal biochemistry and bone turnover data, some studies support the possibility of maternal mineral mobilisation occurring in late pregnancy. Currently data are too few from pregnancy to definitively assert that the maternal mineral reserves are a source of fetal Ca, however, if mineral is mobilised from the maternal skeleton and not replaced later there may be lasting implications for future bone health.

Common clinical densitometry techniques have been of limited use in pregnancy for several reasons: a) typical scans of common axial skeletal sites would involve exposing the abdomen to ionising radiation; b) the position of the fetus would obscure measures of the lumbar spine; c) although the appendicular skeleton can be imaged published data have been inconsistent. If bone mineral mobilisation occurs during pregnancy the extent to which mineral is released may vary due to maternal life stage or could be modulated by environmental factors, combinations of which may determine the maternal skeletal response to pregnancy and potentially increase the pressure on maternal mineral reserves. Newer research techniques allow for the measurement of bone compartments where decreases in bone mineral density (BMD) might be observed in late pregnancy with very low ionising radiation exposure.

The aim of this thesis is to investigate the maternal skeletal response to pregnancy using novel imaging techniques to measure the appendicular skeleton in two distinct populations. The first of these populations was located in rural Sub-Saharan Africa, where Ca intakes are low and the diet is of poor quality (Prentice et al., 1993, Prentice, 1994a), data were collected as part of an add-on to a larger pregnancy dietary supplementation trial in Gambian women aged 18-45 years. The second study recruited pregnant and non-pregnant women in Cambridge, UK, where Ca intakes are higher and dietary quality is high, with a focus on women aged 30-45 years.

The primary objective of this thesis was to determine if total and trabecular volumetric bone mineral density (vBMD, see section 1.5) decreased between mid- to late pregnancy at the distal radius and tibia using novel peripheral QCT techniques. These measures were obtained using

conventional single slice peripheral pQCT in both studies, while the Cambridge study also used High Resolution pQCT (HRpQCT) to measure vBMD and trabecular microarchitecture. Both studies also aimed to explore whether any changes occurred in the cortical compartment using pQCT at the proximal radius and tibia to measure cortical vBMD in addition to measures of bone mass, geometry, distribution and strength. HRpQCT allowed for the investigation of cortical porosity and thickness at the distal radius and tibia during pregnancy in Cambridge women.

Here I will outline the structure of this thesis and briefly describe the contents of each chapter. Chapter 1 introduces a brief background to the basic structure and cytology of bone, prefacing a description of the natural history of bone across the lifecourse: the rapid mineral accrual of childhood and adolescence; the attainment of PBM; maturity and reproduction; and the declines of natural ageing, and osteoporosis. This is followed by a brief section covering the heritability of bone mass and its major environmental determinants. Finally I introduce the bone densitometry techniques used to estimate surrogates of bone strength such as BMD in clinical and research settings. Chapter 2 provides a focused literature review of maternal pregnancy-induced skeletal changes through the examination of Ca homeostasis, bone biochemistry, biomarkers of bone turnover, bone histomorphology, and bone densitometry. Here I have collated the available data to provide detailed tables that summarise the evidence base from which I drew my hypotheses and research questions.

Chapter 3 presents the methodology I applied to pQCT data collected across pregnancy in The Gambia, which I was fortunate to have available to me from the Bone add-on to the Early Nutrition and Immune Development Trial (ENID, ISRCTN49285450). Here I detail my approach from initial data auditing of the pQCT data to longitudinal statistical analysis to describe the pattern of maternal bone homeostasis in this unique Sub-Saharan African cohort. Chapter 4 consists of the findings from the statistical analysis of the ENID Bone study pQCT data. My findings are discussed within the context of the scientific literature, and the potential for further exploration within the cohort is considered.

Chapter 5 introduces the Pregnancy and Bone Study (PABS) where I was responsible for the design, ethical approval and management of the study together with all data analysis and interpretation. With the hindsight of the ENID Bone analysis and my hypotheses clear from my literature review, I attempted to optimise my study design to maximise the ability to measure pregnancy-induced changes in the maternal skeleton. To exclude the possibility of observed changes being the result of natural ageing I recruited a group of healthy non- pregnant non-lactating women. This chapter describes the planning of the study, screening and recruitment process, methodology of data collection, and statistical methods applied to the data. Chapter 6 presents the results from PABS with a focus on the bone mineral changes in the pregnant group; the collection of NPNL data allowed me to control for natural age- related changes that may occur during this stage of the lifecourse. My findings of significant pregnancy-induced changes in the between mid- to late-pregnancy are discussed in the context of the scientific literature.

Chapter 7 is a brief technical comparison of the two pQCT (single-slice pQCT and HRpQCT) techniques used in PABS, here I explore between-scanner correlation and agreement. While somewhat aside from the main focus of the thesis I feel it is a useful exercise, which explains the similarities in the results the two techniques in PABS. This work and the results of the PABS chapters highlight that pQCT is as capable as HRpQCT in detecting pregnancy-induced changes in vBMD and cortical thickness outcomes. Chapter 8 discusses my findings from both populations in the context of the scientific literature. Finally supporting documents and supplementary materials are provided in the appendices.

1.2 Bone

Bone is a highly mineralised connective tissue capable of adapting to changes in its biomechanical environment to maintain a bone that is "fit-for-purpose" (Ward, 2012). The key roles of the skeleton are:

1. **Mechanical:** provides a scaffold for the body, together with skeletal muscles it facilitates movement;

2. **Protective:** shields internal organs against external injury;

3. **Metabolic:** supports metabolic processes; hematopoietic processes by red and yellow marrow within the medullary cavity of long bones and interstices of trabecular bone; storage of fat as yellow bone marrow. Storage of minerals such as calcium and phosphorus needed for acid–base balance by absorbing or releasing alkaline salts to maintain ionic homeostasis (Wojnar, 2010, Ward, 2012).

1.2.1 Determinants of bone strength

The major determinants of bone strength are its material properties, bone mass, structural design (shape, geometry, and distribution), and the loading conditions to which it is subjected (ESHRE-CAPRI, 2010, Cointry et al., 2014). Bone mass encompasses all the matter within whole bone including the collagen fibres, mineral crystals, and extracellular fluid. The mineral mass of a bone refers to the amount of mineral within the tissue, this mass or bone mineral content (BMC, g or mg/mm) is the product of the volume and density of the bone (Figures 1.1, 1.2, & 1.3) (Rauch and Schoenau, 2001).

1.2.2 Material properties

Bone's unique material properties result from its composition of both organic (collagen and ground substance) and inorganic phases (hydroxyapatite or HA) (Figures 1.1, Figure 1.2), which allow bone to be stiff yet tough. Stiffness is the mechanical quality of the bone (i.e to withstand strain without permanent deformation), while toughness refers to the energy required to break the bone (Currey, 1984). A healthy bone requires a balance between these two defining attributes to resist damage. When loaded the collagen within the bone can absorb and disperse the energy (elasticity) induced

through loading by deforming; when the load abates the bone can return to its original shape and length (Ward, 2012). The stiffness of bone is determined by the quantity of HA crystals within the collagen matrix. However, a bone can be too stiff, if mineralisation exceeds that required for strength, the bone becomes increasingly brittle. At lower loads than usual it will behave plastically with a reduced ability to deform and absorb the energy from loading leading to the accumulation of micro-fractures (Rauch, 2006). Conversely when stiffness is reduced due to under mineralisation, toughness increases as more strain is placed upon the collagen fibrils which can result in softening of bone tissue. An example of where bones might have a high toughness/low stiffness can be found in children with vitamin D/Ca deficiency rickets. These children have bowed legs due to decreased stiffness (low mineralisation) with "softening" of the skeleton and poor skeletal support but without increased fragility fracture risk (Pettifor and Prentice, 2011).

Figure 1.1 The mineral and organic components of human bone

Mineral component and collagen (organic) component of bone sample compared following the removal of the other in two respective fragments. Source: https://www.slideshare.net/anr3664/skeletal-system-slides

1.2.3 Distribution of mineralised tissue

Bone comprises two distinct compartments (Figure 1.2) the shape, size, spatial distribution and internal organisation of which contribute to the overall strength (Ward et al., 2005c, Laskey et al., 2010, Ward, 2012). Fractures occur when loading exceeds bone strength (Ammann and Rizzoli,

2003, Ward, 2012). However, the properties contributing most significantly to fracture risk depend on the skeletal site (Gordon et al., 2017). Cortical bone provides a compact protective outer layer with a mechanical function, while trabecular bone provides internal structural strength and in part due to its greater surface area is more metabolically active (Fleisch, 1996). In trabecular bone mineralized tissue makes up 10 - 35% of the total tissue volume compared to >90% of the volume of cortical bone (Petit et al., 2005). Cortical bone accounts for about 80% of bone within the mature skeleton (Wojnar, 2010, Seeman, 2013) and consists of osteons centred on extracellular fluid-filled Haversian canals (Figures 1.2) (Seeman, 2013). Haversian canals and the perpendicular Volkmann canals account for most of the cortical porosity in a healthy bone, and form the intra-cortical surface area (Carter et al., 2000).

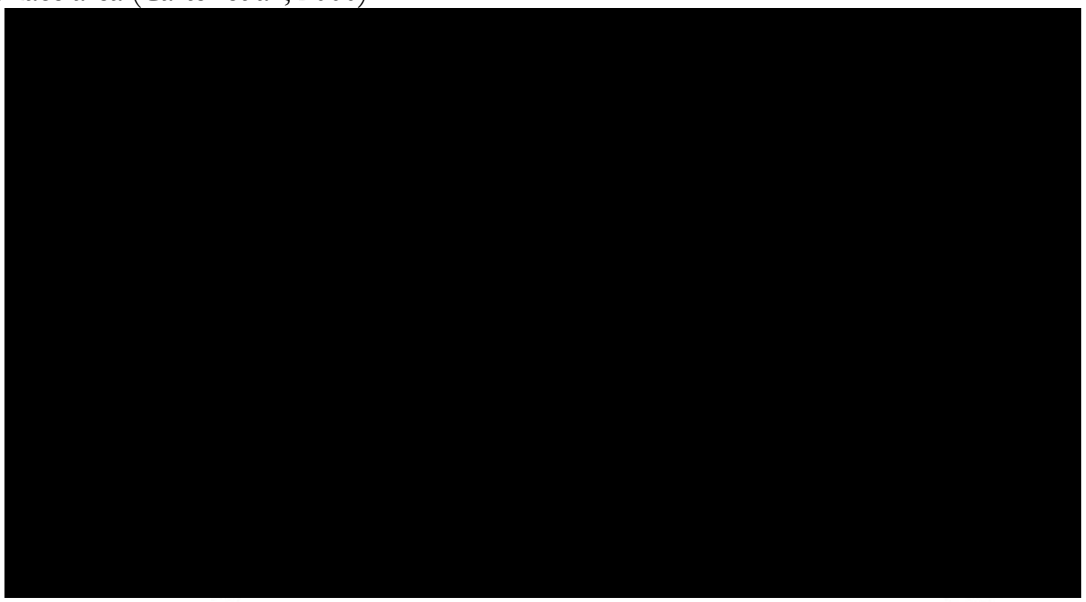

Figure 1.2 The composition of a human long bone.

Diagram of the femur showing both the cortical and trabecular compartments, their distribution and composition from whole bone to the bone ultrastructure. All components pictured, in addition to other factors, ultimately influence bone strength. Image source: Boskey and Coleman (2010)

Trabecular bone accounts for the other 20% of the skeleton and, due to its high metabolic activity, 80% of bone turnover occurs here (Fleisch, 1996). Trabecular bone is primarily located within the medullary cavity at the ends of long bones and vertebral bodies but also occurs through the length of shorter bones (e.g. carpals), in large flat bones (e.g. scapula), and in protuberances such as the muscle attachment sites (Currey, 2012, Seeman, 2013). The distribution, arrangement, and

interconnectivity of this porous cancellous network of trabeculae is vital to trabecular bone strength (Bouxsein and Seeman, 2009). Trabeculae change through the lifecourse; during growth and development they thicken and change orientation, while during ageing they thin and are lost (Section 1.2.4). Adaption to this loss includes the preferential conservation of the most supporting trabeculae to prevent fracture (i.e. in the direction of load).

1.2.4 Geometry and shape

Bone cross-sectional area (CSA) is a major determinant of bone strength. CSA may change in response to growth, ageing, changes in mechanical loading, and general health (Garn et al., 1991, Rantalainen et al., 2013, Warden et al., 2014). Cross-sectional bone strength increases the further from the central axis of the bone that mineral is placed (Schoenau et al., 2001). For two long bones with equal amounts of mineral but differing CSA, their moments of inertia (CSMIs, i.e. resistance to bending) differ greatly (Figure 1.4) because resistance to bending and torsional loading is proportional to CSMI, which is influenced by the distribution of mineral from the central axis. If bone stiffness is reduced, due to low mineralisation, strain may be over-estimated leading to bone mineral being distributed further away from the axis thus increasing CSA through the laying down of mineral on the outer perimeter (i.e. periosteal apposition). Periosteal apposition of mineral would reduce fracture risk as an increase in CSMI improves resistance to bending and torsion. Mineral redistribution within a bone with an unaltered CSA may also confer an increase in bone strength, however, but not as great as that achieved by periosteal apposition (Warden and Fuchs, 2009). Particularly for tubular bones such as the radius maintaining a consistently large cortical CSA at the midshaft through the lifecourse is advantageous as CSA is a major determinant of resistance to bending and breaking strength (Figure 1.4) (Garn et al., 1991)

Figure 1.3 An explanation of bone mineral densities.

The above diagram explains the definitions of the various types of mineral density. BMDmaterial and BMDcompartment (A and B) in trabecular and (C and D) in cortical bone. The mass of mineral (darker) determining BMDmaterial and BMDcompartment is identical (mass 1 = mass 2), but the volume (encircled by blue lines) differs (volume 2 > volume 1). Therefore, BMDmaterial is higher than BMDcompartment. (E) BMDtotal is defined as the mass of mineral divided by the volume enclosed by the periosteal envelope. This definition can be applied to the entire bone, part of the bone (e.g., the distal or proximal end), or a cross section as illustrated above. Source: Rauch and Schoenau (2001)

Figure 1.4 Schematic of long bone cross-sections and the effect of bone geometry and size on measures of bone strength.

Cross-sectional view of two long bone diaphysis: Both have equal cortical thickness and cortical volumetric bone mineral density, but the diameter of the right bone is 50% greater. As a result total CSA of the right bone is more than twice that of the left. Cortical CSA (i.e., the area between the periosteal and endocortical surfaces) and bone mineral content are about 50% higher in the right bone. Section modulus (a parameter reflecting resistance to bending forces) is almost three times higher in the right bone, even though the total vBMD is lower by almost a third. Source: Rauch and Schoenau (2008)

1.2.5 Bone Cells

Cells make up 2-5% of the total volume of bone, they are surrounded in an extracellular matrix (ECM) composed of ground substance and collagen fibres (Sims and Baron, 2000). Ground substance includes proteinaceous components (proteoglycans, matrix proteins, and water) excluding collagen. The remaining volume is composed of non-living material (Heaney, 2014), which can be described as "a fibrous collagen scaffolding reinforced in, around and within its fibres by crystal platelets and needles of calcium hydroxyapatite mineral" (Seeman, 2007). This material component is constructed and maintained by bone cells including: osteoblasts, osteoclasts, osteocytes, and their progenitor cells, each of which have discrete and interconnected roles in the maintenance of a healthy bone.

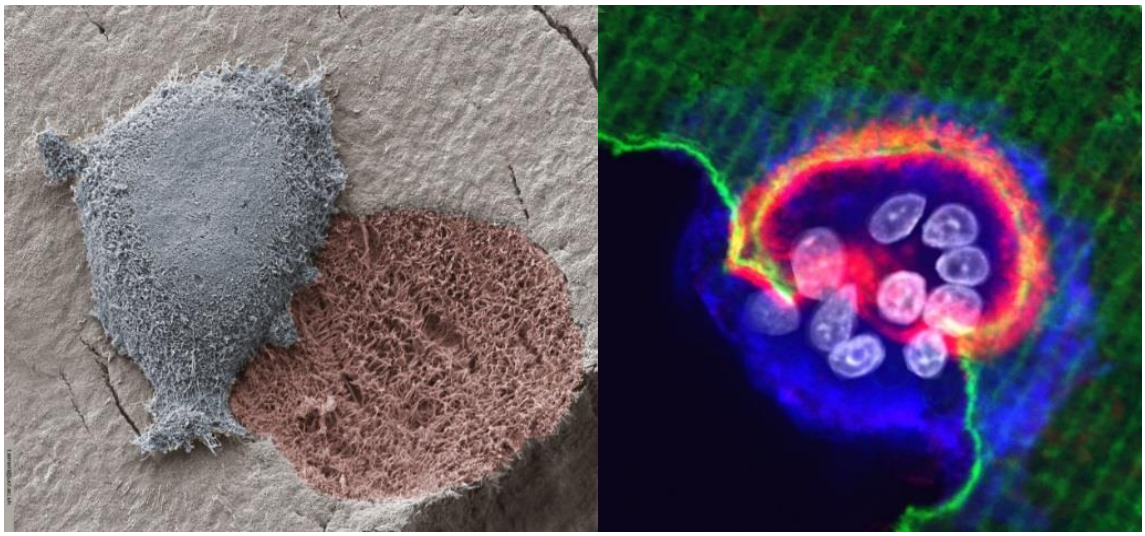

Figure 1.5 Osteoclastic resorption of bone mineral.

Left scanning electron microscope image of osteoclast and resorption pit, Right in vitro resorption. Sources Prof Tim Arnett UCL (left) and Dr Fraser Coxon, University of Aberdeen (right). © Bone Research Society 2015-2018

Osteoclasts are large multinucleated bone-resorbing cells that differentiate from precursors in the myeloid/monocyte lineage and are uniquely capable of resorbing mineralised bone matrix (Miyamoto and Suda, 2003, Bonewald, 2004, Boyce et al., 2009, Raggatt and Partridge, 2010, Bonewald, 2013). Under appropriate conditions osteoclast precursor cells (OCP) are attracted to sites on the bone surface, where they fuse to form multinucleated osteoclasts (Boyce et al., 2009), which primarily resorb bone mineral and create resorption pits (Figure 1.5). They play a regulatory

role in bone turnover by regulating the differentiation of osteoprogenitor cells (Boyce et al., 2009, Cappariello et al., 2014). Osteoblasts are bone- forming cells that differentiate from pluripotent mesenchyme stem cells in the periosteum (Raggatt and Partridge, 2010). They operate in clusters along the bone surface and are found lining unmineralised organic bone matrix (osteoid) prior to its calcification (Sims and Baron, 2000). Osteoblasts produce bone matrix proteins (BMPs), that mineralise osteoid, forming new bone matrix where osteoclasts have resorbed the bone surface. They secrete type I collagen (90% of total bone protein), in addition to calcium binding proteins (e.g. osteocalcin and osteonectin), multiadhesive glycoproteins (e.g. bone sialoproteins I and II, ostepontin and thrombospontin), various proteoglycans and alkaline phosphate (AP). They also express osteoclastogenic factors macrophage colony stimulating factor (M-CSF) and receptor activator of NF-$\varkappa$B ligand (RANKL) for the differentiation of OCP into osteoclasts (Boyce and Xing, 2008, Raggatt and Partridge, 2010). RANKL/OPG expression ratio determines the degree of osteoclast differentiation and function (Zupan et al., 2010). A small number of mature osteoblasts become engulfed within the mineralised osteoid leading to their terminal differentiation into osteocytes (Guo and Bonewald, 2009, Bonewald, 2013). Protein production changes: cytoplasm declines and numerous dendritic processes form (Guo and Bonewald, 2009) and they begin to produce AP and matrix gla protein. Eventually collagen production slows and is followed by osteocalcin (OC) and osteopontin production. Osteocytes inhabit lacunae, fluid filled cavities, deep within the bone where they produce BMPs at a reduced rate. Osteocytes can sense mechanical forces and can instigate bone remodelling through the regulation of osteoclast and osteoblast activity (Figure 1.6).

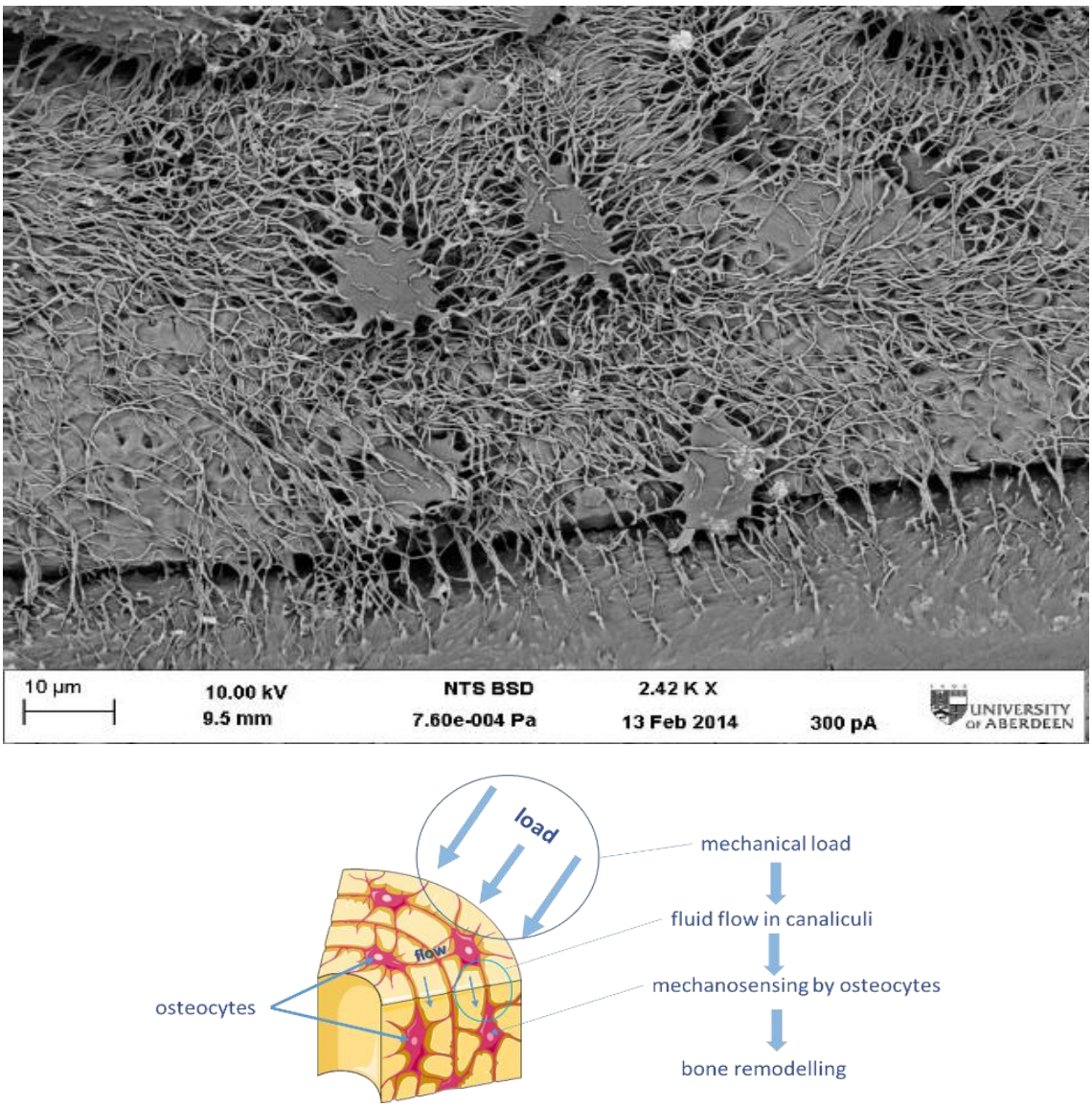

Figure 1.6 The osteocyte a mechanosensing cell.

Electron microscope image of several osteocytes (top) with a schematic overview (bottom) of the role of the osteocyte in mechano-transduction in bone. Left electron scanning microscope image of osteocytes, note the interconnected dendritic links between cells facilitating communication between cells. A schematic diagram of the stages involved in bone mechanotransduction is presented to the right, to which osteocytes are vital. Sources: Left Kevin Mackenzie. © Bone Research Society 2015-2018. The schematic was prepared with the use of images from Servier Medical Art through Creative Commons Attribution 3.0

1.2.6 Bone modelling and remodelling

During adulthood the mature skeleton is continuously renewed and regenerated through remodelling (Figure 1.7), this maintains the integrity of the skeleton by removing old and damaged bone with a high prevalence of fatigue micro-fractures, replacing it with newly formed bone which when fully mineralised will have better mechanical properties (Marie and Kassem, 2011). The basic multicellular unit (BMU) is responsible for resorption and includes osteoclasts, osteoblasts and

their precursor cells (Frost, 1987). Bone-lining cells and osteocytes are also known to contribute to the process. BMUs consist of 9-10 osteoclasts and several 100 osteoblasts and act in microscopic trenches (resorption lacuna) on the surfaces of bone formed by the BMU. These pits are refilled by the osteoblasts which secrete bone matrix into the cavity (Sims and Baron, 2000, Marie and Kassem, 2011). Many factors can modulate bone remodelling including, but not limited to, mechanical strain (Section 1.3.2), cytokines, hormones and growth factors (Frost, 1987, Boyce et al., 2009). Remodelling times can vary but Figure 1.7 provides a schematic over view of the processes of resorption and formation.

Figure 1.7 A schematic overview of bone remodelling.

Bone remodelling and the actions of bone cells in the process. (a & b) Remodelling of bone by a BMU starts with osteoblastic activation of osteoclast differentiation, fusion, and activation. (c) Resorption lacunae are formed, the osteoclast leaves the area, and mono-nucleated cells of uncertain origin "clean up" the organic matrix remnants left by the osteoclast, also possibly forming the cementum line (dotted line) at the bottom of the lacunae. (d) During resorption, coupling factors, including IGF-I and TGF- β, are released from the bone extracellular matrix and contribute to the recruitment and activation of osteoblasts to the resorption lacunae. (e) The osteoblasts fill the lacunae with new bone. Remodelling ends when an equal amount of bone is deposited as was resorbed, and the mineralized extracellular matrix will be covered by osteoid and a one-cell layer of osteoblasts. Adapted from Lerner (2006)

1.3 Bone through the lifecourse

1.3.1 Development and Growth

At birth the skeleton contains 25-30 g of Ca, the majority of this is accrued late in pregnancy when Ca transfer to the fetus peaks (Hosking, 1996, Olausson et al., 2012), within a year this increases to about 90 g (Abrams, 2003). Rapid growth continues from childhood into puberty, almost 90% of adult bone mass is gained in the first two decades of life (Binkley et al., 2008). Peak bone mass (PBM), which is achieved at the end of longitudinal growth (late teens or early twenties), can be regarded as a "bone bank" or mineral reservoir for later life (Figure 1.8) (Bachrach, 2001, Kuh et al., 2013). Failing to reach an optimal PBM is associated with an increased risk of osteopaenia and osteoporosis in later life (Cooper et al., 2006, Zhu and Prince, 2012). Twin studies suggest that genetic factors explain up to 80% of the variability within PBM (Clarke and Khosla, 2010), in contrast a vast array of environmental factors influence the residual variability of PBM and subsequent bone health through the lifecourse, most with relatively modest effects.

Two of the most important environmental factors related to achieving an optimal PBM are adequate Ca intake in childhood and sufficient mechanical loading through physical activity (Ward et al., 2007). Within the skeleton, site- and compartment-specific differences in the timing of when PBM is reached are influenced by sex, maturational timing, and lifestyle factors (Teegarden et al., 1995, Gordon et al., 2017). Pre-pubertal growth follows a pattern of increased growth and mineral acquisition in the appendicular skeleton. However, during the pubertal growth spurt (~10 years in girls) accelerated growth occurs in the axial skeleton (Clarke and Khosla, 2010). During puberty between 30-40% of PBM is gained (Bonjour et al., 1991, Slemenda et al., 1994, Holroyd et al., 2012)

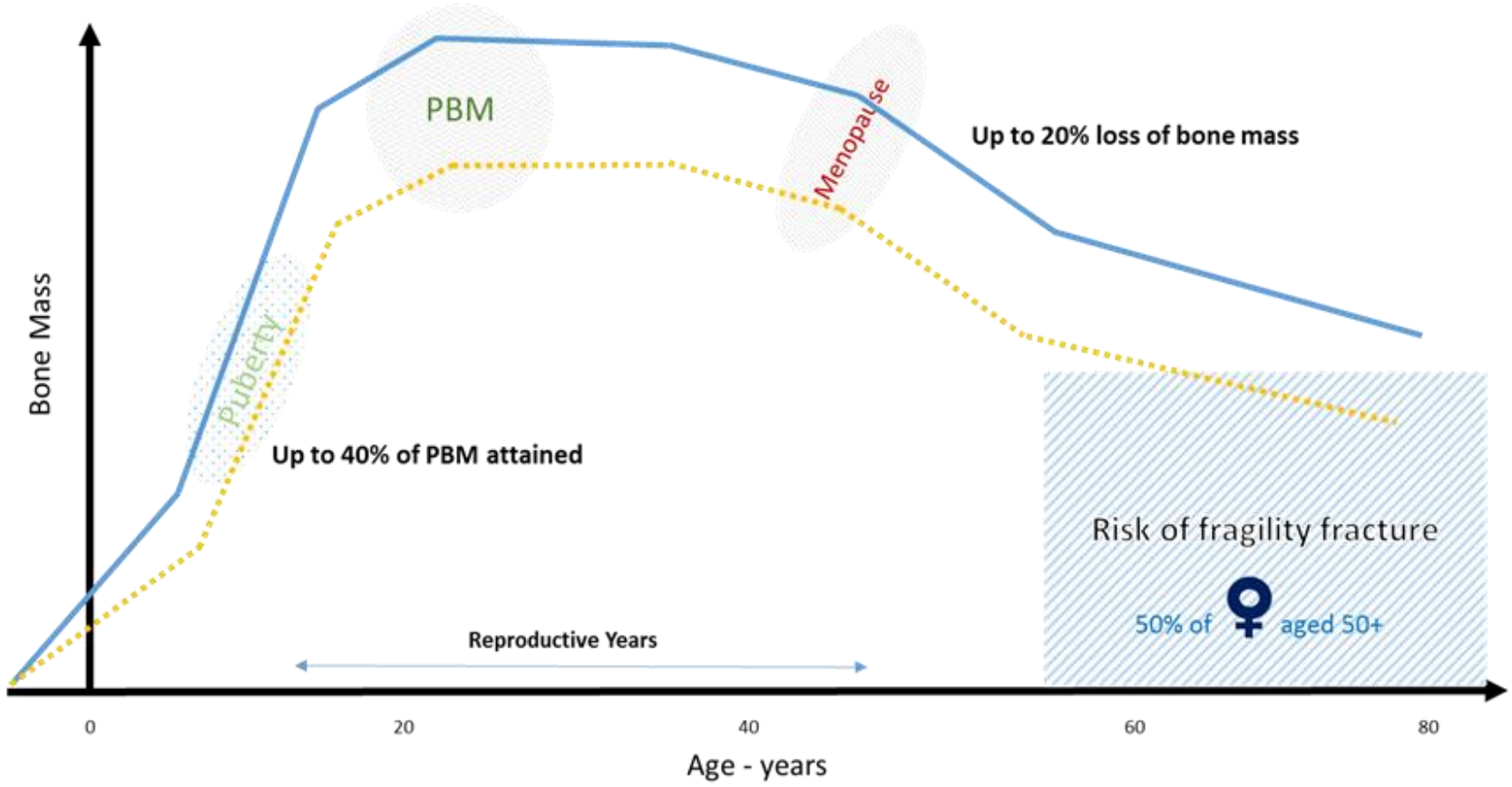

Figure 1.8 Female bone mass across the life course.

Healthy aging is represented by the solid blue line. If a low PBM is attained at the end of longitudinal growth the individual will continue through life with a low bone mass (orange dashed lined), this may put them at risk of fragility fracture in later life. A failure to achieve an adequate PBM can be the result of genetic or environmental factors. Adapted from Ward (2012)

Major pubertal events in females are: 1) the onset of puberty; 2) peak height velocity (PHV); and 3) menarche. The onset of puberty is marked by the development of breast tissue (thelarche), while PHV is the highest height velocity observed during the pubertal growth spurt (Karapanou and Papadimitriou, 2010). The onset of menarche (age of first menstruation) occurs rather late in puberty typically 6 months after PHV. These events are often investigated in the context of PBM attainment and later life BMC however, many such studies are retrospective wih data collected decades after these events. This is particularly true of age of menarche where an earlier age of menarche has been hypothesised as protective for the future bone health (Fox et al., 1993, Tuppurainen et al., 1995, Gerdhem and Obrant, 2004, Ho and Kung, 2005, Chevalley et al., 2008, Kuh et al., 2016a). Longer lifetime exposure to endogenous oestrogens is proposed as a possible mechanism. However, more recent data suggest that a lower PBM may not be the main conduit for increased osteoporosis risk associated with later menarche, because both low peak bone mass and later puberty share genetic determinants (Chevalley et al., 2009).

1.3.2 Adolescent mineral accrual and sexual dimorphism

Three distinct phases of mineral acquisition are documented during adolescence (Baxter- Jones et al., 2003):

1. Increase in height velocity indicates accelerated longitudinal growth
2. Increase in bone CSA
3. Increase in BMC mineralisation of the skeletal envelope during peak mineral accretion

These peak velocities are asynchronous, bone area continues to increase 4 years post peak height velocity, and BMC increases up to 6 years post PHV. During adolescence sex-specific adaptations occur, bone mass increases more rapidly in girls than boys with similar lean body mass (LBM) in addition to sex-specific changes in bone mineralisation and geometry (Rauch et al., 2004) (Figure 1.9), rising oestradiol levels are believed to influence mineral accrual.

Figure 1.9 Schematic overview of changes in cortical bone geometry and distribution with aging by sex in the peripheral skeleton.

Cortical bone through the lifecourse in men (above) and women (below) greater compensatory periosteal apposition in men (highlighted orange) than in women shown with aging. Periosteal apposition increases bone cross sectional area and increases resistance to torsional forces. Source: Seeman (2003)

Stanley Garn and others from the 1970s onwards hypothesised that the additional mineral laid down on the endosteal surfaces (mainly researching the metacarpals) in the female skeleton could form a mineral reserve mobilised during pregnancy and lactation. (Frisancho et al., 1970, Greer and Garn, 1982, Frost, 1987, Schiessl et al., 1998, Schoenau et al., 2001, Ward et al., 2005c). High resolution imaging techniques have also shown that it is a surface from where mineral is lost during the years following the menopause. A 12-year longitudinal HRpQCT study in boys and girls supported sex specific adaptations, finding that accelerated periosteal apposition during adolescence was more evident in boys than girls (Gabel et al., 2015). The authors suggested that rather than endosteal apposition, girls experience diminished endocortical resorption than boys (Gabel et al., 2015). However, these data were obtained from distal long-bone sites (radius and tibia) rather than the midshaft of the bone where Garn and colleagues had hypothesised a mineral reservoir may exist.

1.3.3 Maturity to mid-life

From PBM, remodelling maintains "fit for purpose" bones, new BMUs are formed at a slow but constant rate with an equal amount of BMUs involved in the forming and resorbing of bone (Seeman, 2013). However, during midlife, bone remodelling shifts slowly in favour of bone resorption; this change is referred to as uncoupling. This imbalance of bone formation and resorption sees the volume of bone deposited by BMU decrease and though the volume of bone resorbed also falls, the reduction in the former exceeds the latter, producing a net negative balance (i.e. mineral loss) (Lips et al., 1978, Vedi et al., 1983, Seeman, 2013). Garn et al. (1991) reported that cortical CSA attained by the fourth and fifth decades (in metacarpals II to IV) is highly predictive of cortical CSA two decades later. Newer techniques allow for more detailed exploration of compartmental change from mid-life onwards. Axial and peripheral QCT data (cross sectional and longitudinal) of an age- and sex-stratified US population aged 20–97 years (n=553) reported that until midlife, cortical vBMD remained stable at multiple sites with cortical bone loss beginning in midlife in women, while decreases in trabecular vBMD began earlier in young adulthood and accelerated during perimenopause (Riggs et al., 2004, Riggs et al., 2008).

A 5-year longitudinal pQCT study of Finnish pre-menopausal women (n=79) also reported an annualised reduction of ~0.3% in both trabecular and cortical density (Uusi-Rasi et al., 2007). Data from studies using HRpQCT techniques have added greater detail to our understanding of premenopausal bone mineral maintenance and loss. A study of pre- menopausal and post-menopausal Danish women, reported small but statistically significant yearly increases in total and cortical vBMD in pre-menopausal women at both the distal radius and tibia, while significant increases in cortical thickness and cortical porosity were reported at the tibia only (Shanbhogue et al., 2016). These data may seem at odds with the reductions found with pQCT by Uusi-Rasi but in this study the age range in the premenopausal arm at baseline was much wider (20 years until age at onset of menopause). However another study, reporting longitudinal data for women stratified into 10-year age bands, also reported small yet statistically significant annual increases in cortical

and trabecular vBMD at the distal radius (0.3% and 0.6% respectively per year) in women around mid-life (aged 30-39 years n=23), further changes in cortical structure were observed with changes from baseline in cortical thickness (-0.6%), cortical porosity (-3.7%) and cortical area (-0.6%). At the tibia small changes in cortical thickness (-0.7%), cortical area (-0.6%), and trabecular area (0.2%) were reported in this age band.

1.3.4 The reproductive years: pregnancy and lactation

One of the major sex-specific roles of the skeleton in women is considered to be its function as a source of Ca for the fetus in pregnancy and the infant during lactation. Childbearing years can form a significant portion of the lifecourse and although there are physiological limits to when a woman can conceive, this time frame is wide, close to three decades. Studies suggest that optimal fertility is maintained until 30 years of age and decreases sharply thereafter (Tatone, 2008), however, the proportion of women delaying childbearing beyond 35 years of age has increased greatly in recent decades in high income countries (HIC) (Balasch, 2010); pregnancies >35 years are defined as occurring at advanced maternal age (Harrison et al., 2017). The number of women becoming pregnant at an advanced maternal age is increasing in England and Wales (Figure 1.10, grey and yellow lines) and in 2016 54% of all live births in England and Wales were to women aged 30 years and above (ONS, 2016) (Figure 1.10).

Pregnancy places many physiological demands on a variety of maternal body systems and given that we know changes take place in maternal Ca economy it is not unreasonable to hypothesise some skeletal involvement. At birth the fetus contains up to 30 g of Ca, though the sources of this Ca are not yet fully established (maternal dietary intake, maternal skeletal reserves, etc.). In the context of the maternal skeleton, 30 g Ca is 2-3% of total Ca stores. Limited bone densitometry data at present cannot fully confirm pregnancy-induced changes in the maternal skeleton. However, longitudinal data during lactation have consistently shown transient mobilisation of maternal bone

mineral to satisfy the Ca demands of the infant via breastmilk. Mobilisation has been observed mainly from trabecular-rich axial sites with repletion occurring following weaning (Kalkwarf and Specker, 1995, Krebs et al., 1997, Laskey and Prentice, 1997, Laskey et al., 1998, Laskey and Prentice, 1999, Kalkwarf and Specker, 2002, Holmberg-Marttila et al., 2003). Newer techniques move beyond density and can determine changes in bone microarchitecture (appendicular sites), and add further nuance to existing knowledge by questioning the nature of repletion with suggestions of lasting micro-architectural adaptation (Bjornerem et al., 2017). Should the maternal skeleton be established as a source of fetal Ca, and with further mineral mobilisation in lactation, we need to consider whether full repletion is possible prior to the onset of menopause in women of varying maternal ages.

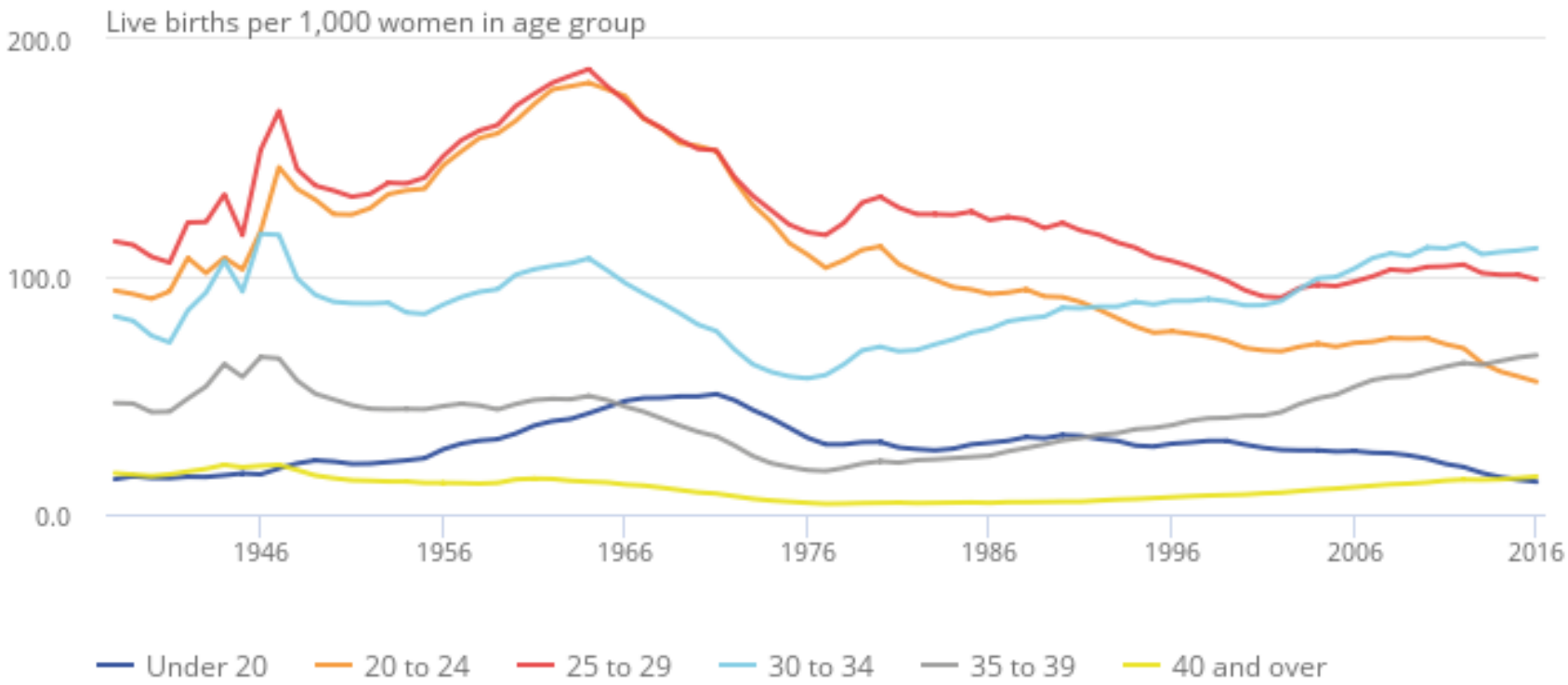

Figure 1.10 Age-specific fertility rates, 1938 to 2016 England and Wales.

These data show the number of live births per 1000 women in each age group, note the increases in bands 30-34 (blue), 35-39 (grey), and 40 and over (yellow). In 2016 54% of all live births were to women aged 30 years and over. Source: Office of National Statistics (2016) available under the Open Government Licence v3.0

1.3.5 Menopause and Ageing

The mean age of natural menopause ranges from 48-52 years in US and European populations (Sowers and La Pietra, 1995). The skeletal changes associated with menopause are complex, multifaceted, and ultimately foreshadow the trajectory of bone strength into advanced age. During the menopause transition bone mineral is lost from both compartments. However, importantly, compartmental mineral loss can differ in magnitude and timing. Also it is vital to consider that decreased bone strength is not just the result of the absolute quantity of mineral lost but also from where within the skeleton mineral is lost e.g. whether from cortical or trabecular sites, or from axial or appendicular, and even from where within the compartment loss occurs such as the endosteal or periosteal surface in the cortical compartment, or rods or plates in the trabecular compartment (Seeman, 2013). An important difference between the compartments is the ratio of their surface area to mineralised bone matrix volume. As the surface area of the trabecular compartment is large relative to its mineralised bone matrix volume (Petit et al., 2005), unbalanced remodelling (net negative) results in thinning, and perforation of these structures (Figure 1.11) (Seeman, 2013). Perforation irreversibly reduces the number of trabeculae and thus the mechanical properties and strength of the bone (van der Linden et al., 2001, Marie and Kassem, 2011, Fonseca et al., 2014).

Age related-deterioration of cortical bone in the female skeleton is not only limited to decreasing density but also cortical micro-architectural alterations; cortical porosity increases and endosteal resorption, without compensatory periosteal apposition reduces the thickness of the cortex (Figure 1.12) (Seeman, 2013, Shanbhogue et al., 2016). Haversian canals may coalesce creating large pores, which at the endosteal surface lead to "trabecularisation" of the cortex (Figure 1.12) (Augat and Schorlemmer, 2006, Zebaze et al., 2010). This makes it difficult to define the transition of the junction between compartments, and can obscure both the age associated increase in cortical porosity and the decrease in trabecular density with QCT based technologies.

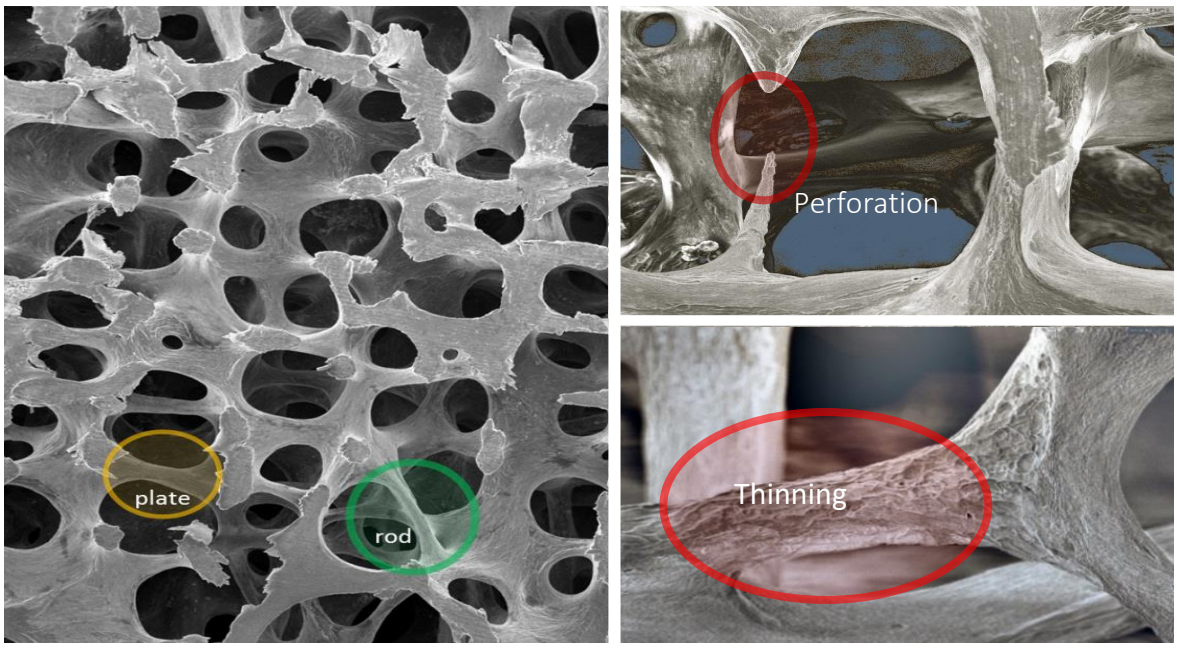

Figure 1.11 Normal trabecular architecture and age associated trabecular thinning and perforation at the 3rd lumbar vertebra.

Left, the 3rd lumbar vertebrae of a 30 year old woman with trabecular rods and plates highlighted. Right trabecular thinning (bottom) and perforation (top) in a 71 year old woman. Source Tim Arnett, University College London. © Bone Research Society 2015-2018

Figure 1.12 Scanning electron micrographs of female anterior subtrochanteric specimens at different ages:

(A) 29-year-old: Pores are regular in shape and evenly distributed in the cortex; (B) 67-year-old: Pores are large, irregularly shaped, and have coalesced in cortex adjacent to the marrow producing cortical remnants; (C) 90-year-old: Trabecularisation of the cortex – large and coalesced pores; (D) 78-year-old woman – extensive intracortical porosity, associated with cortical thinning due to transformation of the inner cortex into a trabecularised structure. Cortical remnants (white arrows); (E) Cortex adjacent to periosteum – enlarged pores without trabecularisation; (F) Preserved endocortical envelope (arrows) suggests that cortical thinning occurred from within the bone; (G) 90-year-old woman – cortex largely porous. Source: Zebaze et al. (2010)

1.3.5.1 The menopause transition

Cross-sectional data from the OFELY cohort in French women aged 31–89 years (n=1,039) compared DXA measured areal BMD from total body, hip, anteroposterior and lateral spine, and forearm scans by age, and the number of years post-menopause. Cross-sectionally, significant trabecular bone loss premenopause at the lateral spine and Ward's triangle was reported, with accelerated bone loss reported during the 10 years following the onset of menopause. Accelerated bone loss continued thereafter at all sites except the anteroposterior (AP) spine, up to 25 years postmenopause (Arlot et al., 1997). When compared to reference data from young healthy adults (T-scores) at various sites they found the percentage of postmenopausal women identified as osteoporotic varied widely depending on site (T score -

1.6 to -3.4) with the greatest bone loss at Ward's triangle and the mid- and distal radius (Arlot et al., 1997).

Figure 1.13 Bone mineral (aBMD) changes across the menopause transition in the axial skeleton.

Annual rate of change in BMD of the lumbar spine and total hip in premenopausal (red bars), early perimenopausal (blue bars), late perimenopausal (yellow bars), and postmenopausal (green bars) women (n = 1902). Rates of change were estimated from multivariable linear mixed models and adjusted for multiple covariates. Error bars represent 95% confidence limits. Comparisons were made across status categories: early peri- vs. premenopausal, P < 0.001 (spine) and P = 0.002 (hip); late peri- vs. early perimenopausal, P < 0.001 (spine) and P < 0.001 (hip); and post- vs. late perimenopausal, P = 0.002 (spine) and P < 0.001 (hip). Source: Finkelstein et al. (2008)

Longitudinal data from SWAN (Study of Women's Health across the Nation) a multi-centre study of the menopause transition in a community-based sample from multiple ethnic groups in the USA, provided further evidence of bone mineral loss across the transition from premenopause to

postmenopause (Finkelstein et al., 2008). Significant annual reductions in areal BMD (aBMD) at the spine and hip showed increasing mineral loss as women progressed from early to late menopause, with further increased mineral loss postmenopause at both sites (Figure 1.13). Changes in the axial skeleton as measured by DXA are clinically relevant but do not provide a compartment specific description of age-related skeletal deterioration.

Figure 1.14 Total and compartmental volumetric bone mineral density across the lifecourse in both sexes

Cross-sectional volumetric bone mineral density at the radius and tibia in men and women aged 20-80 obtained with HR-pQCT Source: Dalzell et al. (2009)

The use of quantitative computed tomography (QCT) can also be used to image the axial skeleton allowing for the assessment of age- and sex-specific changes in vBMD, in addition to bone size, geometry, and structure. Central QCT allows for highly detailed scans to be obtained at sites in the axial skeleton similar to DXA measurement sites (i.e. lumbar spine and femoral neck) but can assess change in the trabecular and cortical compartment. pQCT allow for compartment specific changes in the appendicular skeleton during aging to be documented also. Age-related decline in the axial (QCT: femoral neck and lumbar spine) and peripheral skeleton (pQCT Densiscan: radius) was documented cross-sectionally in both sexes by Riggs and colleagues from midlife into older adulthood reporting that women experienced 37% (men 42%) of their total lifetime trabecular

bone loss before 50 years of age compared with 6% (men 15%) for cortical bone (Riggs et al., 2004, Riggs et al., 2008). Dalzell and colleagues also showed the later decline in cortical bone postmenopause in the appendicular skeleton with newer HR-pQCT (Figure 1.14).

1.3.5.2 Postmenopausal bone loss in the appendicular skeleton

Menopausal bone mineral loss and postmenopausal bone mineral loss differ in both their annualised rate of loss and the sites from where mineral is lost, particularly in the peripheral skeleton where cortical bone loss has been documented with HRpQCT (Zebaze et al., 2010, Seeman, 2013). Shanbhogue et al. (2016) reported postmenopausal women had significant decreases in both trabecular vBMD (radius) and cortical vBMD (radius and tibia), accompanied by increasing cortical porosity at both sites and decreasing cortical thickness at the radius. This was in contrast to men, who had a lower rate of bone loss with advancing age with smaller increases in cortical porosity at both the radius and tibia (Shanbhogue et al., 2016). Burt et al. (2017) found statistically significant annualised changes in postmenopausal women aged 60-69 years at the radius; cortical vBMD (-0.4%), cortical porosity (9.8%), cortical area (-1.1%), and trabecular area (0.4%); and at the tibia total vBMD (-0.4%), cortical vBMD (-0.7%), cortical porosity (6.5%), and trabecular area (0.2%).

Bjornerem et al. (2018) and colleagues documented age related change in 199 monozygotic and 125 dizygotic twin pairs aged 25 to 75 years. Annual increases in tibial cortical porosity differed between women remaining premenopausal (0.44%), transitioning to perimenopause (0.80%), and from perimenopausal to postmenopause (1.40%). Porosity increased in all regions of the cortex. Annual decreases in the ratio of trabecular bone volume to total (BV/TV) also accelerated in the 3 groups 0.17%, 0.26%, and 0.31%, respectively, due to a decrease in trabecular number ($p<0.001$). The greatest mineral loss occurred in women transitioning from peri-menopause to postmenopause and, of this, 80% was cortical. Similar observations were made at the distal radius but despite earlier microarchitecture changes, no significant mineral loss was observed before menopause (Bjornerem et al., 2018).

HRpQCT data of postmenopausal cortical bone loss are in line with epidemiological data showing that fractures occurring in advanced age predominantly occur at cortical-rich peripheral sites, with women having a higher incidence of non-vertebral fractures than men at any given age (Schuit et al., 2004, Zebaze et al., 2010). Data suggests that combining variables derived from HRpQCT may with DXA give useful information regarding fracture risk (Litwic et al., 2018). Research suggests the measures of cortical porosity or thickness parameters are associated with non-vertebral fracture independent of FRAX® and Garvan estimates in postmenopausal women which may help to improve identification of women at risk for fracture (Kral et al., 2017).

The underlying physiology and biochemistry behind these stark changes are complex. However, one of the primary drivers of mineral loss is the abrupt reduction of circulating oestrogen during menopause that has repeatedly been associated with accelerated bone turnover (Seeman and Delmas, 2006, de Villiers, 2009, Marie and Kassem, 2011). The protective role of oestrogen in bone health is apparent during menopause, where deficiency influences bone remodelling, osteoclastogenesis, osteoblastogenesis, osteoclast and osteoblast numbers, as well as bone resorption and formation (Manolagas et al., 2013). Resorption exceeds formation leading to bone mineral loss. Oestrogen is known to act via oestrogen receptors (ERα) (Khosla, 2010) and stimulates osteoclast apoptosis while suppressing osteoblast and osteocyte apoptosis (Tomkinson et al., 1997, Falahati-Nini et al., 2000, Chen et al., 2005, Nakamura et al., 2007, Martin-Millan et al., 2010). In a state of oestrogen sufficiency the rate of bone remodelling is maintained as formation and resorption are balanced. Oestrogen replacement therapy (ORT) alone or as part hormone replacement therapy (HRT) is recognised as protective against bone loss (Cauley et al., 1995, Seeman and Delmas, 2006, de Villiers, 2009). However, HRT was withdrawn as a first line treatment for osteoporosis following findings that indicated a number of serious adverse effects including increased risk of venous thromboembolism (VTE), stroke and breast cancer after five years of HRT (de Villiers, 2009).

1.3.5.3 Osteoporosis – a disease of the ageing skeleton

The cumulative effects of our lifecourse from in utero onwards can determine how our bones will adapt through life and into aging. At the extreme end of age related mineral loss is osteoporosis. This is a disease of the bones, which occurs when too much bone is lost, too little bone is made, or both (NOS, 2014). Bones become weakened due to decreasing mineral mass and micro-architectural deterioration and may break from minor falls resulting in a fragility fracture (NOS, 2014, NOF, 2015). These result in pain, loss of independence, increased morbidity and mortality, and significant healthcare costs. Osteoporosis is estimated to affect 200 million women globally (Kanis, 2007), and in the UK one in two women will suffer from osteoporosis after the age of 50 years (van Staa et al., 2001). Clinically osteoporosis is diagnosed using Dual-energy Photon Absorptiometry (DXA) to compare femoral neck or total hip aBMD to that of a healthy young adult reference population. Despite DXAs role as the clinical gold standard for diagnosis of osteoporosis through the measurement of femoral neck BMD (aBMD) and its use in FRAX® to calculate future fracture risk, clinically not all individuals who fracture have a low BMD or BMC, which are related to fracture risk in populations at high risk of fracture (Marshall et al., 1996). In addition to this there are world- wide differences in age-adjusted fractures (Lau et al., 1990, Johnell et al., 1992, Zebaze and Seeman, 2003, Dhanwal et al., 2011, Curtis et al., 2017).

1.4 Determinants of bone health during the lifecourse

While the previous sections aim to provide a brief overview of bone as we move through the lifecourse, it is obvious that individual's do not all follow the same trajectory from birth to advanced age. Both genetic and environmental determinants modulate our journey across the lifecourse. While PBM has been found to have a strong genetic basis, it can modulated by environmental determinants beginning in utero and continuing throughout the lifecourse. This section will briefly summarise the evidence for the strong heritability of bone (mass) and will subsequently touch on

two of the main environmental factors associated with reaching an optimal PBM: adequate childhood Ca intake and musculoskeletal loading.

1.4.1 Bone and heritability

Studies of families have proved very useful as genetic factors can be identified more easily due to the shared environmental factors. A study by Krall and Dawson-Hughes (1993) followed up 160 adult members of 40 families in the USA using dual-energy (DXA: total body, femoral neck, and lumbar spine) or single-photon absorptiometry (SXA: radius and os calcis) finding in the study population that 46-62% of aBMD variance was hereditable. An Australian study of mother-daughter pairs, including postmenopausal women with osteoporotic compression fractures (n=25) and their premenopausal daughters (n=32) reported evidence for the heritability of reduced bone mass compared to normal controls (Seeman et al., 1989). Mothers with osteoporosis had lower BMC in the lumbar spine, femoral neck, and femoral midshaft (all $p<0.001$) compared to postmenopausal controls but crucially their daughters had statistically significant lower BMC at the lumbar spine and femoral midshaft compared to premenopausal controls. These data suggested that achieving a relatively low PBM rather than excessive loss through aging alone may partly explain osteoporosis (Seeman et al., 1989).

Twin studies have provided the most compelling evidence for genetic variance being the major determinant of BMD and PBM. Pocock et al. (1987) measured lumbar spine and proximal femur aBMD and forearm BMC by dual photon absorptiometry (DPA) in 38 monozygotic and 27 dizygotic Australian twin pairs, mostly female, and reported strong correlation between bone outcomes at spine, proximal femur, and forearm. These data suggest a substantial genetic component in adult bone mass, with further studies confirming these findings with DPA, DXA, and qualitative ultrasonography (QUS)(Christian et al., 1989, Kelly et al., 1993, Flicker et al., 1995,

Seeman et al., 1996, Slemenda et al., 1996, Howard et al., 1998, Knapp et al., 2003, Videman et al., 2007).

More recent studies have benefited from higher resolution imaging and advances in the field of genetics. Yang et al. (2018) in an Australian study of 177 mother-offspring pairs from 162 families measured trabecular and cortical bone outcomes with HRpQCT and Oligogenic Linkage Analysis Routines (SOLAR) software to conduct quantitative genetic analyses. Heritability estimates ranged from 24% to 67% at the radius and from 42% to 74% at the tibia. Most interestingly they found the relationship for most bone geometry measures was significantly stronger in mother-son pairs (n=107) compared with mother-daughter pairs (n=70) ($p<0.05$), whereas the heritability for most vBMD and microarchitecture measures were higher in mother-daughter pairs (Yang et al., 2018). Although genetic determinants influence mineral accrual during growth in both sexes, a failure to achieve an optimal PBM may be very detrimental to later bone health in females. Accumulating evidence suggests that diminished bone accrual in girls is the basis for the lower PBM in young women, a recent review article cited this as a major determinant of the 2- to 4-fold higher incidence of vertebral fractures vs men in later life (Gordon et al., 2017).

1.4.2 Environment and its effect on bone

Professor David Barker initially hypothesised developmental origins of ischaemic heart disease which led to many other adult health outcomes being associated with the earliest stages of the lifecourse (Barker et al., 1989). Strong evidence has accumulated to support developmental origins of bone health in later life and several large epidemiological birth cohorts in the UK such as the Hertfordshire Cohort Study (HCS) and the MRC National Survey of Health and Development (NSHD) have collected longitudinal data at various stages of the lifecourse to explore the determinants of osteoporosis and healthy bone ageing. The HCS cohort demonstrated a positive association between weight at 1-year and aBMD in late adult life (Oliver et al., 2007) in addition to

more recent data demonstrating that muscle strength and function decrease faster than muscle mass with aging and reporting further evidence that changes in bone structure with age differ by sex (Patel et al., 2018). NSHD followed a cohort born in the early post-war period finding higher birth weight, gaining weight and height faster than others, particularly through the prepubertal and postpubertal periods, was positively related to bone strength in late adulthood mainly as a result of greater bone CSA (Kuh et al., 2014). Furthermore the cohort revealed the association between later puberty and lower BMD persists into early old age (Kuh et al., 2016b). More recent prospective British studies such as the Southampton Women's Study (SWS) and the Avon Longitudinal Study of Parents and Children (ALSPAC) have collected data from mothers and their offspring. These studies have demonstrated the necessity of taking a lifecourse approach to bone health and also interactions of our intrauterine environment, early life environmental exposures, and later lifestyle can shape our skeletal phenotype with inter-individual differences observable during growth and still detectable into advanced age (Fall et al., 1998, Godfrey et al., 2001, Backstrom et al., 2005, Javaid et al., 2006, Cole et al., 2009, Dennison et al., 2009, Harvey et al., 2010a, Harvey et al., 2010b, Steer and Tobias, 2011). From in utero to later-life the skeletal phenotype is modulated by its environment, the greatest effects can be found during childhood growth and development which ultimately influence PBM. Two of the most important factors during this period are mechanical loading (via physical activity) of the skeleton and adequate nutrition (primarily Ca intake) (Bonjour et al., 1997, Ward et al., 2005c, Gunter et al., 2008a, Gunter et al., 2008b, Burrows et al., 2009, Gunter et al., 2012, Ward, 2012).

1.4.2.1 Mechanical loading

The mechanostat model is a refinement and expansion of Wolff's law (i.e. bone adapts to mechanical stress) described by its greatest proponent Harold Frost as "the bone itself, plus mechanisms that transform its mechanical usage into appropriate signals, plus other mechanisms that detect those signals and then direct the above three biologic activities" (Frost, 1987). This

feedback system is led by mechanosensing osteocytes detecting strains in the bone's structure by mechanical loading of the skeleton.

Figure 1.15 The mechanostat model: A biomechanical feedback loop

Schematic highlighting external modulators and the regulatory feedback loop. Refined version of Frost's mechanostat functional model of bone development and strength Source: Rizzoli et al. (2014).

Osteocytes regulate osteoblastic and osteoclastic processes to respond as determined principally by regional muscle contractions and impact forces (Frost, 1987, Cointry et al., 2004). Physiological set points act as thresholds for the initiation or inhibition of bone modelling and remodelling (Figure 1.15). With increased strain (i.e. physical activity) the mechanostat may modulate bone strength in several ways to distribute the mechanical strain over a larger area throughout the bone by 1) increasing BMC, 2) widening the bone via periosteal apposition, and 3) increasing trabecular thickness, improved trabecular connectivity (Warden and Fuchs, 2009, Ward, 2012). It has long been established that the opposite also holds true as is demonstrated with prolonged immobilisation (Uhthoff and Jaworski, 1978) where strain decreases there is: 1) increasing cortical porosity, 2) endosteal resorption, and 3) trabecular thinning, and a deterioration of trabecular connectivity (Lee et al., 1997, Modlesky et al., 2004).

Much research, including randomised controlled trials (RCTs) and longitudinal cohorts, has focused on the muscle-bone unit during childhood development and adolescence, with weight

bearing exercise, which appears to enhance bone mineral accrual in children (especially in early puberty), though it is difficult to define an optimal exercise programme (Hind and Burrows, 2007). Tan et al. (2014) in a systemic review of activity PA interventions and observational studies, including organised sports participation studies with child/adolescent bone strength as the main outcome, concluded that pre-puberty and peri-puberty may be the most opportune time in both sexes to enhance bone strength through PA. The authors also highlighted a lack of data in more mature groups, and that few studies were able to discern the specific contribution of muscle function to bone strength (Tan et al., 2014). Conversely where there is disuse of the musculoskeletal system, the role of mechanical loading as a determinant of skeletal size, shape and mass has been also been observed, in children with osteopenia due to immobilisation, adequate nutrition cannot reverse abnormal bone shape and thin cortices (Ward et al., 2007). Non-mechanical factors can modulate the mechanostat such as nutritional deficiencies, hormonal imbalance, medication, and lifestyle choices (Figure 1.15) (Frost, 1987, Schoenau and Fricke, 2008, Ward, 2012).

1.4.2.2 Nutrition and bone health

Dietary factors associated with bone strength and longitudinal growth include bone-forming minerals (Ca, P, Mn, and Zn), vitamins (D, K, and C involved in Ca–P homoeostasis and/or bone metabolism), energy, amino acids, and ions (e.g. Cu, Mn, CO3 and citrate) (Prentice and Bates, 1994, Ward, 2012). Nutrients can alter the bone matrix and its composition (i.e. Ca, P, vitamins; D, C and K), while deficiencies in single nutrients or food groups can lead to inadequate bone development and poor longitudinal growth (e.g. protein, Ca and Zn) (Prentice, 2004, Ward, 2012). Some nutrients (e.g. B vitamin complex) indirectly influence bone health regulating the metabolism of other key nutrients that play a more direct role in bone health (Dai and Koh, 2015). In addition to deficiencies or malnutrition, chronic illnesses affecting the absorption of nutrients along the gastro-intestinal tract such as inflammatory bowel disease (IBD) or coeliac disease may be associated with abnormal bone mineral accrual or deficits in bone microarchitecture (Stein et al., 2015, Zanchetta et al., 2015, Haschka et al., 2016). Conditions that alter nutrient intake such as

anorexia nervosa are associated with decreased fat, muscle, and bone mass (Faje and Klibanski, 2012). While increased body mass was once considered protective against fractures, growing evidence is showing that obesity has no protective effect and may be associated with fractures in growing children (Seeley et al., 2014) and fragility fractures in later life (Leslie et al., 2014).

1.4.2.3 The importance of calcium as a bone forming mineral

Adequate Ca intake is required for optimal bone health across the lifecourse but the ability to absorb Ca depends on endogenous factors including the efficiency of absorption in the gut and gastric acid production, and exogenous factors including vitamin D status and the consumption of dietary components that enhance or inhibit Ca absorption (Kerstetter et al., 2005, Wigertz et al., 2005). Active Ca transport is vitamin D dependent intestinal absorption of Ca is facilitated by mediating active Ca transport across the intestinal mucosa (Heaney, 2008). Active Ca transport involves; the synthesis of the Ca transport protein calbindin, which shuttles Ca from the brush border across to the basolateral side of the mucosal cell (Heaney, 2008). It is beyond the scope of this background chapter to delve into the intricacies of population Ca requirements but it must be noted that there is a paradox that countries with the highest Ca intakes (and requirements) have the highest rates of fracture (Dhanwal et al., 2011, Warensjö et al., 2011). Some have suggested that a relationship with other-macro or micronutrients of this may help to explain the apparently higher Ca requirement of HIC vs LMIC (Nordin et al., 1998). Studies have suggested a positive correlation between dietary protein and urinary Ca (Linkswiler et al., 1981, Nordin et al., 1998), for 40 g of animal protein (representing ~ 800 mg (26 mmol) of elemental phosphorus) ~ 40 mg (1 mmol) of Ca is lost in the urine (Nordin et al., 1998), though there are few data in LMIC. How this influences Ca requirements depends on concurrent Ca intake due to the curvilinear relationship between Ca ingestion and Ca absorption (Nordin et al., 1998). In addition sodium (Na) and Ca excretion are also strongly positively correlated (Nordin et al., 1998), for every 2.3 g (100 mmol) of Na ingested, 40 mg (1 mmol) Ca is lost in the urine (Nordin and Polley, 1987, Nordin et al., 1993).

Globally Ca intakes vary widely and are particularly low in populations where milk and dairy products are not major dietary components, which may be due to the local unavailability (Prentice et al., 1993, Prentice, 2002, Prentice, 2004, Prentice, 2011), a lack of regional food fortification, or lactose intolerance (Erinoso et al., 1992, Heaney, 2013). In countries with populations primarily of European ancestry Ca rich foods of dairy origin are consumed throughout childhood into adulthood, while in other parts of the world, a mix of low bioavailability, no traditional place in the diet, and to a lesser extent lactose maldigestion mean little to no dairy is consumed with the notable exception of some fermented products (Scrimshaw and Murray, 1988, Wittenberg and Moosa, 1990, Erinoso et al., 1992).

In HIC, Ca is a threshold nutrient and adult bone mass has a linear relationship with intake until a physiological threshold is reached, however in these replete populations, once reached the two are unrelated meaning that the skeletal response will occur when Ca intake is increased from deficiency levels to a threshold zone (Ilich and Kerstetter, 2000) and that increasing intake when the level of dietary Ca already exceeds the threshold will most likely not produce further gains in bone mass (Ilich and Kerstetter, 2000). UK Ca dietary requirements were set by Committee on Medical Aspects of Food Policy (COMA) in 1991 (COMA, 1991) with a further report of Ca, Vitamin D and Bone health was published by COMA in 1998 (COMA, 1998).

Given the importance of Ca intake for skeletal development, much research has focused on the adequacy of Ca intakes and the effects of supplementation during periods of growth. Despite the evidence of Ca intake being important for bones evidence from trials is less conclusive. In a RCT of UK children (pre-pubertal gymnasts) meeting their Ca RNI (Reference Nutrient Intake) no additional benefit of Ca supplementation was found (Ward et al., 2007). A large US multi-centre 6-year longitudinal assessment of Ca intake on bone in (n=1743), reported that dietary Ca had a positive effect on bone accrual at the lumbar spine in non-black females only, while no effect was found in other ethnic groups or males (Lappe et al., 2015). To control for genetic variability in

aBMD another study recruited twin pairs (monozygotic n=27, and dizygotic n=24) of premenarcheal Australian females into a randomized, single- blind, placebo-controlled trial with one twin of each pair receiving 1200 mg of CaCO3 (Caltrate) over a 24 month study period (Cameron et al., 2004). Adjusted total body BMC was higher vs placebo at all points to 24 mo ($p<0.001$). Supplementation was associated with increased aBMD adjusted for age, height, and weight at the total hip up to 18 mo (2.4%), lumbar spine 12 mo (1.0%), and femoral neck up to 6 mo (1.9%) (all $p<0.05$). While supplementation appeared to be effective in increasing regional aBMD in the first 12-18 months these gains were not maintained to 24 months of supplementation (Cameron et al., 2004). There is conflicting evidence whether increased Ca intakes via supplementation translate into sustained benefits for skeletal health, particularly in non-white populations and especially those with habitually low Ca intakes (Ward et al., 2007, Lambert et al., 2008, Jarjou et al., 2010, Sawo et al., 2013). In Gambian children increasing Ca intake from 300 to 700 mg/d increased BMC and size adjusted BMD, but not bone width (possible endosteal rather than periosteal apposition) (Dibba et al., 2000). At one year follow up, differences in size adjusted BMC remained despite normalisation of bone turnover (Dibba et al., 2002, Prentice et al., 2012). By young adulthood, no positive lasting effects of supplementation were observed, the amount of bone accrued (mineral or size) or the rate of bone growth did not differ compared to controls (Ward et al., 2014).

Possible mechanisms for the lack of sustained bone changes following Ca supplementation may be: 1) A remodelling transient (Heaney, 1994, Heaney et al., 1997, Parfitt, 2004); through a slowing of bone turnover due to increased Ca results in fewer bone sites undergoing resorption and more mineralised/mature bone being measured temporarily increasing BMD/BMC. When normal turnover resumes, an increased number of bone sites undergo resorption and the less mineralised immature bone would result in a lower BMD/BMC (Prentice et al., 2006); 2) Extra Ca is deposited on the endosteal surface, however, once the source of Ca is removed the effects are lost (Specker et al., 2004). Apposition on periosteal rather than endosteal surface would confer a greater bone

strength; 3) Method of supplementation; the maintenance of sustained bone changes have been observed where dairy sources of Ca were used (Specker, 1996, Cadogan et al., 1997, Eastell and Lambert, 2002, Prentice et al., 2006), however, these contain growth factors such as IGF-1 and protein so observed intervention effects might be due to increases in intakes of all of these factors (Prentice et al., 2006, Ward, 2012).

1.5 Bone densitometry and the estimation of bone strength and quality

The aim of bone densitometry is to measure parameters that contribute to bone strength, to predict risk of low trauma fractures, to diagnose osteoporosis, and to differentially assess effects of growth, aging, disease, and treatment on these parameters (Baim et al., 2008). It is not possible to directly measure resistance to fracture load in-vivo as a measure of ultimate failure load would be required. Non-invasive X-ray based techniques measure the attenuation of X-ray beams of known energy through the body with one or multiple detectors measuring the beams' energy loss on the opposite side. Figure 1.26 and Table 1.4 at the end of this chapter summarise the most common research techniques and their strengths and limitations. Tissues of varying densities such as bone, muscle, and fat cause the beam to attenuate to greater or lesser extents. Denser tissues such as bone lead to greater attenuation because they contain heavier elements such as calcium and phosphorus (Petit et al., 2005). In contrast soft tissues contain lighter elements, such as carbon, hydrogen, oxygen, and nitrogen, which cause little attenuation compared to bone (Petit et al., 2005). In the following sections methods to assess and estimate bone mineral density, mass, geometry, distribution, and strength will be described.

1.5.1 Dual-Energy X-Ray Absorptiometry (DXA)

Dual Energy X-Ray Absorptiometry (DXA, Figure 1.16) evaluates a 2D projection of a 3D object and as such is unable to measure the volume itself provides but provides an areal measure of bone

mineral density (aBMD, g/cm^2). Two X-ray beams of differing photon energies are used to separate mineralised tissues (bone) from soft tissues (fat and muscle), low energy photons are attenuated by the fat and fat-free mass surrounding the bone whilst the high energy photons are attenuated by both bone and soft tissue. Each picture element (i.e. pixel) has a linear attenuation coefficient. The value of a single pixel is the sum of the mineral mass of the bone material between the X-ray source and the detector. The unit of this is expressed in g/cm^2 of hydroxyapatite e.g. a pixel value of 0.5 g/cm^2 is the attenuation equivalent to a layer of 0.5 g of HA over a 1 cm x 1 cm area (Petit et al., 2005). DXA cannot evaluate the distribution of bone mass within the bone envelope and is therefore size dependant i.e. for two bones of equal physical density but of different size the larger bone will be projected as denser than the smaller bone (Figure 1.17). DXA can only estimate cortical aBMD at sites where little trabecular bone is present, e.g. midshaft of the femur and radius.

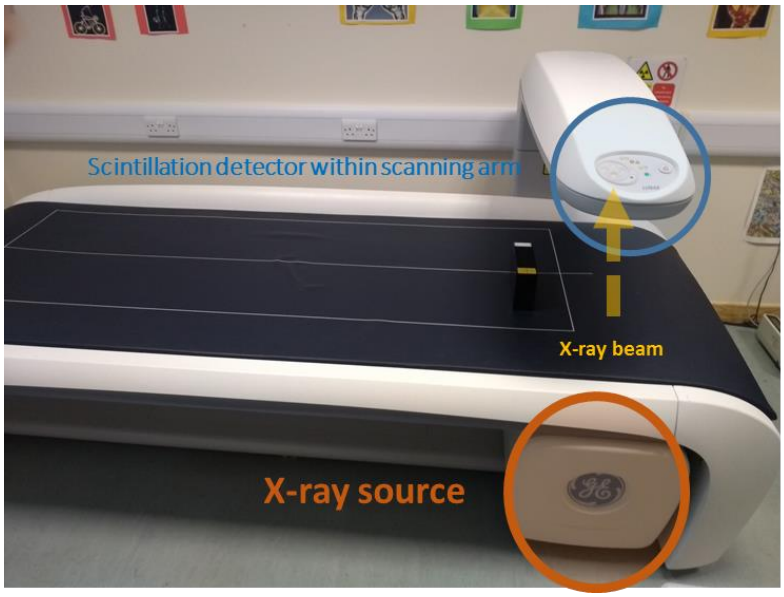

Figure 1.16 Dual-energy X-ray DXA scanner

The location of x-ray source is highlighted in orange, with the yellow arrow showing the path of the beam to the scintillation detector located within the scanning arm.

During scanning the scanner arm sweeps over the region of interest, with the X-ray beam passing through the subject in a posterior-anterior direction projected upwards to the scintillation detector, the collimator directs the X-ray beams in the direction of the participant reducing radiation scatter and improving image resolution. Early devices used highly collimated beam of X-rays (pencil beams), with a single detector that moved in a raster pattern (thin parallel lines across the subject).

Older pencil beam systems produced an image with minimal magnification but with long scan times (whole body 10-20 mins) (Cullum et al., 1989, Mazess et al., 1989). Newer techniques use fan (linear array detector) beams (Boudousq et al., 2003). Fan beam systems use a slit collimator to generate a beam that diverges in two directions with a linear array of detectors (Boudousq et al., 2003).

Total aBMD can be obtained for the whole body and particular regions of interest in axial and peripheral skeleton (Table 1.1)(Ward, 2012). DXA measurements of aBMD and BMC have been shown to be related to fracture risk in populations at high risk of fracture (Marshall et al., 1996, Goulding et al., 2000, Clark et al., 2006). Clinically osteoporosis is defined as a DXA T- score of -2.5 SD below the average score of young healthy adults in the reference population. Osteopaenia is diagnosed as a T-score of between -1 and -2.5 SD compared to peak bone mass (Kanis et al., 1994).

Figure 1.17 A comparison of physical density vs areal bone mineral density (aBMD).

Both bones have the same physical density, however, the larger bone appears denser when projected on to a screen. The reason is that it absorbs more radiation because of the longer path length of the radiation beam through the bone. Source: Rauch and Schoenau (2002)

aBMD is used as a predictor of fracture risk and osteoporosis within the FRAX® tool. More recently other DXA derived parameters such as Trabecular Bone Scone (TBS) have also been included in FRAX as it may improve FRAX® prediction accuracy for major osteoporotic fractures (Iki et al., 2015). Despite the role of DXA as the clinical gold standard for the measurement of BMD, clinically not all who fracture have a low aBMD or BMC, studies have revealed that in some populations i.e. the obese, those who fracture do not have a low aBMD (Compston, 2013, Leslie

et al., 2014, Premaor et al., 2014). Also not all those with a low aBMD or BMC fracture (Schuit et al., 2004). Research in females from The Gambia and China found that though low in comparison with an age-matched UK population, these populations are known to have lower fracture risk (Aspray et al., 1996, Yan et al., 2004). Although aBMD accounts for approximately 60–70% of bone strength (Ammann and Rizzoli, 2003) more than half of fragility fractures occur at a T-Score not classified as osteoporotic (Schuit et al., 2004).

Although DXA measures of aBMD or BMC are strong predictors of fracture risk in older people and children in populations at high risk of osteoporosis they may be of limited value in different populations and at different stages of the lifecourse (Prentice et al., 2006). While BMC provides information about the amount of mineral within the measured area of the skeleton, bone strength is also determined by factors that DXA cannot measure (i.e. bone shape, geometry, and loading conditions) (Slemenda et al., 1996, Currey, 1999, Schoenau and Fricke, 2008, Nikander et al., 2010). This view is supported by the International Society for Clinical Densitometry (ISCD) which has stated that the inclusion and measurement of additional parameters that, together with aBMD may better account for bone strength improve fracture predication (Simonelli et al., 2008, Zemel et al., 2008). Also there are issues in large or obese subjects as the lean and fat tissues have a higher X-ray attenuation than in a thin subject leading to an over estimation of aBMD and bone size due to the large projected area, this may be accompanied by poor image quality with blurred bone edges (Brownbill and Ilich, 2005, Silver et al., 2010).

Table 1.1 Common bone parameters that can be assessed by Dual-Energy X-ray Absorptiometry (DXA)

Bone mineral content (BMC, g/cm)	Mineral mass component of bone (hydroxyapatite), does not include the mass of bone's organic components
Bone area (BA, cm^2)	Projected area of the bone onto the image plane
Areal bone mineral density (aBMD, g/cm^2)	The mineral mass of bone per unit image area

1.5.2 Quantitative Computed Tomography (QCT)

Quantitative Computed Tomography (QCT) is a 3D technology that provides detail beyond DXA measured BMD or BMC (Engelke et al., 2008), that are size-independent measures of volumetric

BMD (vBMD) (g/cm^3) for both trabecular and cortical compartments (Ferretti, 1999). The accuracy of measurement by standard clinical QCT methods are limited by resolution of the technique (Ward et al., 2005a), and while microcomputed tomography (μCT) is the gold standard for bone microstructure, it is invasive requiring a bone biopsy (Krause et al., 2014). In contrast QCT is relatively non-invasive but is expensive, time intensive, and involves a higher exposure to ionising radiation than DXA.

1.5.3 Peripheral QCT

pQCT scanners allow for the non-invasive measurement of parameters of bone strength in the appendicular skeleton (Table 1.2) (Grampp et al., 1997, Laib et al., 1997, Ferretti, 1999) and involve low exposure to ionising radiation with quicker scan durations than clinical axial QCT as they only measure at peripheral sites. Single-slice conventional pQCT scanners operate on a rotate translate basis, during scanning, the forearm or lower-leg is placed into the gantry and the frame rotates the X-ray tube and detector around the limb. A fan beam generates a 2D cross-sectional CT slice (1-2 mm thick) of the limb with a resolution of 0.2 – 0.8 mm (Ulrich et al., 1999), measures are based on linear X-ray absorption coefficients of the mineralised and unmineralised tissues. This attenuation can be expressed using the Hounsfield scale which is a linear transformation of the original linear attenuation coefficient measurement. The Hounsfield scale is defined as the attenuation value of the X-ray beam in a given voxel, minus the attenuation of water, divided by the attenuation of water, multiplied by 1000 (Kamalian et al., 2016). While pQCT resolution is far poorer than that of ex-vivo μCT systems and newer *in-vivo* HRpQCT scanners, it offers a non-invasive relatively inexpensive way to provide useful information in addition to BMD such as indices of bone strength and geometry (Cointry et al., 2004, Capozza et al., 2010, Cointry et al., 2014).

In addition, to mineralised tissue (material), trabecular and cortical bone contain unmineralised soft tissues (Ferretti, 1999, Ward et al., 2005a). pQCT estimates only the mineral portion of bone and does not account for the material properties determined by the unmeasured collagen (Petit et al., 2005) (Figure 1.3). As vBMD represents the amount of mineral averaged over the volume, and analysed regions also include unmineralised soft tissue, the tissue BMD is always lower than the material BMD. In the trabecular compartment, vBMD is mainly determined by the thickness of trabeculae and the average spacing between them, although it is also influenced by trabecular tissue mineralization (Petit et al., 2005). In addition as the trabecular compartment contains bone marrow, unlike the cortical compartment, trabecular vBMD is lower than cortical vBMD, despite having a similar, though not identical, material BMD (Petit et al., 2005). Cortical vBMD is determined by the number and average size of osteonal canals (porosity) and the mineralised tissue density (Figures 1.18 & 1.19). At cortical-rich sites the resolution of single-slice pQCT is not high enough to exclude small pores, making it difficult to distinguish between differences in tissue porosity and mineralization.

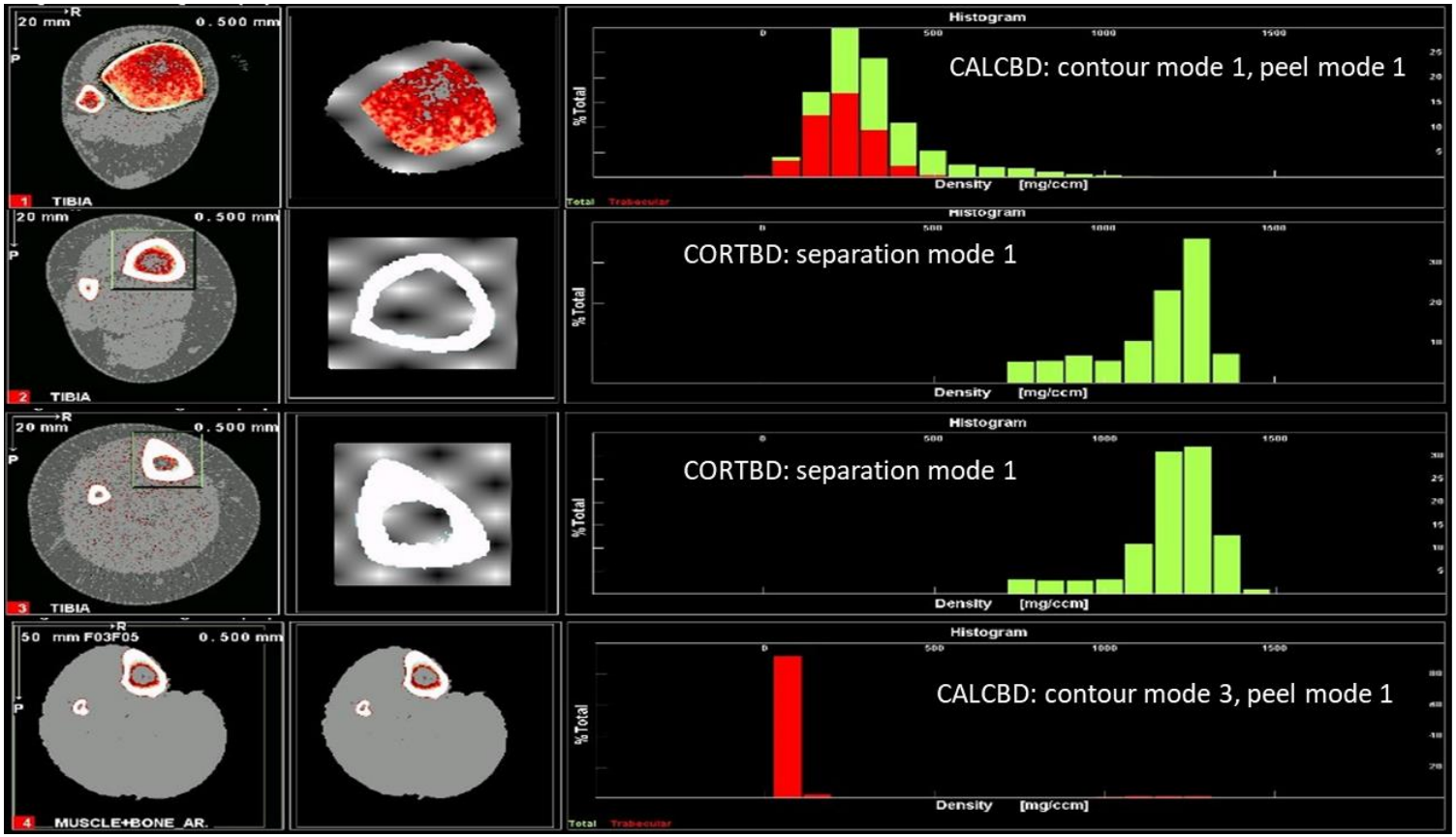

Figure 1.18 Contouring and thresholding of pQCT slices to obtain volumetric bone mineral density outcomes and body composition data.

pQCT scan slice at 4%, 14%, 38% and 66% tibia are displayed to the left, analysis of the region of interest (ROI) for each slice is shown to its right, with its density profile on the far right. At the 66% slice a muscle smoothing filter has been applied, from this slice muscle and bone area data are obtained. At the 66% site a total area and bone area ROI are also applied, these are similar to the muscle and bone ROI but different thresholds are applied. Data from these 3 ROIs at the 66% are used to subsequently generate (through subtraction) measures of cross-sectional muscle and fat area. Muscle density parameters can be calculated.

Table 1.2 Parameters of bone density, mass, geometry, distribution and strength as obtained by single slice pQCT.

Parameters of bone mineral	
Total vBMD (mg/cm^3)	Total BMC divided by total CSA
Total BMC (mg/mm)	Mass of mineral per unit of axial bone length
Trabecular vBMD (mg/cm^3)	Mean vBMD of 45% of the total CSA
Cortical subcortical vBMD (mg/cm^3)	Mean vBMD of the remaining 55% not included in trabecular vBMD
Cortical vBMD (mg/cm^3)	Determined by application of a threshold of 710 mm/mg3 to the scan image
Cortical content (BMC) (mg/mm)	Total BMC minus Trabecular BMC.
Parameters of bone geometry	
Total CSA (mm^2)	The surface area of the whole bone cross-section, including cortical and trabecular bone. This is directly measured.
Trabecular CSA (mm^2)	Usually measured as 45% central area of the total CSA. Depends on the peel mode settings.
Cortical CSA (mm^2)	The surface area of the cortical bone cross-section, equivalent to total CSA minus the cross-sectional size of the marrow cavity.
Cortical thickness (mm)	The average thickness of the cortical shell.
Marrow CSA (mm^2)	Total CSA minus cortical shell (i.e. trabecular and bone marrow).
Periosteal circumference (mm)	The outer diameter of bone: mathematically derived from total CSA by assuming total CSA is circular. PC = (Cross-sectional area / π)½
Endosteal circumference (mm)	The inner diameter of bone: mathematically derived from trabecular CSA. EC = (Trabecular bone area / π)½
CSMI (mm^4)	Taken with reference to the "neutral axis" resulting from given bending conditions: i.e. resistance to torsion
Polar moment of inertia (mm^4)	The sum of the bone-filled voxel areas multiplied by the square of this distance for each voxel.
Section modulus (mm^3)	The ratio between the polar moment of inertia and the maximal distance of a bone-filled voxel from the centre
SSI (mm^4)	Represents the distribution of bone from the diaphyseal axis, weighted by density (i.e. density-weighted polar section modulus)
Body composition	
Muscle CSA (mm^2)	Used as a surrogate of muscle force, determined at the widest CSA of the limb. Bone area is subtracted from whole image area
Muscle density (mg/cm^3)	Muscle density derived by dividing total muscle mass by MCSA
Subcutaneous Fat (mm^2)	Bone mass and muscle mass subtracted from whole image to derive subcutaneous fat mass.

vBMD = volumetric bone mineral density, BMC = bone mineral content, CSA = cross-sectional area, CSMI = Cross-sectional moment of inertia, SSI = Stress strain index

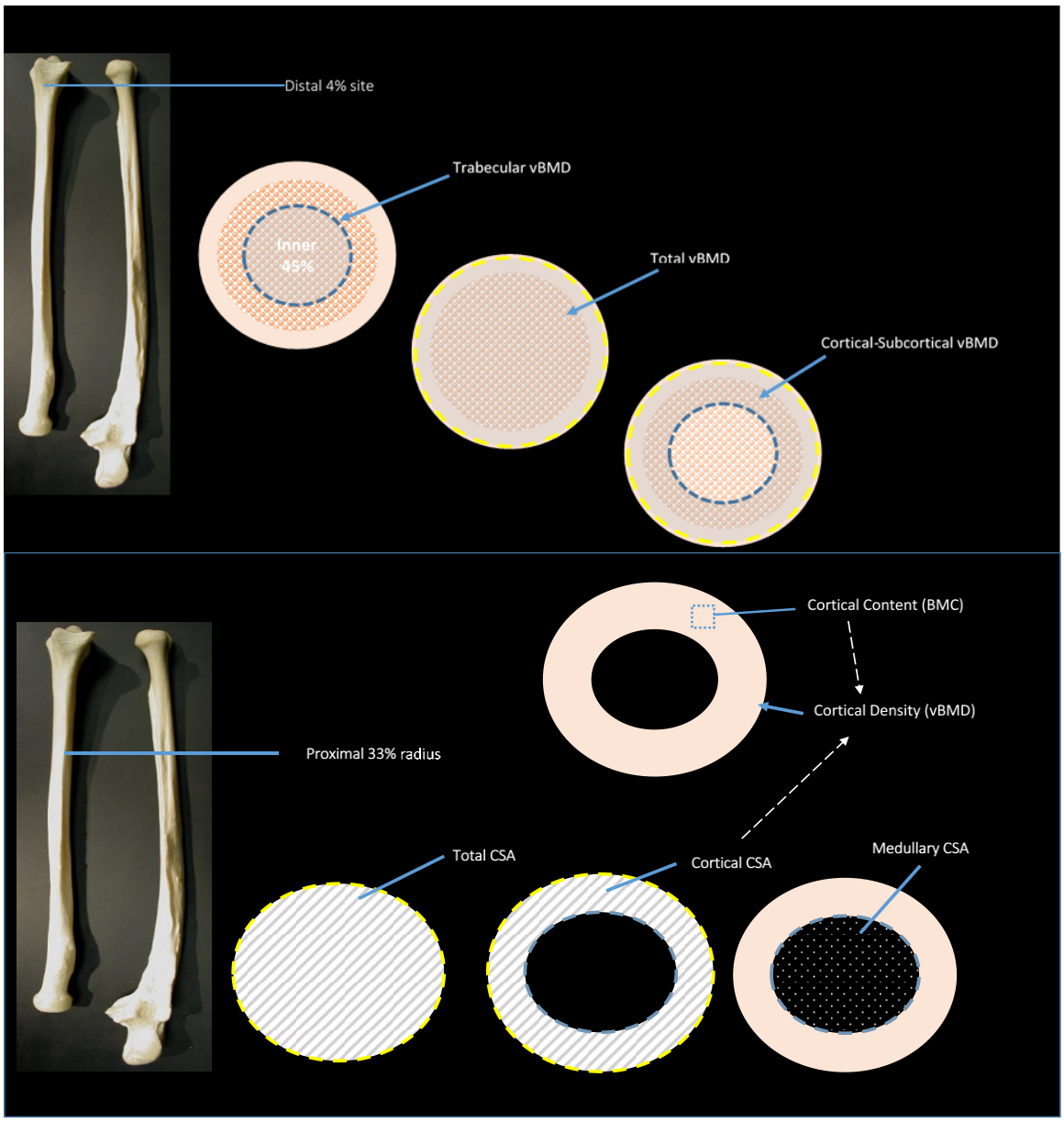

Figure 1.19 Basic schematic of parameters of bone area, mass, and density as obtained by single-slice pQCT

The examples in the top pane are at the 4% distal radius a site rich in trabecular bone, and below the 33% proximal radius a site containing almost exclusively cortical bone. Equivalent sites are measured at the tibia, however, pQCT can obtain data from any user defined site along the radius or tibia. Source Ó Breasail 2018

Single slice pQCT methods used at different research centres may vary in the selection of site for scanning, selection of thresholds for analysis, and the use of manufacturer or third party software for scan analysis these factors can make it difficult to directly compare studies in the literature. There are no official guidelines for using pQCT clinically, it is therefore difficult to compare reference datasets acquired under different device settings and at different regions of interest (Adams et al., 2013). pQCT is restricted to the appendicular skeleton and cannot provide data from

axial osteoporotic fracture sites such as the spine or hip. Consistent positioning is vital for longitudinal measurements, inaccurate accurate positioning will mean that the site of interest is incorrect and can result in large errors, especially in trabecular bone (Marjanovic et al., 2009). Participant movement can result movement artefacts, good quality scans require the participant to remain still throughout the procedure, which can be difficult for the very young, elderly, groups with learning difficulties or involuntary body movements. Scan times are dependent on the number of sites being measured, each individual CT slice takes approximately 1 minute, as typically 3-4 sites are measured along the tibia length (total scan time = ~5 minutes) and 3 sites along the radius (total scan time = 3 minutes). However, scan acquisition can be time intensive as in addition to scanning time, time is spent to ensure the participant is correctly positioned in the scanner.

In images acquired by pQCT the 2D pixels correspond to 3D volume-elements (voxels) that extend through the slice thickness (2 mm) of the cross-section, like a grid (Figure 1.20) (Hangartner and Gilsanz, 1996). The accurate measurement of bone by pQCT (and all other techniques) is limited by voxel size, if a voxel contains a mixture of tissues these attenuate the x-rays to different extents and average attenuation of the voxel will be lower than that of true bone and the bone within these voxels will not be counted. This is referred to as partial volume effect (PVE) and it is very problematic when measuring small bones or bones with thin cortices (<2.5 mm) which will have a greater proportion of voxels that are close to the bone edge and will consequently appear to have a lower density (Prevrhal et al., 1999).

Figure 1.20 Partial volume effect (PVE) and the role of thresholds in pQCT imaging

(A) Partial volume effect (PVE) explained with pQCT radius scans and schematic diagrams. At the bone boundary due structures that cover only part of a pixel will be represented at that location with a density value that is an average of all the densities of the various materials covering that pixel (right). An example of why cortical vBMD is not analysed at the 4% distal radius due to the large number of voxels that are partially filled much of the cortex is not found on analysis (above). When cortical thickness is greater, the relative influence of these partially filled voxels becomes smaller. PVE is a limitation of both pQCT and HR-pQCT imaging. Adapted from (Schoenau et al., 2002) and (Hangartner, 2007) (B) Appropriate threshold selection to accurately determine cortical volumetric density and bone geometry. The hatched area denotes where density is maximal and the dotted area where the partial-volume effect is influencing the results. Adapted from Hangartner (2007)

To segment bone from the surrounding soft tissues, specific thresholds of attenuation are selected. Figure 1.20 based on work by Hangartner and colleagues shows the varying density across the actual bone boundary (superimposed pQCT bone scan), density increases from the periosteum towards the medullary cavity to its maximal cortical value and then decreases again as it crosses the bone boundary (Ward et al., 2005a, Hangartner, 2007). PVE will influence the accurate contouring and segmentation of the bone from the surrounding soft tissues as the edge voxels contain both bone and soft tissue, with the subsequent measured density being an average of these two tissues. Thus the error in the final density depends on the proportion of values used in the averaging process that suffer from PVE (Hangartner, 2007). Altering the threshold at which scans are analysed allows for the selection of only voxels above a user defined threshold, which is optimised to ensure that tissues of interest can be measured (Figure 1.20) (Ward et al., 2005a). The analysis of cortical bone parameters with pQCT (Figure 1.20) requires specific attenuation thresholds in order to analyse the image appropriately for the density or geometry based outcome measures (Ward et al., 2005a). Thresholds have a significant effect on the assessment of these parameters and selecting an optimum threshold for cortical thickness is based upon full width at half maximum (FWHM) of the difference between the densities of cortical bone and immediately adjacent tissues (Hangartner and Gilsanz, 1996). Whereas the ideal threshold to accurately obtain cortical vBMD is the peak of the density profile across the cortex, however if the cortical width is not great enough this peak is artificially lowered due to PVE (Hangartner and Gilsanz, 1996, Ward et al., 2005a).

1.5.4 High Resolution-pQCT

HRpQCT allows for the simultaneous measurement of vBMD and microarchitecture (Table 1.3, Figure 1.21) at the distal radius and tibia with an isotropic voxel size of 82 μm (largest trabeculae are ~100 μm). This is much greater than that of conventional pQCT (0.2 – 0.5 mm) but still far below that of ex-vivo μCT (Krause et al., 2014). HRpQCT has been validated against μCT reporting moderate to high correlation coefficients in individuals without a known metabolic bone

disorder or only slightly reduced bone quality (Burghardt et al., 2007, MacNeil and Boyd, 2008, Liu et al., 2010). Krause et al. (2014) compared the accuracy of in-vivo HRpQCT vs µCT in cadaveric ex-vivo samples from postmenopausal females (n=34) with osteoporosis finding HRpQCT provides acceptable in-vivo accuracy for bone volume (BV/TV). The major difference between these two techniques is a matter of image resolution; modern µCT systems can generate ex-vivo images with a voxel size as low as 0.5 µm with a spatial resolution of less than 2 µm. This greatly exceeds the standard in-vivo imaging resolution of both XtremeCTI and XtremeCTII scanners (60.7 µm) (Tjong et al., 2012, Manske et al., 2017). The resolution influences how HR-pQCT assesses the trabecular compartment. As images are of lower resolution, only trabecular number is directly measured and thus an indirect analysis method is used for standard clinical analysis. The other parameters, such as trabecular thickness and trabecular separation, are derived using various assumptions (Laib et al., 1998, Laib and Ruegsegger, 1999, Tjong et al., 2012, Cheung et al., 2013). Recent improvements in HRpQCT technology have improved the measurement of trabecular thickness (Agarwal et al., 2016, Manske et al., 2017) and cortical porosity (Agarwal et al., 2016) while there is high agreement between the XtremeCTI and XtremeCTII for parameters of vBMD (Agarwal et al., 2016, Manske et al., 2017).

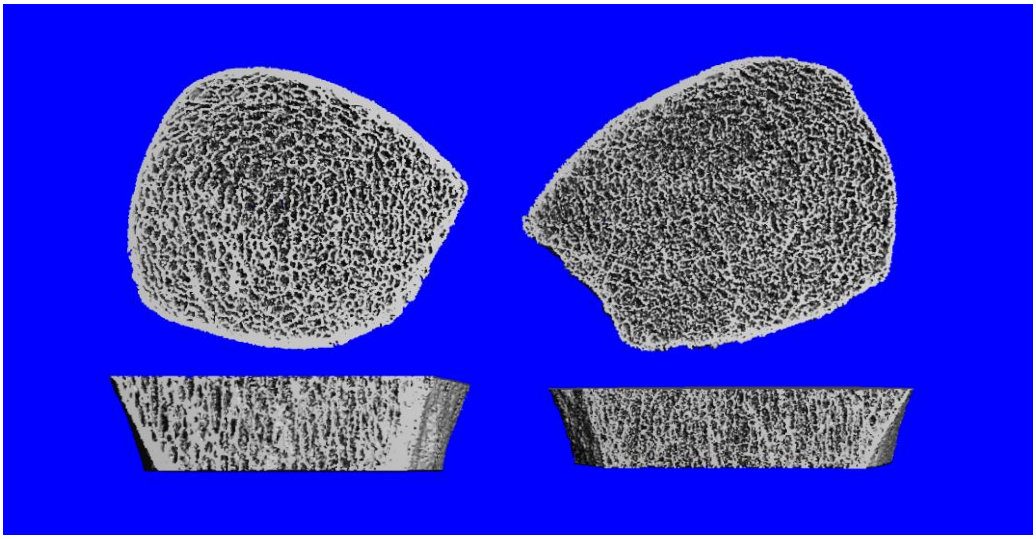

Figure 1.21 HRpQCT tibia scans from pre-menopausal and post-menopausal women

HRpQCT scans at the tibia highlighting age-related changes in the appendicular skeleton. Note the healthy trabecular structure seen left compared to the thinner trabecular structures right. Additionally the greater porosity of the cortical structures on the right are association with aging.

The ability to assess trabecular microarchitecture is a major benefit of HRpQCT imaging over other in-vivo bone densitometry techniques. While conventional pQCT can provide trabecular vBMD measures, it lacks the resolution to measure the spatial distribution of trabeculae within the compartment. Additionally HRpQCT measures the entire trabecular compartment within the acquired volume of interest (VOI), while conventional pQCT trabecular vBMD is acquired only from the inner 45% of the CSA. Burghardt et al. (2010) developed a cortical imaging script to provide improved measures of cortical density and cortical microarchitecture such as cortical thickness and cortical porosity. As with the standard analysis protocol a semi-automatic contour algorithm locates the periosteal surface with an additional contour placed on the endocortical surface. This process consists of three distinct stages to mask the bone structure to define the cortical bone compartment: 1) Segmentation of the cortical bone compartment – cortical image; 2) Identification of Haversian canals – porosity image; 3) combination of the segmented cortical bone and porosity images to generate a refined cortical compartment region (Figure 1.22).

Figure 1.22 Diagram illustrating the additional cortical analysis of Burghardt and colleagues

End result of the additional cortical analysis as described in Burghardt et al. (2010). A, above shows the radius both at the most distal and most proximal slice and the corresponding 3 dimensional visualisation obtained following the analysis. B, tibia shows the corresponding analysis from the tibia. Cortical porosity is illustrated by the red.

The limitations of HRpQCT overlap considerably with other pQCT techniques such as: sensitivity to participant movement, only scanning the distal regions of the peripheral skeleton (pQCT can be

used at the diaphysis and metaphysis), difficulty in positioning for longitudinal measurements, the indirect calculation of parameters (i.e. trabecular thickness), and PVE (Figure 1.20). HRpQCT, at present does not provide any body composition data as there are no imaging scripts available to assess soft-tissue. Limitations unique to HRpQCT compared to conventional pQCT include 'ring artefacts' which are prominent in third generation CT scanners that use multiple detectors. Ring artefacts occur if a detector is out of calibration when it provides a consistently inaccurate reading at each angular position forming a ring (Figure 1.23).

As with all CT techniques beam-hardening negatively effects HRpQCT imaging through distortion causing the edges of the bone to appear brighter than the centre, even if the material is the same throughout. This is the result of the preferential attenuation of low-energy radiation, which "hardens" the energy spectrum of the beam as it passes through the limb, shifting the energy spectrum towards higher energy photons (Sekhon et al., 2009). vBMD varies between the cortical and trabecular compartments. Therefore it is difficult to differentiate between beam hardening artefacts and actual material variations (Barrett and Keat, 2004, Sekhon et al., 2009).

Further issues that are encountered during scan analysis relate to the contouring of the bone edges. The HRpQCT software applies a semi-automated contour algorithm to select the outer cortical edge of the whole bone. Contouring time is reduced by using these algorithms. However, the algorithm may attach contours to unwanted regions and as such the operator must correct these errors. In participants with thin porous cortices (<2 mm) the automated contouring will often select trabecular over adjacent cortical pores (Buie et al., 2007). The operator is required to subjectively verify each slice to ensure that the correct contour has been selected and if not the operator is required to amend the contour. This process is time consuming and subjective and can introduce further error in both precision and bias. To reduce this, all scans should ideally be analysed by one researcher (Buie et al., 2007).

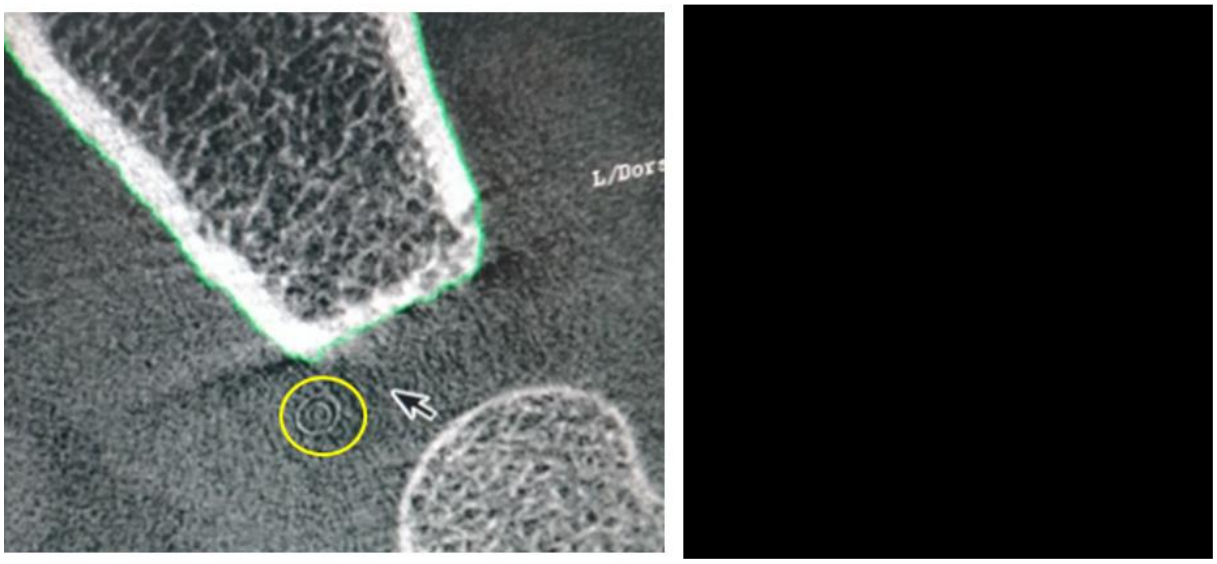

Figure 1.23 Ring artefacts on HRpQCT scans and a schematic explanation of how these artefacts occur:

Ring artefact (highlighted, left) present on a HRpQCT forearm scan to the left of the mouse pointer, schematic explaining this phenomenon (right) from Artul (2013).

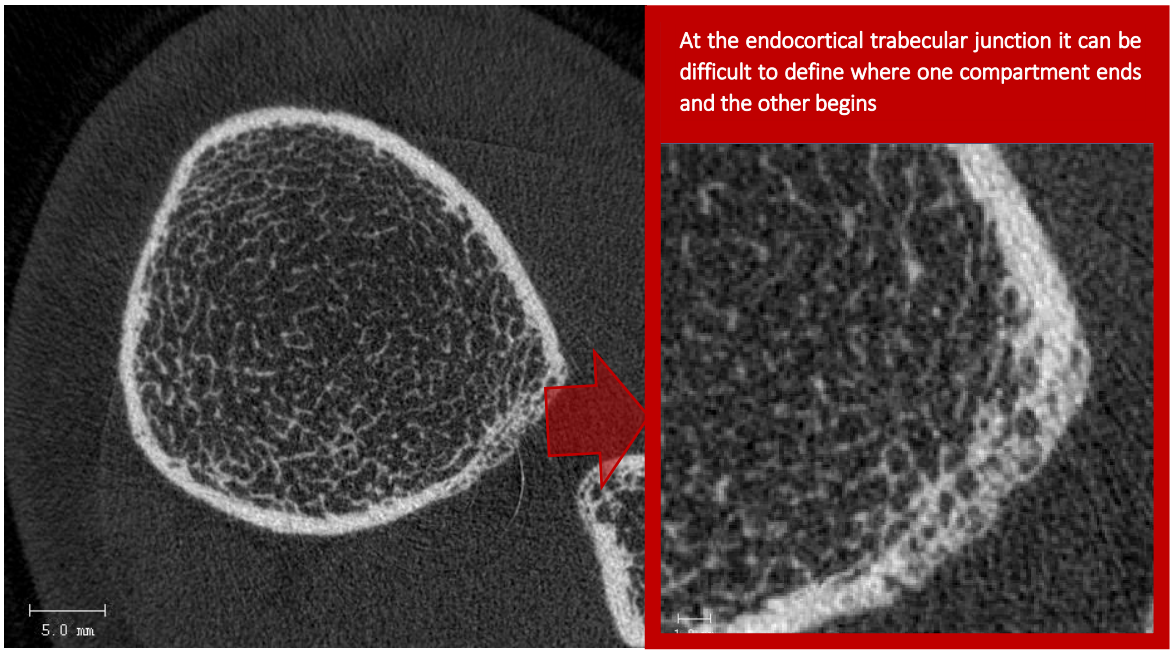

Figure 1.24 The junction of the trabecular and cortical compartments is difficult to measure even with high-resolution in-vivo scanning techniques.

Table 1.3 HRpQCT parameters from standard analysis and additional cortical analysis

Standard analysis: Parameters of bone mineral	
Total vBMD (D100, mg/cm^3)	Represents both trabecular and cortical compartments and quantified based on the periosteal segmentation. i.e. mean value of all voxels within the whole bone.
Cortical vBMD (mg/cm^3)	Whole bone minus trabecular compartment and is a direct measure
Trabecular bone volume to tissue volume (BV/TV)	Derived from trabecular density assuming fully mineralised bone to have a mineral density of 1.2 g HA/cm^3
Standard analysis: Parameters of bone geometry	
Total bone area (Tt.Ar, mm^2)	Mean total area defined by the imaging contours of the middle 104 slices analysed. Tt.Ar includes all bone tissue and marrow contained within the periosteal envelope.
Cortical area (CtAr, mm^2)	Tt.Ar minus trabecular area.
Trabecular area (Tb.Ar, mm^2)	Tt.Ar minus cortical area.
Standard analysis: Parameters of bone structure	
Trabecular number (Tb.N, mm^{-1})	The inverse of the mean spacing of the mid-axes. Does not depend on a priori assumptions regarding the plate- or rod-like nature of the underlying structure.
Trabecular thickness (Tb.Th, mm)	Derived from BV/TV and TbN using standard methods from histomorphometry. TbTh = (BV/TV) /TbN
Trabecular separation (Tb.Sp, mm)	Derived from BV/TV and TbN using standard methods from histomorphometry. TbSp = 1 - BV/TV)/TbN
Cortical thickness (Ct.Th, mm)	Mean cortical volume divided by the outer bone surface.
Additional cortical analysis: Parameters of bone mineral	
Cortical bone mineral density (Ct.BMD, mg/cm^3)	Mean mineralisation of the cortical volume of interest.
Cortical tissue mineral density (Ct.TMD, mg/cm^3)	Mean mineralization of the segmented cortical bone voxels after exclusion of pores.
Cortical total volume (Ct.TV, mm^3)	The volume of all voxels (i.e. bone and pore) contained within the cortical volume of interest.
Cortical bone volume (Ct.BV, mm^3)	The volume of all bone voxels within the cortical volume of interest.
Additional cortical analysis: Parameters of bone geometry	
Total bone area (Tt.Ar, mm^2)	The average cross-sectional area of the whole bone circumscribed by the periosteal contour.
Cortical bone area (Ct.Ar, mm^2)	The average cross-sectional area of the cortical compartment between the periosteal and endosteal contours.
Trabecular bone area (Tb.Ar, mm^2)	The average cross-sectional area of the trabecular compartment circumscribed by the endosteal contour.
Additional cortical analysis : Parameters of bone structure	
Cortical thickness (Ct.Th, mm)	Mean cortical thickness
Cortical pore volume (Ct.Po.V, mm^3)	Direct voxel-based measure of the volume of the intracortical pore space.
Cortical porosity (Ct.Po, %)	Relative voxel-based measure of the volume of the intracortical pore space normalised by the sum of the pore and cortical bone volume.

1.5.5 Non X-ray based techniques to measure bone mineral

Quantitative Ultrasound (QUS) is a method based on the transmission of a sound wave through bone, energy from the sound wave (frequency 20 kHz to 100 MHz) propagates though the bone tissue with some of this energy transferred to the bone tissue, through attenuation (Gluer et al., 2004). The shape, intensity, and speed of the sound wave are quantified by the receivers as speed of sound (SOS) and broadband ultrasound attenuation (BUA). SOS, BUA and stiffness index BMD equivalent (SI, a linear combination of BUA and SOS) show moderate correlations with DXA in some studies (Clo et al., 2015) but not in others (Laskey and Prentice, 2004). QUS is used at peripheral sites which have little soft tissue (Figure 1.25) (Baroncelli, 2008). However, in the past many manufacturers have produced these devices and there are difficulties in comparing studies without cross-calibration. A multicentre study by Gluer and colleagues tested five different commercially available QUS devices tested and found significant age-adjusted differences between subjects with and without vertebral fracture (i.e. 20% height reduction). They also claimed to show QUS of the calcaneus worked comparatively well as central DXA for the identification of women at high risk for prevalent osteoporotic vertebral fractures (Gluer et al., 2004). However, in conclusion they cautioned that the high correlation between QUS methods was due to rigorous quality assurance measures that should also be applied in clinical practice (Gluer et al., 2004). QUS is a non-invasive, safe, and radiation free modality which is easy to use (Guglielmi et al., 2009), in addition to being inexpensive and portable. This has allowed for data to be collected in large reference populations. However, despite the advantages of QUS there are a number of major limitations. They are sensitive to temperature change. Some QUS techniques that measure the calcaneus use a fixed sized foot well as such participant foot size may affect the region of the bone being observed. In conditions where oedema is common such as pregnancy, additional soft tissue may interfere with the attenuation perhaps leading to erroneous measures at the calcaneus and phalanges. Although today very few manufacturers continue to produce and market QUS devices, historically they have been widely used by many studies in pregnancy as they are non-ionising and

have allowed for the collection of QUS data from large numbers of pregnant women at multiple time points across pregnancy. As such QUS warrants a brief mention in this chapter and data from studies during pregnancy with be discussed in the next chapter.

Figure 1.25 Schematic showing qualitative ultrasound (QUS) devices and their respective scan sites.

QUS devices (probes: light blue, transducers: red), X-ray films left used to represent the sites of measurement, and the approximate ROI (yellow box). The yellow arrows indicate the principal pathways of the ultrasound waves from the emitter transducer(s) to the receiving transducer(s). Below Hologic Sahara (Os Calcis) and miniOmnisense (Radius) QUS devices. Source: Baroncelli (2008).

Figure 1.26 Overview of the interrelated biological determinants of bone stiffness and strength and the available techniques to measure bone and muscle.

The production of anisotropy (different stiffness / strength in different directions) at tissue and organ levels of structural complexity of bones derived from the material and geometric properties, respectively, is shown. The ability of some current methodologies (DXA, QCT, and QUS) to determine some suitable indicators of those properties is also indicated. Adapted from Cointry et al. (2004)

Table 1.4 Summary of the primary advantages and limitations of main x-ray modalities used in bone mineral research along with the exposure to ionising radiation from standard imaging investigations. Typical precision estimates are also given.

Advantages	Limitations	Radiation Exposure	Precision
	DXA		
Rapid scan acquisition Low cost Low radiation dose High precision Clinical gold standard Flexibility – can assess the total body, lumbar spine, forearm, femoral neck and body composition (lean and fat mass).	Size dependent measurements Sensitivity to body composition changes Software and reference data changes. Unable to quantify trabecular and cortical bone compartments separately	whole body <1.0µSv, hip scan 3.1µSv, lateral spine 12.09µSv AP spine 3.37µSv	AP spine: 1-2% Hip: 2-3%
	pQCT		
Size independent Low radiation Can quantify cortical and trabecular bone compartments Can assess bone geometry and shape Can assess indices of bone strength Can assess fat and muscle Low radiation dose Low cost	Relatively long acquisition time Peripheral sites only Sensitive to participant movement Research tool – little clinical use Less reference data vs DXA Imaging artefacts Partial Volume Effect. Reproducibility of scan location.	<1.6 µSv per scan	Radius: 0.9– 5.7% Tibia: 0.4 – 2.8%
	HR-pQCT		
Size independent Low radiation Can quantify cortical and trabecular bone compartments Can assess bone geometry and shape Can assess indices of bone strength Very detailed images due to high resolution Can image the trabecular bone structure	Relatively long acquisition time Peripheral sites only Sensitive to participant movement Research tool – little clinical use Less reference data vs DXA Imaging artefacts. Partial Volume Effect High cost and maintenance	<3 µSv per scan	Radius: 1.1 – 12.7 % Tibia: 0.2 – 5.2 %

DXA = dual-energy x-ray absorptiometry ,pQCT = peripheral qualitative computed tomography , HR-pQCT = high-resolution peripheral qualitative computed tomography, AP= anteroposterior

2 Literature Review: Pregnancy-Induced Bone Mineral Changes in the Maternal Skeleton

2.1 Introduction

The new-born skeleton contains 20–30 g calcium, 16 g phosphorus, 750 mg magnesium and 50 mg zinc, this represents 98%, 80%, 60% and 30%, respectively, of total body stores (Givens and Macy, 1933, Prentice, 1994b, Prentice and Bates, 1994). Very little of this fetal mineral accrual occurs before mid-pregnancy; Ca accretion increases thereafter, peaking in the third trimester (Prentice, 2000a). The total Ca accretion rate of the fetus increases from approximately 50 mg/day at 20 weeks to approximately 330 mg/day at 35 weeks (Forbes, 1976). As pregnancy and lactation place increased pressure on the maternal mineral economy to supply these essential nutrients, there needs to be potential biological strategies that allow the mother to meet these extra nutritional demands of pregnancy and later lactation (Prentice, 1994b, Prentice, 2000a, Prentice, 2000b). Possibilities include increases in mineral intake, physiological adaptations via increased gastrointestinal absorption and decreased urinary/faecal mineral excretion, and/or mobilisation of mineral from tissue stores (Kalkwarf and Specker, 1995, Prentice, 2003, Olausson et al., 2012).

This final possibility has been well documented in lactation (Laskey and Prentice, 1997, Laskey and Prentice, 1999, Moller et al., 2012, Brembeck et al., 2014) but as there are few and conflicting densitometry and bone turnover data in pregnancy, questions remain as to whether mineral mobilisation from the maternal reserves occurs, and, if so, its possible magnitude. Although pregnancy is not considered a risk factor for osteoporosis (Kalkwarf and Specker, 1995) if mobilisation occurs and is not restored after pregnancy, some individuals and populations may be more vulnerable and subsequently at increased risk of fracture in later life.

It helps to contextualise the potential for increased risks of bone mineral mobilisation during pregnancy for women at different ages by considering the changes in bone mass through the lifecourse (Figure 1.8). Framing the question as such we can consider whether: 1) mobilisation

influences PBM attainment in the still-growing skeleton (i.e. adolescent pregnancies); 2) future skeletal health is affected in women who become pregnant after PBM and at the start of skeletal decline in midlife; 3) sufficient time is available to fully replenish any mobilised mineral prior to the onset of menopause. These final two points are of growing relevance in 2016 because 54% of live births in the UK were to women aged 30 years and older (ONS, 2016), while the latest ONS data shows that births to women aged ≥40 years are at their highest number since 1949 (ONS, 2017).

There is conflicting evidence about whether pregnancy is associated with changes in bone, from studies using a variety of outcome measures, including circulating hormone levels, bone turnover markers (BTM), bone histomorphology (via bone biopsies), QUS, and a handful of studies using DXA and pQCT. Limitations of the available technologies for the direct measurement of maternal bone, such as the invasiveness of bone biopsies and the concerns over the use of ionising radiation during pregnancy, make it challenging to quantify maternal bone mobilisation during pregnancy.

The following sections present a review of the literature to address these points. Pubmed was searched using the keywords pregnancy AND Bone to identify papers that may be relevant, these were filtered to only include those carried out in humans. As well as original research, reviews and systematic reviews of pregnancy/lactation and bone mineral metabolism were also consulted to help identify suitable papers for inclusion. I chose to include only prospective studies prospective studies, as retrospective studies assessing bone mineral density in postmenopausal women are difficult to interpret due to factors affecting bone since pregnancy and the need to rely on participants recall of such variables as previous weight, diet and vitamin D status, hormonal contraceptives use, and intervening lactation and infant feeding practices (Olausson et al., 2012). While osteoporosis and fractures in pregnancy can occur rarely in some women (Dunne et al., 1993, Herath et al., 2017, Laroche et al., 2017), there are no data to suggest that osteoporosis of pregnancy is either an exaggerated metabolic response to pregnancy or the result of dietary deficiencies (Prentice, 2000a). The fact that osteoporosis can occur in pregnant women cannot be taken as

evidence that bone mineral loss is a necessary corollary of normal pregnancy (Prentice, 2000a); for this reason I have not included literature relating to osteoporosis during pregnancy in this review.

2.2 Normal calcium metabolism and pregnancy-related adaptations

Ca metabolism is tightly regulated, even in cases of dietary Ca deficiency circulating levels of serum ionised Ca are maintained within a narrow normal range. The reason for such tight regulation is that Ca^{2+} ions act as an important electrolyte in many body systems and are vital to the regulation of muscles contraction, nerve conduction, and the clotting of blood. For these reasons the consequences of serum Ca being too low (hypocalcaemia) or too high (hypercalcaemia) can be life threatening and the maintenance of adequate levels of blood ionised Ca (1.1–1.3 mM) is vital. The skeleton at maturity contains approximately 1 kg Ca, of which more than 99% is found in within bone and teeth. This Ca stored in the skeleton can be released into the plasma when required to supply the body with Ca ions required by the other body systems.

Although in my review and PhD research I have focussed predominantly on methods used to assess whether bone mineral changes occur in the maternal skeleton during pregnancy, I have also provided a brief summary of normal Ca homeostasis and a consideration of how metabolic changes may initiate bone mineral changes during pregnancy. This tightly regulated metabolic pathway is controlled by a number of hormones including parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxyvitamin D (1,25(OH)2D) metabolically active form of vitamin D), and fibroblast factor 23 (FGF23) which permit serum calcium and phosphate to be maintained within very narrow limits over a wide range of dietary Ca intakes (Figure 2.1). PTH is one of the key players in the regulation of circulating Ca levels, in response to reductions in ionised Ca it can: 1) promote bone resorption and releasing Ca from the skeleton; 2) reduce urinary Ca losses and increase phosphate excretion; 3) enhance intestinal Ca absorption (indirectly via renal 1,25(OH) 2 vitamin D production).

Subsequent increases in blood ionised Ca and the active form of vitamin D contribute to the negative feedback inhibition of PTH secretion, while serum phosphate increases PTH secretion.

Simplified overview of the relationships between Ca2+ and Pi and their regulatory hormones, PTH, FGF23, and 1,25(OH)2 vitamin D are displayed above as a network of endocrine feedback loops that govern mineral homeostasis. The effect of calcium to increase serum FGF23 levels, a low Pi level to increase serum 1,25(OH)2D, and a high Pi level to decrease serum 1,25(OH)2D is not shown. FGF23, fibroblast growth factor 23; PTH, parathyroid hormone. Source: Silver and Naveh-Many (2009)

While the actions of PTH and 1,25(OH)2D work together to increase blood Ca levels, calcitonin (produced in the C glands of the thyroid), is a hypocalcaemic hormone which reduces plasma Ca (Figure 2.1). Compared to the other calciotropic hormones calcitonin is relatively poorly understood, its primary action being the inhibition of osteoclast activity (Friedman and Raisz, 1965, Chambers et al., 1984, Samura et al., 2000). Interestingly calcitonin is produced in large amounts in the mammary gland and in the pituitary gland, which has fuelled speculation of a potential role in protecting the maternal skeleton during reproduction (Miller, 2006, Davey and Findlay, 2013). It is thought that during pregnancy, calcitonin may have a role in promoting renal Ca excretion (Kovacs, 2001) and in protecting the maternal skeleton from excessive resorption (Stevenson et al., 1979) though at present the evidence base is insufficient. Finally, FGF23 is another hormone that contributes to the regulation of calcium and phosphate homeostasis by promoting renal phosphate excretion, which in turn reduces the circulating levels of 1,25(OH)2D, thus diminishing intestinal Ca absorption (Figure 2.1).

In non-pregnant, non-lactating (NPNL) women, the absorption of Ca from dietary sources depends on many intrinsic and extrinsic factors. Efficiency of intestinal absorption of Ca is

important, active transport of Ca occurs in the duodenum and proximal jejunum, with passive absorption in the distal jejunum and ileum accounting for the majority of Ca absorption (Diaz de Barboza et al., 2015). As such, conditions (coeliac disease, Crohn's disease, irritable bowel syndrome) that afflict the gut can result in intestinal Ca malabsorption (Melvin et al., 1970, Walters, 1994). The production of sufficient gastric acid also has a role in Ca absorption (Kopic and Geibel, 2013). While exogenous factors such as vitamin D (dietary or skin synthesis after UVB exposure) and dietary components can enhance or inhibit Ca absorption (Heaney and Skillman, 1964, Heaney and Skillman, 1971, Heaney, 1990, Heaney et al., 2001, Rafferty and Heaney, 2008, Heaney, 2013). Ca is excreted through faeces, urine and sweat (Heaney and Recker, 1994).

2.3 Changes in Ca metabolism and calciotropic hormones during pregnancy

Data from longitudinal studies across pregnancy demonstrate that total Ca plasma concentration (i.e. both ionised and protein(albumin)-bound) decreases during pregnancy versus pre-pregnancy or early pregnancy (Ritchie et al., 1998, Black et al., 2000, Ulrich et al., 2003, Wisser et al., 2005). However, this is likely to be due to the expansion of the intravascular volume, as indicated by the decrease of plasma albumin (Pitkin and Gebhardt, 1977, Black et al., 2000). Ionised Ca, the tightly regulated fraction in circulation, remains unchanged between early and late pregnancy (Pitkin et al., 1979, Seki et al., 1991, Dahlman et al., 1994, Wisser et al., 2005). Between individuals there is large variability in PTH concentrations during early gestation (Hemmingway et al., 2018a). Within individuals, PTH concentration has been reported to be unchanged (Ritchie et al., 1998, Casanueva et al., 2004), or significantly decreased across pregnancy (Black et al., 2000, Naylor et al., 2000). Studies have reported 25-hydroxyvitamin D (25(OH)D) levels remain stable during pregnancy (Brooke et al., 1980, Ritchie et al., 1998, Hollis et al., 2011, Roth et al., 2013, Wagner et al., 2013) or slightly increased (Morley et al., 2006). Increases in plasma 1,25(OH)2D relative to NPNL controls have been reported in early pregnancy, with further increases in 1,25(OH)2D observed by the 3rd trimester. Increased concentrations of 1,25(OH)2D reported from cross- sectional studies

can be several-fold greater than pre- and early-pregnancy levels (Fleischman et al., 1980, Whitehead et al., 1981, Bikle et al., 1984, Moller et al., 2013). Vitamin D Binding Protein (DBP) and concentrations of free 25(OH)D and 1,25(OH)2D (regarded as the biologically active forms) also increase during the same period (Bikle et al., 1984, Seki et al., 1991, Ardawi et al., 1997, Ritchie et al., 1998), although it is not until late pregnancy that the proportion of free to bound 1,25(OH)2D increases. However, it is possible that free 1,25(OH)2D may be raised across gestation but only detectable in the third trimester due to the decreases in albumin (Kovacs, 2016). The importance of adequate calcium and vitamin D intake to maternal and fetal health is known, though research is emerging to suggest that maternal calcium metabolic stress rather than low calcium intake or insufficient vitamin D alone has an adverse influence on fetal growth (Scholl et al., 2014), however, further evidence for this is needed. The role of calcitonin during pregnancy is not fully determined (see section 2.2), findings from observational studies have varied widely reporting unchanged concentrations during gestation compares to postpartum levels (Ritchie et al., 1998, Wisser et al., 2005), or longitudinal measures at multiple points across pregnancy (Pitkin et al., 1979, Cruikshank et al., 1980, Seki et al., 1991). Conversely some studies have found several-fold increases from early to late pregnancy compared to non-pregnant values (Samaan et al., 1975, Hillyard et al., 1978, Stevenson et al., 1979, Kovarik et al., 1980, Silva et al., 1981, Whitehead et al., 1981, Woloszczuk et al., 1981, Dahlman et al., 1994, Ardawi et al., 1997).

Figures 2.2 summarises how Ca metabolism in (i) early pregnancy and (ii) late pregnancy differ from NPNL women with hypothesised bone mineral changes highlighted. For context within the reproductive cycle of pregnancy and lactation, Figure 2.3 is an overview of changes occurring in the maternal skeleton during (iii) lactation and (iv) post-lactation. Large shifts in Ca homeostasis and bone mineral changes have been widely documented during these periods (iii & iv) with several comprehensive reviews available (Olausson et al., 2012, Kovacs, 2016). Briefly, mobilisation of maternal bone mineral has been documented consistently from trabecular-rich sites (Hayslip et al., 1989, Sowers et al., 1993, Cross et al., 1995, Kolthoff et al., 1998, Laskey and Prentice, 1999, Polatti

et al., 1999, Akesson et al., 2004, Pearson et al., 2004), with repletion documented at several skeletal sites following the cessation of lactation (Laskey and Prentice, 1999, Polatti et al., 1999, Akesson et al., 2004) and the resumption of menses (Holmberg-Marttila et al., 2000). Recent HRpQCT data suggest potential lasting reorganisation of trabecular and cortical microarchitecture up to 5 years postpartum (Bjornerem et al., 2017).

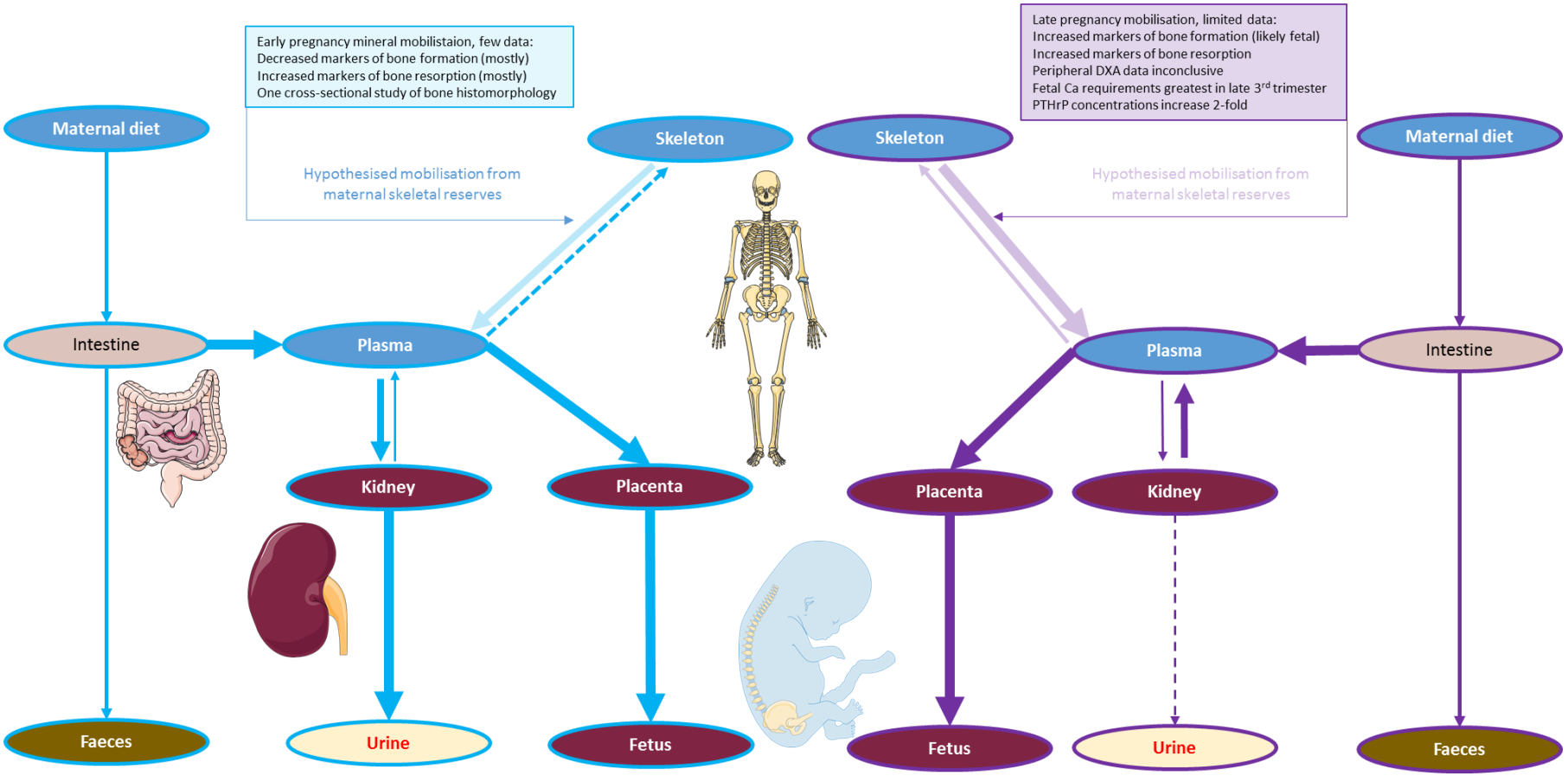

Figure 2.2 Summary of known and possible changes in maternal calcium homoeostasis during early gestation.

Thicker arrows denote increases from NPNL, while dashed lines denote decreases from NPNL, lighter arrows denote few data or conflicting evidence for mechanisms of change. During early pregnancy calcium absorption from the small intestine increases, urinary excretion also increases. Limited data suggest some changes may occur in the maternal skeleton however data are few and inconsistent. See Tables 2.2 & 2.3 for data summarised here. The schematic was prepared with the use of images from Servier Medical Art through Creative Commons Attribution 3.0

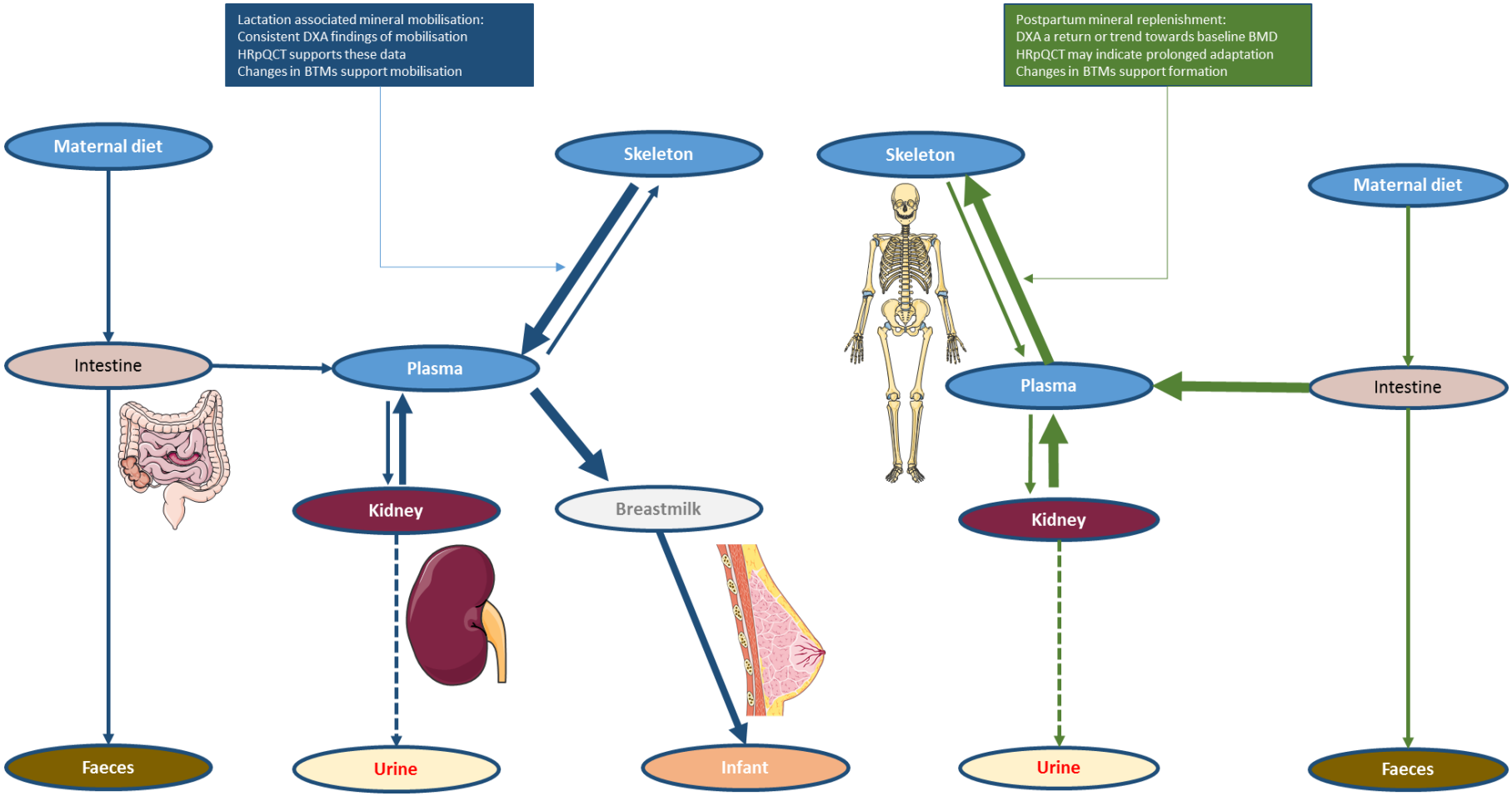

Figure 2.3 Summary of differences in calcium flux during lactation and the period following weaning compared with non-pregnant non-lactating women (NPNL).

Thicker arrows denote increases from NPNL, while dashed lines denote decreases from NPNL.. The well-documented changes in maternal bone mineral during lactation are supported by biochemical data. Data from DXA for the most part support a trend towards repletion, near total repletion or as in several studies higher aBMD at the spine (depending of course on the baseline). However, HRpQCT data may indicate lasting adaptations in bone microstructure despite the repletion of mobilised bone mineral. The schematic was prepared with the use of images from Servier Medical Art through Creative Commons Attribution 3.0

2.3.1 Bone turnover markers during pregnancy

Bone turnover markers (BTM) are a useful method of indirectly investigating mineral changes in the skeleton. BTMs reflect mainly osteoblast (formation) or osteoclast (resorption) activity rather than osteocyte function (Table 2.1). Changes in BTM are measurable closer to the initiation of changes in bone formation and resorption than the physiological manifestations of such events observed through changes in BMD (Vasikaran et al., 2011). BTM levels reflect global skeletal turnover and cannot distinguish the metabolic activity of the trabecular or cortical bone compartments (Chapurlat and Confavreux, 2016). A number of studies have used BTMs alone or alongside densitometry in pregnant women, however appropriate BTM selection is crucial, as some historically widely-used BTMs have inadequate bone specificity or may enter or leave the maternal circulation from/to the placenta, fetus, or other tissues (e.g. mammary gland). (Chapurlat and Confavreux, 2016). This is discussed in more detail in sections 2.3.1.2 and 2.3.1.3. Other limitations of BTMs such as haemodilution which is common (but not confined to) in early pregnancy, and while BTMs are known to have diurnal variation in NPNL there are few data from across pregnancy. Haemodilation can be overcome to some extent through correcting for albumin (Kaur et al., 2003a).

Collagen type 1 comprises 90% of the total protein in our skeletons making collagen formation or degradation products useful as BTMs. Early techniques measuring hydroxyproline required dietary restriction (i.e. meat and other collagen-containing foods). Newer collagen derived BTM used to assess bone formation and resorption are more robust as their secretion is not influenced by diet and they are produced by the metabolism of mature collagen and are secreted without being re-utilised in collagen formation (Black et al., 2000). These include: cross-linked telopeptides of type I collagen (DPD) and pyridinoline (PYD), are measured in urine, while both NTx and CTx may be measured in either urine or blood (Olausson et al., 2012) all 4 have been widely to investigate bone turnover in pregnancy (Table 2.2). DPD is more specific for bone resorption than PYD, but neither is absolutely bone-specific (Yamaga et al., 1997). BTMs used to assess formation include products

of osteoblastic synthesis of new bone matrix, such as N and C-propeptides of type I collagen (P1NP and P1CP respectively) these are released when collagen type I collagen is synthesised (Black et al., 2000, Olausson et al., 2012). Proteins involved in osteoblast function such as alkaline phosphatase (BAP is produced by osteoblasts and reflects osteoblast activity, while only a fraction of TAP be reflective of osteoblast activity) and serum osteocalcin (OC) have also been widely used (also see sections 2.3.1.2 and 2.3.1.3).

Table 2.1 Commonly used bone turnover markers (BTM) of bone formation and resorption

Formation markers (i.e. osteoblast activity)	Resorption markers (i.e. osteoclast activity)
Total Alkaline Phosphatase (TAP) in serum	C – Terminal Crosslinks (CTX)* in serum and urine
Bone Specific Alkaline Phosphatase (BAP) in serum	N – Terminal Crosslinks in serum and urine (NTx)
Osteocalcin (OC) in serum	Free Crosslinks: pyridinoline (PYD) and deoxypyridinoline (DPD) in urine
N - propeptides of type I collagen (P1NP)* in serum	Telopeptide of type 1 collagen (ICTP) in serum
C-propeptides of type I collagen (P1CP) in serum	

* International Osteoporosis Foundation (IOF) recommended reference markers for clinical trials

As the inconsistent use of the various BTMs makes it difficult to directly compare studies, the International Osteoporosis Foundation (IOF) has recently recommended particular reference BTMs to be reported in all clinical trials to ensure that outcome measures are comparable between studies. These are C-terminal telopeptide of type 1 collagen (CTx) for bone resorption, and procollagen type 1 N propeptide (P1NP) for bone formation (Vasikaran et al., 2011). A detailed review of the evidence for collagen-derived BTM, Alk phos and OC follows in sections 2.3.1.1, 2.3.1.2 and 2.3.1.3. In general there appears to be an increase in resorption markers throughout pregnancy (highest levels in late-pregnancy) while evidence for formation markers is much more conflicting with some indications of potential increases in late pregnancy.

Table 2.2 Summary of studies using biomarkers of bone resorption across gestation.

Authors	Country, n	Age*	Controls	T1	T2	T 3	PP
Cross-linked telopeptides of type I collagen C-Terminal (CTx)							
Yamaga et al. (1997)	Japan (n=230)	P: 28.9 (3.4) NP: 26.2 (2.5) P-FU: 28.1 (2.7)	18 NP	-	-	↑ vs NP	-
Naylor et al. (2000)	UK (n=16)	29 (20–36)	Pre-preg	-	↑	↑	↓
Kaur et al. (2003a)	UK (n=41)	31 (5.0,	Pre-preg	↓*	-	↑	↑
Haliloglu et al. (2011)	Turkey (n=60)	P: 28.1 (3.7) NPNL: 29.2 (4.2)	NPNL	-	↑ vs NPNL	↑ vs NPNL	↑ vs NPNL
Cross-linked telopeptides of type I collagen N-Terminal (NTx)							
Akesson et al. (2004)	Sweden (n=254)	31 (20–45)	NA	-	-	↑ vs T1	↑ vs T1
Moller et al. (2013)	Denmark (n=153)	29 (25–35)	NPNL & Pre-Preg	↑	↑	↑	↑
Ulrich et al. (2003)	USA (n=15)	32.7±3.4	NP & Pre-Preg	-	-	↑ vs Prepreg	↑ vs Prepreg
Naylor et al. (2000)	see above	29 (20–36)	Pre-preg				↓
Ettinger et al. (2014)	Mexico (n=563)	26	NA	-	↑ vs T1	↑ vs T2	↓ vs T3
Yamaga et al. (1997)	see above	see above	NP	-	↑ vs NP	↑ vs NP	↑ vs NP
Black et al. (2000)	USA (n=10)	30 (23–40)	Pre-preg	↑	↑	↑	
Telopeptide of type 1 collagen (ICP)							
Ulrich et al. (2003)	See above	32.7 (3.4)	NP & Pre-Preg	-	-	↑	↑
Free Crosslinks: pyridinoline (PYD) and deoxypyridinoline (DPD)							
Yamaga et al. (1996)	Japan (n=18)	27.5 (3.2)	NA	-	↑ vs 5 wks	↑ vs 5 wks	↑6 mo BF
Yamaga et al. (1997)	see above	see above	NP	-	-	Both ↑	Both ↑
Black et al. (2000) (both)	see above	see above	Pre-preg	↑	↑	↑	
Naylor et al. (2000) (both)	see above	see above	Pre-preg	-	-	Both ↑	Pyd ↑
Yoon et al. (2000)	South Korea (n=41)	30 (0.9) 29.5 (0.7)	NPNL	↑ vs NPNL	↑ vs NPNL	↑ vs NPNL + 8wks	↑ vs NPNL, 8 wks
Ritchie et al. (1998)	USA (n=14)	29.4 ± 2.3	Pre-preg	-	-	↑ vs T1	↑ vs T1
Wisser et al. (2005)	Switzerland (n=43)	28.4 (0) NP 34.0 (4.7))	NP	-	-	↑ Vs T1	-
Cross et al. (1995)	USA, (n=10)	28.4 ± 1.0	NPNL/Pre-Preg	- Vs NPNL	- Vs NPNL	↑ Vs NPNL	↑ Vs NPNL
Paoletti et al. (2003)	Italy (n=44)	27.6±2.1, NPNL 28.2±1.8	NPNL	both ↑ vs NPNL	both ↑ vs NPNL	both ↑ vs NPNL	both ↑ vs NPNL

Statistically significant increase or decreases in each marker are indicated by arrows. Where possible it is reported whether a control group was recruited or whether women had pre-pregnancy baseline data from which the differences are expressed. Post-partum data are shown only when considered relevant and not beyond 6 months postpartum. *range given if mean (SD) not available, T1 = 1st trimester, T2 = 2nd trimester, T3 = 3rd trimester, PP = postpartum, NP = non-pregnant, NPNL = non-pregnant non-lactating, BF = breastfeeding

Table 2.3 Summary of studies using biomarkers of bone formation across gestation.

Authors	Country, n	Age *	Controls	T1	T2	T3	PP
			Procollagen type 1 N Propeptide (P1NP)				
Naylor et al. (2000)	UK (n=16)	29 (20–36)	Pre-preg	-	-	↑	-
Kaur et al. (2003a)	UK (n=41)	31 (5.0)	Pre-preg	↓*	↓*	↑	↑
			Procollagen type 1 C Propeptide (P1CP)				
Naylor et al. (2000)	See above	29 (20–36)	Pre-preg	-	-	↑	-
Black et al. (2000)	USA (n=10)	30 (23–40)	Pre-preg	-	-	↑	
Cross et al. (1995)	USA (n=10)	28.4 ± 1.0	NPNL/Pre-Preg	↓ Vs NPNL	↓ Vs NPNL	↑ Vs NPNL	Vs NPNL
			Bone-specific Alkaline Phosphatase (BAP)				
Naylor et al. (2000)	See above	See above	Pre-preg	-	-	↑	-
Wisser et al. (2005)	Switzerland (n=34)	28.4±.0 NP 34.0±4.7	NP	-	-	↑ Vs T1	
Ulrich et al. (2003)	USA (n=15)	32.7±3.4	NP & Pre-Preg	↓	-	↑	↑
Black et al. (2000)	See above	See above	Pre-preg	-	-	↑	
Cross et al. (1995)	See above	See above	NPNL/Pre-Preg	- Vs NPNL	- Vs NPNL	↑ Vs NPNL	↑ Vs NPNL
Rodin et al. (1989)	(n=80)	20-36	NP		↑	↑	-
			Total Alkaline Phosphatase (TAP)				
Ulrich et al. (2003)	See above	See above	NP & Pre-Preg	-	-	↑	↑
Yamaga et al. (1996)	Japan (n=18)	27.5 (3.2)		-	-	↑ vs 5 wks	↑ until 6mo PP
Ardawi et al. (1997)	Saudi Arabia (n=280)	26:8 (5:8) 27.8 (5.3)	NP	↑	↑	↑	↑
Black et al. (2000)	See above	30 (23–40)	Pre-preg	-	-	↑	
Rodin et al. (1989)	See above	20-36	NP	-	↑	↑	-
Dahlman et al. (1994)	Sweden (n=24)	Abstract only	N/A	↑	↑	↑	↓ vs T3, ↑ vs T1

Significant increase or decreases in each marker are indicated by arrows. Where possible it is reported whether a control group was recruited or whether women had pre-pregnancy baseline data from which the differences are expressed. Post-partum data are shown only when considered relevant and not beyond 6 months postpartum.*range given if mean (SD) not available, T1 = 1st trimester, T2 = 2nd trimester, T3 = 3rd trimester, PP = postpartum, NP = non-pregnant, NPNL = non-pregnant non-lactating, BF = breastfeeding

Table 2.3 (continued).

Authors	Country, n	Age *	Controls	T1	T2	T3	PP
			Osteocalcin (OC)				
Akesson et al. (2004)	Sweden (n=254)	31 (20–45)	NP	-	-	↑	↑
Ulrich et al. (2003)	See above	32.7±3.4	NP & Pre-Preg	↓	-	↑	↑
Moller et al. (2013)	Denmark (n=153)	29 (25–35) Age matched	NPNL& Pre-Preg	↓	↓	-	↑
More et al. (2001)	Hungary (n=-38)	26 (19–36)	Pre-Preg		-	↑	↑
Ainy et al. (2006)	Iran (n=95)	26.2 (5) NP 24.1 (5.3)	NP	-	↓	↓	
Sowers et al. (2001)	USA (n=962)	21.0 (0.17) NP 19.6 (0.6)		-	↓	-	
Naylor et al. (2000)	See above	29 (20–36)	Pre-preg	↓	-	↓	↑
Yoon et al. (2000)	South Korea (n=41)	30 (0.9) NPNL 29.5 (0.7)	NPNL	-	↓ Vs NPNL ↓ Vs 8wks	- Vs NPNL ↓ vs 8wks	↑ vs NPNL ↑ vs 8wks
Paoletti et al. (2003)	Italy (n=44)	27.6±2.1 NPNL 28.2±1.8	NPNL	-	-	↓ vs 18 wks, pre-preg, NPNL	-
Wisser et al. (2005)	See above	28.4 (0) NP 34.0 (4.7)	NP	↓$	-	↑ Vs T 1	
Ardawi et al. (1997)	See above	26:8 (5:8) NPNL 27.8 (5.3)	NPNL	↓	↓	↑	↑
Cross et al. (1995)	See above	28.4 (1.0)	NPNL/Pre-Preg	↓ Vs NPNL	↓ Vs NPNL	↓ Vs NPNL	Vs NPNL
Seki et al. (1991)	Japan (n=20)	Abstract only		low	↓	↑	
Ritchie et al. (1998)	USA (n=14)	29.4 ± 2.3	Pre-preg	↓vs PrePreg	↓ vs PrePreg	↓ vs PrePreg	↑ vs T1 – T3

Significant increase or decreases in each marker are indicated by arrows. Where possible it is reported whether a control group was recruited or whether women had pre-pregnancy baseline data from which the differences are expressed. Post-partum data are shown only when considered relevant and not beyond 6 months postpartum.*range given if mean (SD) not available, T1 = 1st trimester, T2 = 2nd trimester, T3 = 3rd trimester, PP = postpartum, NP = non-pregnant, NPNL = non-pregnant non-lactating, BF = breastfeeding

2.3.1.1 Collagen-derived markers of bone resorption during pregnancy

Many studies have used pyridinium crosslinks (DPD and PYD) to investigate maternal bone mineral resorption. Despite conflicting results in early pregnancy (vs NPNL/pre-pregnancy values) consistent increases in these markers into the third trimester and during lactation are reported (Table 2.2) (Yamaga et al., 1996, Ritchie et al., 1998, Black et al., 2000, Naylor et al., 2000, Yoon et al., 2000, Black et al., 2003, Paoletti et al., 2003, Wisser et al., 2005). Yamaga and colleagues performed longitudinal measurements of urinary CTx at 5-9, 28-31 and 36-39 weeks of gestation and reported similar changes observed in CTx to their previous work with PYD and DPD (Yamaga et al., 1996), CTx and NTx were reported to increase significantly in the 3rd trimester of pregnancy and remained high during puerperium compared with non- pregnant or early pregnancy measures ($p<0.05$). Several other studies have also reported a similar trend. Naylor et al. (2000) in a comprehensive study, investigated a wide range of biomarkers during pregnancy finding of both CTx and NTx to be elevated by 36 weeks of pregnancy levels in addition to DPD and PYD ($p<0.05$). They also measured insulin-like growth factor-I (IGF1) and suggested it may be implicated for the increased bone turnover in pregnancy as IGF1 levels were significantly correlated with the change in BTMs at 36 weeks (Naylor et al., 2000). Haliloglu et al. (2011) reported significant differences in CTx between 30 NP controls and 30 pregnant women in the second ($p<0.05$) and third trimesters ($p<0.05$) of pregnancy.

Kaur et al. (2003a) also followed up women from pre-pregnancy to post-partum and in contrast to several of the above studies found early pregnancy decreases in CTx. These differences were likely to due to haemodilation and were attenuated when corrected for albumin, like other studies reported a significant increase in CTx by week 36 of pregnancy (Kaur et al., 2003a). Black et al. (2000) followed up 10 women aged 23-40 years from pre-pregnancy taking serial measures at each trimester, finding NTx increased throughout pregnancy. Ulrich et al. (2003) also reported significant increases in NTx during pregnancy during the third trimester compared to pre-pregnancy values, postpartum NTx declined but remained significantly above baseline. A large

study of Swedish pregnant women reported a significant increase in NTx from the first trimester (mean 12 weeks) to the late third trimester (mean 37 weeks), compared to the late 3rd trimester NTx decreased puerperium (mean 3 days) before increasing again during lactation (Akesson et al., 2004). A comprehensive Danish study of women planning to conceive aged 25-35 years (n=153) of whom 92 conceived measured NTx and ICTP from pre- pregnancy through pregnancy (week 11 ± 2, 22 ± 1 and 35 ± 2) and into lactation. In this study those who did not conceive acted as NPNL in addition to the pre-pregnancy data available from all those who became pregnant. They reported that urinary NTx increased significantly as a function of time ($p<0.001$) rising from early pregnancy and remaining elevated throughout pregnancy compared to pre-pregnancy levels and to 52 non-pregnant, age-matched control ($p<0.001$). ICTP was also found to rise significantly by the third trimester of pregnancy (Moller et al., 2013).

2.3.1.2 TAP and BAP as markers of formation during pregnancy

Alkaline phosphatase (AP) reflects osteoblast function, many studies have used total alkaline phosphatase (TAP) as a marker of bone formation during pregnancy finding for the most part that TAP increases throughout pregnancy particularly in the third trimester (Rodin et al., 1989, Dahlman et al., 1994, Ardawi et al., 1997, Black et al., 2000, Ulrich et al., 2003) and remains elevated from early pregnancy levels into lactation (Dahlman et al., 1994, Ardawi et al., 1997, Ulrich et al., 2003). However, caution is required when interpreting TAP as alkaline phosphatase is also derived from extra-skeletal sources and, in pregnancy the placenta produces an isoenzyme that is excreted into the maternal circulation. The use of bone-specific assays of alkaline phosphatase is essential because, unlike TAP, most studies that have measured bone-specific AP (BAP) have reported little significant change until late pregnancy when values are significantly higher than in pre-pregnancy, NPNL, or the first trimester (Table 2.3). However, Ulrich et al. (2003) reported first trimester decreases compared to pre- pregnancy but this may have been as a result of haemodilution. Naylor et al. (2000) reported that levels of BAP increased ($p<0.05$ vs pre-pregnancy levels) by 36 weeks of pregnancy. Moller et al. (2013) also found that BAP increased as a function of time, in early

pregnancy they were lower than in controls ($p<0.001$), however, in late pregnancy they had surpassed control levels.

2.3.1.3 Collagen-derived markers of bone formation during pregnancy

Relatively few studies have used P1NP or P1CP during pregnancy, Naylor et al. (2000) reported that levels of P1CP, and P1NP increased ($p<0.05$ vs prepregnancy) by 36 weeks of pregnancy. Both Kaur et al. (2003a) and Cross et al. (1995) reported reduced P1NP from prepregnancy. This, however, was likely to have been due to haemodilation because the authors reported that correcting for albumin removed this apparent early pregnancy reduction, similar to the finding of Naylor et al. (2000) P1NP was reported to be significantly to be elevated at 36 weeks of gestation. Other studies have also found late pregnancy increases in P1CP (Cross et al., 1995, Black et al., 2000), although one of these studies reported decreases during early pregnancy compared to NPNL controls (Cross et al., 1995).

2.3.1.4 Osteocalcin (OC) as a Marker of Bone Formation during Pregnancy

OC has been widely used as a marker of bone formation in pregnancy (Rodin et al., 1989, Seki et al., 1991, Ardawi et al., 1997, Ritchie et al., 1998, Naylor et al., 2000, Sowers et al., 2000, Yoon et al., 2000, More et al., 2001, Paoletti et al., 2003, Ulrich et al., 2003, Akesson et al., 2004, Wisser et al., 2005, Ainy et al., 2006, Moller et al., 2013, Ettinger et al., 2014). These studies have suggested that OC is reduced in early pregnancy which also may be attributable to haemodilation. Data are inconstant in late pregnancy with a number of these studies reporting increases (Ardawi et al., 1997, More et al., 2001, Ulrich et al., 2003, Akesson et al., 2004, Wisser et al., 2005) and others reporting decreases in OC (Ritchie et al., 1998, Naylor et al., 2000, Yoon et al., 2000). Many studies have historically used OC as a formation marker (Ardawi et al., 1997, Naylor et al., 2000, Paoletti et al., 2003, Akesson et al., 2004, Wisser et al., 2005, Moller et al., 2013) with several accrediting rising OC levels during pregnancy to formation. Late pregnancy increases in OC may need to be interpreted with some caution as they may stem from OC secreted by osteocytes trapped within

the bone matrix released due to the resorption. Although increases in P1NP and P1CP in late gestation cannot be discounted as easily.

2.4 Estimating changes in maternal bone mineral during pregnancy

2.4.1 Direct measurements of maternal bone mineral during pregnancy

Directly measuring changes in maternal bone mineral during pregnancy is invasive as it would involve taking of bone biopsies which are painful. Only one study to date has measured bone histomorphology during pregnancy with bone biopsies from the iliac crest a common biopsy site rich in trabecular bone (Figure 2.4). Purdie et al. (1988) used a cross-sectional study design with iliac crest biopsies from 1) women planning elective 1st trimester terminations (n=15), 2) women with scheduled 3rd trimester C-sections (n = 13), 3) non-pregnant women (n=40: living n=14; cadaveric n=26). From these samples the authors suggested that changes in maternal trabecular bone take place in early pregnancy (Purdie et al., 1988, Shahtaheri et al., 1999). However, their sample was limited by poor age-matching between the groups (1: 22.4 ± 3.0 years; 2: 25.1 ± 5.0; 3: 32.6 ±8.1 years).

Figure 2.4 Potential pregnancy-induced change in trabecular architectural variables of trans-iliac bone biopsies.

Photomicrographs (top) and stylised drawings derived from trabecular architectural variables of trans-iliac bone biopsies of potential modification induced by pregnancy in trabecular bone. Cross-sectional bone biopsies from: (A) Non-pregnant woman with interconnected trabecular network; (B) early pregnancy where there is less bone and thinner, less connected trabeculae; (C) late pregnancy where the bone volume is restored by more numerous finer struts. Study limitations are noted above. Source: Shahtaheri et al. (1999)

2.4.2 DXA and pregnancy-induced changes in aBMD

Much of what we know about bone changes during pregnancy comes from techniques that measure areal BMD (aBMD) before pregnancy and again shortly after term, capturing the cumulative effect of pregnancy and the early post-partum period. Earlier studies used single photon absorptiometry (SPA) and later dual photon absorptiometry (DPA) and more recently studies with similar longitudinal designs to the earlier literature have used DXA (Drinkwater and Chesnut, 1991, Sowers et al., 1991, Holmberg-Marttila et al., 1999, Black et al., 2000, Naylor et al., 2000, More et al., 2001, Butte et al., 2003, Fiore et al., 2003, Kaur et al., 2003b, Ulrich et al., 2003, Pearson et al., 2004, Olausson et al., 2008, Moller et al., 2012). The primary limitation of DXA is that it cannot be used to measure common clinical sites in the axial skeleton due to the position of the fetus obstructing clear imaging of the lumbar spine and concerns over ionising radiation exposure in utero. A further limitation of DXA is that it lacks sensitivity because it measures a two dimensional projection of bone and cannot separate bone compartments.

A number of these studies show statistically significant decreases in whole body, lumbar spine (L1-L4), total hip, trochanter, femoral neck, and radial aBMD (Table 2.4). There are few longitudinal DXA data on the effect of pregnancy in LMIC, although a number of studies based in the USA have included ethnically varied populations. However, the main difficulty with many of the studies discussed in the period 'shortly postpartum' when the follow scans were obtained it is not specified clearly whether the mothers were exclusively breast-feeding or not. With rapid mobilisation occurring in the early stages of lactation, this is an important consideration when trying to attribute any observed changes to pregnancy.

Table 2.4. Mean change (%) using DXA, DPA, and SPA in healthy women pre-pregnancy (PRE) to postpartum (POST) at different sites. Adapted from Olausson et al.2012

Study	Subject details	Modality and measurement	Pre-pregnancy and postpartum timing	Mean age (SD)	NPNL controls	Whole body	Spine	Total hip	Trochanter	Femoral neck	Radius
Moller et al. (2012) ‡	Denmark, Healthy (n=153)	DXA: aBMD	<15 days PP	29 median	Yes (52)	-2.4	-1.8	-3.2	NA	NA	RW -4.2
Olausson et al. (2008) ‡	UK, Healthy (n=114)	DXA: BA adjusted BMC	PRE <13mo (median 5mo) POST 15±5d (10–21d)	31.7 (3.7)	Yes (84)	-1.7***	-2.6***	-2.2***	-3.7***	-1.4*	RW -0.08 RS -0.94 NS
Pearson et al. (2004)‡	UK, Healthy (n=60)	DXA: aBMD	PRE <16mo POST 2wks (median 8d)	30.9 (4.5)	No	NA	-1.5**	-1.2*	-3.9***	0 NS	NA
Kaur et al. (2003b)‡	UK, Healthy (n=76)	DXA: aBMD	PRE <13mo POST <2wks	31 (5)	Yes (30)	NA	-0.9 NS	-1.2 NS	-4.2 NS	-0.7 NS	NA
Butte et al. (2003)§	USA, (n=63)	DXA: BMC¶	PRE 179±184d POST 2wks	31 (4)	No	-2.0 -0.8 -0.5*	In women of pre-pregnant BMI, 19·8 kg/m2 In women of pre-pregnant BMI, 19·8–26·0 kg/m2 In women of pre-pregnant BMI, 26·0 kg/m2				
Fiore et al. (2003)‡	Italy, Healthy (n=16)	D XA: aBMD ‖	PRE <90d POST 2wks	21 - 35	No	-13.4*	-9.2*	NA	NA	-7.8*	NA
Ulrich et al. (2003) ‡§	USA (n=30) 7 attending fertility clinics	DXA: aBMD	PRE <6 months POST <2 weeks	32.7 (3.4)	NPNL (15)		-3.4**	1·8 NS	-4.3***	-1.7 NS	RS 1.3*
More et al. (2003)§	Hungary, Healthy, (n=38) 1st pregnancy	DXA: aBMD	PRE <3mo POST <6d	26 (19–36)	No	NA	-2.1***	NA	NA	NA	RS, RW -3.8***
Naylor et al. (2000)‡§	UK, Healthy (n=16)	DXA: aBMD	PRE <8mo (mean <3mo) POST <4wks (mean <2wks)	29 (20–36)	No	<+1	-4.5*¶¶	NA	NA	NA	NA
Black et al. (2000) ‡§	USA, (n=10) recurrent miscarriage clinic	DXA aBMD	PRE not defined POST 6 weeks	30 (23–40)	No		-2.0* ‖‖	-3.6*	-4.8**	-2.0*	RS -4.2NS RW-3.1NS
Holmberg-Marttila et al. (1999)§	Finland, Healthy (n=5)	DXA: aBMD	PRE <17wks POST 'some days'	27 (2.6)	No	NA	-2.8†††	NA	NA	-0.4*†††	RS -3.5*†††
Ritchie et al. (1998)§	USA, Healthy (n=14)	DXA: aBMD and QCT	PRE 3.5±3·2mo POST 1–2wks	29.4 (2.3)	No	+0.5NS	QCT	NA	NA	NA	NA
Drinkwater and Chesnut (1991)‡	USA, Athletes (n=31)	DPA & SPA: aBMD	PRE 3.3mo POST <6wks	30 (0.5)	Yes (25)	NA	-3.6 NS	NA	NA	-2.4*	RS -2.2*
Sowers et al. (1991)§	USA, Healthy (n=32)	DPA: aBMD	PRE not defined POST <15d	29.6 (3.6)	Yes	NA	NA	NA	-1.2 NS	+1.2 NS	NA

NPNL, non-pregnant, non-lactating; NBF, non-breast-feeding; BA, bone area; BMC, bone mineral content; RS, radius shaft; RW, radius wrist; aBMD, areal bone mineral density; QCT, quantitative computed tomography.
Statistically significant: * P<0·05, ** P<0·01, *** P<0·001, NS, non-significant, as indicated in original papers.
†All data are for measurements without correction for changes in body weight.
‡ Bone mineral data taken from paper. § Bone mineral data derived from tables or figures in paper.
‖ Implausible values?
¶Data adjusted for weight.
‖‖ Note: value of -3·5% cited in abstract is incorrect.
¶¶Derived from the original paper by Olausson et al., 2012 from whole-body scan divided into sub regions.
††† Given the small sample size, the original authors concluded that there is a tendency for a decrease in bone mineral status at the spine, but not at the femoral neck or radial shaft.

Sowers et al. (1991) followed up 32 pregnant women in US from preconception to within 15 days of giving birth, performing scans of the femoral head (i.e. femoral neck, trochanter, Ward's triangle) with DPA (Lunar DP3 bone densitometer). This was a follow-up from a cross-sectional study of maximal bone mass in women aged 20-40 years allowing a large number of potential NPNL controls for age- (±3 years), height- (±3 cm), weight- (±3 kg), and parity- (±1) matching. No significant aBMD differences were observed between pregnant women compared to NPNL at any site. A longitudinal study in USA by Drinkwater and Chesnut (1991) used DPA (Ohio Nuclear Series 84) at 5 sites (lumbar spine Ll-L4, femoral neck, femoral shaft, tibia, and fibula) and SPA (Norland-Cameron Bone Analyzer, Model 178) at the forearm (10%, and 20% proximal from the ulnar styloid process) in 6 pregnant women and 25 non-pregnant women. Significant decreases in aBMD at the trabecular-rich femoral neck and at the cortical-rich radial shaft was reported in the pregnant women (Table 2.4, both $p<0.05$). However, aBMD of the tibia, a loadbearing bone, increased in both groups (both $p<0.05$). Pregnant women had an 8.2% increase in body weight during the course of their pregnancy while no increase in control group weight was reported, which may have partly explained the increase in aBMD in the tibia. (Drinkwater and Chesnut, 1991). Holmberg-Marttila et al. (1999) obtained DXA scans from 5 pregnant Finnish women from before pregnancy until up to a year postpartum, reported a tendency for a decrease in bone mineral status at the spine, but not the femoral neck or radial shaft shortly postpartum. Ritchie et al. (1998) performed repeated DXA (DPX, Lunar Radiation Corporation, WI) and QCT (model 9800, General Electric, Milwaukee) measures on 13 US women from pregnancy to postpartum, collecting BTMs and other biochemistry also (Tables 2.2 & 2.3). No statistically significant changes were observed by DXA or QCT from baseline to shortly postpartum.

One of the most comprehensive studies in the literature was conducted by Naylor et al. (2000) who studied 16 women longitudinally in the UK, with DXA (DPX, Lunar Radiation Corporation, WI) measurements pre-pregnancy and postpartum and supported by biochemistry and BTMs collected at baseline, 16, 26, and 36 weeks of pregnancy, and 2 weeks postpartum. They reported

aBMD decreases at trabecular rich axial sites of the spine ($p<0.01$) and pelvis ($p<0.05$). Total-body scans were divided into sub-regions for analysis of different body sites reporting increases in aBMD at sites where cortical bone predominates in the appendicular skeleton (arms ($p<0.05$), legs ($p<0.01$)). Biochemistry and BTM data supported the densitometry data indicating that elevated bone turnover may explain trabecular bone mobilisation during pregnancy at the lumbar spine (Tables 2.3 and 2.4).

More et al. (2001) used DXA to measure changes in the maternal skeleton (n=38) from pre-pregnancy and throughout lactation until 12 months postpartum. This study recruited only first time mothers. Lumbar spine (L2–L4) and forearm scans (ultra-distal radius and one-third radius) were obtained. During pregnancy DXA was only performed at the forearm. Significant decreases between pre-pregnancy and <6 days postpartum were found at the lumbar spine, ultra-distal radius (trabecular site) and one-third radius (cortical site) (all $p<0.001$, Table 2.4). A study by Butte et al. (2003) to evaluate how changes in gestational weight and body composition affect infant birth weight and maternal fat retention after delivery collected DXA data in underweight, normal-weight and overweight US women. Significant differences in BMC were observed between BMI groups before pregnancy ($p=0.001$). After delivery maternal whole body aBMD had significantly decreased in each BMI category from pre-pregnancy (Table 2.4).

Kaur et al. (2003b) explored longitudinal aBMD changes from pre-pregnancy to shortly postpartum in the same population on which they previously published BTM changes during gestation (Kaur et al., 2003a). DXA data were collected from 46 women who became pregnant and 30 controls who did not conceive. Non-significant declines in aBMD were observed at the spine (L1–L4), total hip, femoral neck, and trochanter. However, a significant difference was seen at the trochanter between those who did and did not become pregnant ($p<0.01$). The same group subsequently reported that in 60 women measured from preconception to 2 weeks postpartum significant decreases in aBMD occurred at the lumbar spine ($p<0.01$), total hip ($p<0.05$) and

trochanter ($p<0.001$) while change at the femoral neck was not statistically significant (Kaur et al., 2003b, Pearson et al., 2004)(Table 2.4).

Two studies stand out in the literature due to their large sample size and large number of representative NPNL controls. Olausson et al. (2008) scanned 34 pregnant participants pre-pregnancy and at 2 weeks postpartum, 84 NPNL participants were also scanned with a similar time difference between visits. BA-corrected BMC in this study was used to allow for the known relationship between BMC and scanned bone area (BA) that is not fully accounted for in the measure of aBMD (Dibba et al., 1999, Olausson et al., 2008). Pregnant participants had statistically significant decreases in BA-adjusted BMC for total body ($p<0.001$), spine ($p<0.01$), total hip ($p<0.001$), trochanter ($p<0.001$), femoral neck ($p<0.05$) (Table 2.4 below). However, the authors noted that there was considerable between-subject variation in bone change. These findings are supported by those of Moller et al. (2012) who obtained longitudinal DXA measures at the lumbar spine, hip and whole body from pre-pregnancy (with forearm scanned during pregnancy) and then in multiple time points during lactation, additionally 75 NPNL women were followed up concurrently. Compared with NPNL participants the pregnant women showed decreased aBMD at all sites 15 days after delivery ($p<0.01$) (Moller et al., 2012). Furthermore, aBMD measures were taken at the distal, one-third proximal, and total forearm during pregnancy with decreases reported ($p<0.001$) (Table 2.4). As noted above Moller et al. (2013) also measured BTMs during pregnancy finding that BAP was significantly lower in the pregnant women compared to NPNL while urinary NTx/Creatinine ratio was significantly higher (both $p<0.05$), adding further support to the possibility of skeletal changes occurring during pregnancy in this population (Moller et al., 2013).

The largest significant mean change in maternal aBMD between pre-pregnancy and 2 weeks postpartum was reported by Fiore et al. (2003), ($n=16$). Significant decreases aBMD of total body (- 13.4%), the spine (-9.2%) and hip (-7.8% at the femoral neck and -9.2 % at Ward's triangle) were reported and while such changes appear implausibly large compared with other studies (perhaps suggesting technical problems), the same study obtained QUS data and showed a large BUA

decrease (-14.5%) concurrent to the DXA findings. Given that DXA and QUS typically have weak to moderate correlation, it is difficult to confirm the validity of these data and even in the context of mineral mobilisation during lactation these changes seem very large.

Two additional studies found similar differences to those described above but were conducted in two slightly different groups of women that potentially differ from the general population. It is important to consider that an underlying hormonal problem can affect normal bone turnover and may confound pregnancy-induced bone mineral changes. Black et al. (2000) followed 10 patients attending recurrent (≥2 previous) miscarriage clinics. The authors reported oestrogen levels, thyroid function, follicle-stimulating hormone, and luteinising hormone were all normal in the study population. Significant decreases were found by 6 weeks postpartum at the spine, total hip, trochanter, and femoral neck. However with such a lengthy follow-up postpartum these data are highly confounded by lactation ($p<0.05$). Ulrich et al. (2003) recruited patients attending fertility clinics (n=7), some of whom were on treatment, and healthy, normally-menstruating women (n=8) planning a pregnancy through natural conception with no previous history of infertility. NPNL controls (n=15) were also recruited. By two weeks postpartum statistically significant decreases at the spine ($p<0.01$) and trochanter ($p<0.001$), while an increase was reported at the radial shaft ($p<0.05$). The authors did not investigate whether any differences could be found between the women with a history of fertility problems and those who did not (Ulrich et al., 2003).

In summary, the fact that these studies have consistently shown decreases in trabecular-rich bone sites from preconception to shortly postpartum, with the women acting as their own controls or with an NPNL group scanned at concurrent time points, seems to support that maternal bone mineral is released from the maternal skeleton. However, these data unfortunately cannot tell us whether the changes observed occur during the pregnancy or occur in the immediate postpartum period because mineral is lost from the maternal skeleton at the onset of lactation. Despite this common limitation, these studies, especially those with accompanying measures of BTM, provide at present some of the best evidence for changes in maternal bone mineral during pregnancy.

Olausson and colleagues reported a decrease of approximately 2% in whole body aBMD which would equate to ~25 g of Ca being mobilised from the maternal skeleton, which, interestingly, is close to the approximate Ca content of the fetal skeleton at term (Olausson et al., 2008).

2.4.2.1 DXA of the appendicular skeleton during pregnancy

The second important body of densitometry data comes from studies that used DXA and its precursors to image the appendicular skeleton during pregnancy. There is some overlap with the studies listed in Table 2.4, however, several of these studies of the peripheral skeleton lack pre-pregnancy or postpartum data. Data discussed here are from forearm SPA, DPA, and DXA scans (Figure 2.5) and should not be confused with studies performing sub-regional analysis of whole body scans. Data are presented as change in aBMD, with data from forearm scans reported from the ultra-distal radius, one-third distal radius and/or the mid radius (radial shaft). The terminology used to describe these sites differs between studies but simply put the distal radius contains a relatively high proportion of trabecular bone and a thin cortex, progressing proximally the cortex thickens and the amount of trabecular bone diminishes greatly (Figure 2.6). Importantly, the distal radius is a common fracture site in later life

The data are inconclusive with most studies finding no significant change across pregnancy (Table 2.5). However, the findings of both Kolthoff et al. (1998) and Moller et al. (2012) in Danish women suggest changes could take place during pregnancy at the radius. Kolthoff et al. (1998) reported changes at the ultra-distal radius during pregnancy amounting to a 2% reduction in aBMD within 2 weeks postpartum. Moller (Moller et al., 2012) also investigated several sites at the forearm (ultra-distal, proximal 1/3 distal forearm, and total forearm) during pregnancy. At the ultra-distal forearm, measurements performed during pregnancy showed a progressive aBMD reduction compared with the NPNL group ($p<0.001$). Total forearm aBMD was also reported to decline in late pregnancy however this is likely a reflection of the changes at the ultra-distal radius. This highlights the limitations of aBMD at the total forearm as measured by DXA because it is unable to distinguish between cortical and trabecular bone compartments.

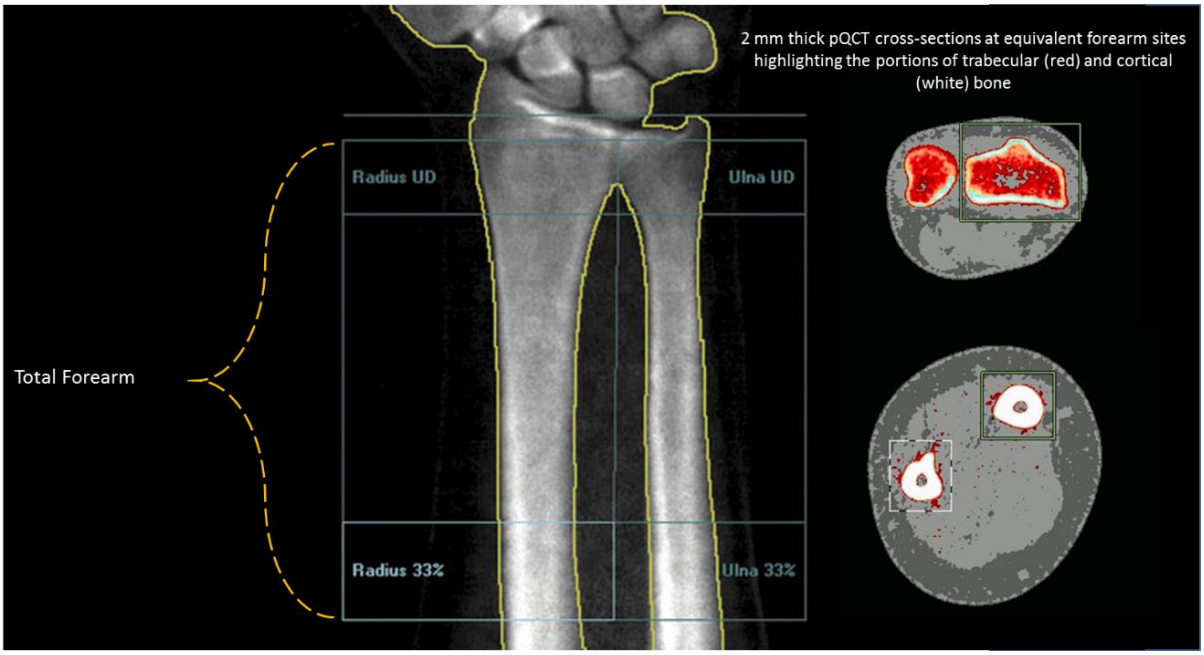

Figure 2.5 DXA scan of the forearm, highlighting the regions of interest (ROI) that are analysed.

pQCT scans are shown adjacent for context and to highlight the relative proportions of each type of bone at these sites. The distal radius is a relatively trabecular-rich site (top right), while the 1/3 proximal radius, is a cortical-rich site (bottom right). The analysis of the total forearm ROI includes both these regions and as such is an admixture of both compartments.

2.4.3 Peripheral QCT techniques and pregnancy-related changes in vBMD

pQCT techniques provide measures and estimates of bone size, shape, mineral content, while HRpQCT additionally assesses cortical and trabecular microarchitecture. Furthermore the data acquired are not as size-dependant as aBMD from DXA, and provide a compartment-specific measures (i.e. cortical and trabecular bone) of density, mass, bone size, shape and distribution. Despite these advantages, to date only one study has used a pQCT technique (Densiscan 1000, Scanco Medical) in pregnancy (Wisser et al., 2005). Their method differs from the single slice systems discussed previously (see section 1.5.3) and was the precursor of the modern Scanco HRpQCT systems. The Densiscan had a voxel size of 0.3mm is similar to that of single-slice pQCT techniques, but rather than a single slice a stack of tomograms are acquired (slice thickness 1mm, interslice distance 1.5mm). In-keeping with single-slice pQCT the resolution of this technique is too low to make simultaneous measurements of trabecular and cortical bone: trabecular vBMD is obtained at the ultra-distal radius; cortical vBMD was measured closer to the bone diaphysis.

(Dambacher et al., 1998). The authors reported wide inter-individual variation in trabecular vBMD with significant decline between the 1st and 3rd trimesters ($p<0.001$). No significant changes were noted in the cortical compartment of the radius. Whilst no data have been published to date using single-slice pQCT or HR-pQCT in pregnancy, HRpQCT has recently been used in a number of studies during lactation where changes in trabecular and cortical microarchitecture in the appendicular skeleton are reported. These studies add to the already large body of literature of lactation related bone changes and are beginning to provide a better understanding of whether full repletion of lactation-mobilised bone mineral occurs (Brembeck et al., 2014, Bjornerem et al., 2017).

2.4.4 QUS to measure surrogates of BMD during pregnancy

Qualitative Ultrasound (QUS) has been used to quantify changes in bone during pregnancy as it is non-invasive, non-ionising, and inexpensive. QUS is used at peripheral sites that have little soft tissue to interfere with attenuation such as the calcaneus, phalanges, tibia, and radius. Several studies suggest there are decreases in QUS measured SOS (speed of sound) and BUA (bone ultrasound attenuation) parameters of bone quality during pregnancy at the calcaneus and phalanges (Table 2.6) (Yamaga et al., 1996, Akesson et al., 2004, Javaid et al., 2005, To and Wong, 2011, Kraemer et al., 2012, Hellmeyer et al., 2014, Whisner et al., 2014). However, Fiore et al. (2003) who also used DXA pre- and post-pregnancy (Section 2.4.2) used calcaneal QUS pre-pregnancy and at 16, 26, and 36 weeks of gestation and <2 weeks postpartum. They found no significant changes across pregnancy but reported a significant BUA decrease of 14.5% postpartum (Fiore et al., 2003). The lack of change during the pregnancy time points observed may suggest that if losses occur in gestation they may be quite late towards the second half of the 3rd trimester or postpartum. However, it may also be the case that perhaps the calcaneus is not truly representative of the changes occurring elsewhere in the skeleton.

QUS parameters from the phalanges have been reported to progressively decrease from the first trimester into the second and third trimesters. Hellmeyer et al. (2006) reported AD-SOS (amplitude

dependant-SOS) was significantly lower in the second and third trimesters compared to the first ($p \leq 0.001$) and that bone transmission time (BTT) decreased significantly in second and third trimesters ($p \leq 0.001$). Della Martina et al. (2010) also reported a progressive decrease of BTT from the first to the third trimester, however, AD-SOS remained unchanged between the first and second trimester only dropping in late pregnancy. Pluskiewicz et al. (2004) performed in each trimester reporting significant differences between 1st and 2nd AD-SOS measurements ($p<0.01$), 2nd and 3rd, and 1st and 3rd (both $p<0.001$). A significant difference between 1st and 2nd trimester diameter ($p<0.001$) and 2nd and third trimester finger diameter ($p<0.001$), would indicate changing soft tissue composition at the scan site. Similarly, as oedema around the lower limbs is common during pregnancy, additional soft tissue may interfere with sound wave attenuation and resulting QUS data. While this may affect techniques that scan at the calcaneus or phalanges, techniques that scan the radius or tibia are less effected. An important caveat regarding the usefulness of QUS during pregnancy comes from the literature of bone mineral changes in lactation. During lactation, bone mineral mobilisation from the lumber spine was detected with DXA, but QUS did not find a concurrent change at the calcaneus in the same women (Laskey and Prentice, 2004). This cautions against extrapolating from QUS measures in the peripheral skeleton to those that may occur in the axial skeleton with DXA.

Table 2.5 Investigation of bone mineral changes in the maternal appendicular skeleton (radius) during pregnancy

Authors	Country, n	Modality and measurement	Pre-preg/NPNL	T1	T 2	T3	PP
Christiansen et al. (1976)	Denmark, n=13	SPA: UD, One-third distal, RS	No		-	-	
Kent et al. (1993)	Australia, n=37	SPA: UD, One-third distal, RS	No		-	-	
Cross et al. (1995)	USA, n=10	SPA: UD, One-third distal	NPNL	-	-	-	
Kolthoff et al. (1998)	Denmark, n=59	DXA: UD, One-third distal	Yes		-		2% ↓ vs 18wks
Black et al. (2000)	USA, n=10	DXA: UD, One-third distal, RS	Yes	-	-	-	
More et al. (2001)	Hungary, n=38	DXA: UD, One-third distal	Yes		NS		↓ at 1wk vs 6 mo
Moller et al. (2012)	Denmark, n=153	DXA: UD, RS	NPNL	progressive ↓ in UD BMD vs NPNL (p<0.001)			

Pre-preg = Pre-pregnancy,T1 = 1st trimester, T2 = 2nd trimester, T3 = 3rd trimester, PP = postpartum, DPA = Dual Photon Absorptiometry, DXA = Dual-energy X-ray Absorptiometry, SPA = Single Photon Absorptiometry, RS = radial shaft, UD = ultra-distal

Table 2.6 Longitudinal studies using QUS at two or more time points during pregnancy to measure surrogates of bone mineral density

Author	Country	Site	T1	T2	T3	PP
Hellmeyer et al. (2014)	Germany, n=125	calcaneus	-	-	↓	
Whisner et al. (2014)	USA, n = 156	calcaneus	Significant ↓ in SOS, BUA, and QUI across pregnancy			
Kraemer et al. (2012)	Germany, n=200	calcaneus	-	-	-	Median ↓ SOS vs T1
Della Martina et al. (2010)	Finland, n=59	phalanges	-	BTT ↓	BTT & AD-SOS↓ at term	
Hellmeyer et al. (2006)	Germany, n=60	phalanges	-	BTT & AD-SOS ↓ vs T1	BTT & AD-SOS ↓ vs T1	
Javaid et al. (2005)	UK, n=307	calcaneus	-	-	↓ SOS & BUA vs early preg	
Akesson et al. (2004)	Sweden, n=254	calcaneus	-	-	NS vs early preg	Stiffness index ↓ vs T1
Tanquilli et al. (2004)	Italy, n=200	phalanges	AD-SOS ↓	AD-SOS ↓	AD-SOS ↓	
Pluskiewicz et al. (2004)	Poland, n=48	phalanges	↓	↓	↓	-
Fiore et al. (2003)	Italy, n=16	calcaneus	No significant change during pregnancy			↓
To et al. (2003)	China, n=780	calcaneus	Significant ↓in simulated BMD across pregnancy			
Yamaga et al. (1996)	Japan, n=18	calcaneus	-	-	SOS ↓ vs T1	

Note BMD cannot be measured with QUS and simulated BMD is not a true measure of bone mineralisation. T1 = 1st trimester, T2 = 2nd trimester, T3 = 3rd trimester, PP = postpartum, QUS = qualitative ultrasound, BUA =bone ultrasound attenuation, SOS = speed of sound, AdSOS = amplitude dependant speed of sound, BMD = bone mineral density, BTT = Bone Transit Time

2.5 Discussion and conclusions

The evidence of pregnancy-induced maternal bone mineral mobilisation comes from studies using diverse techniques including bone turnover and biochemistry (section 2.3.1), bone densitometry and QUS (sections 2.4.2, 2.4.3 & 2.4.4), and bone histomorphology (section 2.4.1). When these data are carefully considered in the context of known pregnancy-induced adaptions in Ca homeostasis (section 2.3), there appears to be a reasonable case for pregnancy-induced maternal skeletal adaptation to satisfy at least a proportion of fetal Ca requirements. While few data at present support early-pregnancy bone mineral mineralisation when fetal Ca accrual and requirement are low (Purdie et al., 1988, Shahtaheri et al., 1999, Whisner et al., 2014), a strong case can be made for transient mineral mobilisation from the maternal skeletal reserves in late gestation when Ca transfer and accretion peak (section 2.3). Due to a lack of densitometry during pregnancy the primary question of this work remains unanswered: Is maternal bone mineral mobilised during pregnancy?

If mobilisation does occur few data are available to explain from where in the maternal skeleton (axial vs appendicular sites; trabecular vs cortical compartment) this increased resorption happens due to the global nature of BTM and our present inability to make axial measures during pregnancy. Data presented in Table 2.4 reflect the cumulative effects of pregnancy and early-lactation, losses occur mostly at trabecular-rich skeletal sites, in-keeping with data from studies of breastfeeding. It is impossible to determine to what extent the initiation of lactation has confounded these data. The data presented in Tables 2.5 and 2.6 from peripheral DXA scans of the forearm and QUS of the calcaneus and phalanges during pregnancy are inconclusive, in part due to the limitations of the techniques used. Peripheral DXA scans provide an aBMD measurement without additional context of bone shape or mineral distribution, the inability to make independent measures of the respective bone compartments could potentially cloud any redistribution of mineral from one to the other. Interestingly Moller et al. (2012) reported significant progressive decreases in aBMD at the trabecular-rich distal radius (but not the radial shaft) during pregnancy, which is in keeping with the pattern from trabecular sites in Table 2.4. Body composition changes during pregnancy may

interfere with the precision and accuracy of QUS, while how well changes in QUS parameters reflect any changes in the axial skeleton is questionable.

pQCT techniques although not widely used in the literature overcome a number of the limitations of DXA and QUS. Wisser et al. (2005) using an early pQCT modality (limited to measures of vBMD only) reported compartment-specific changes during pregnancy, with wide inter-individual variance found in trabecular vBMD without change in cortical vBMD. However, the cortical site scanned with this technique is still relatively distal and more accurate measures would be obtained if the cortex was measured at its thickest closer to the midshaft of the bone. Single-slice pQCT scanners can provide additional parameters beyond vBMD such as bone mass, geometry, distribution and strength at both trabecular- (distal) and cortical-rich (proximal) sites. Another advantage is that any site along the length of the bone can be selected for scanning. However, single-slice pQCT does not have sufficient resolution to detect changes in bone microstructure, such adaptations could occur without overt changes in vBMD. HRpQCT has been used to show changes in both cortical and trabecular compartments during lactation (Brembeck et al., 2014) and provides micro-architecture data such as trabecular thickness, number and separation in addition to cortical thickness and porosity.

There is a genuine need to establish whether maternal skeletal adaptations occur across gestation. Ca is a vital mineral to bone health and were pregnancy to temporarily disrupt maternal reserves it is important to understand to what extent it may happen. Pregnancy and lactation are not considered risk factures for osteoporosis, and lactation associated maternal bone mineral mobilisation has been repeatedly found to be mostly transient in nature (Laskey and Prentice, 1999, Olausson et al., 2008, Laskey et al., 2011, Moller et al., 2012), however, a recent study which followed up women over five years has hinted towards the possibility of lasting skeletal micro-architectural adaptations following lactation (Bjornerem et al., 2017). In the context of maintaining optimal bone health across the lifecourse, if changes occur as a result of pregnancy and indeed lactation, the replenishment of any mineral mobilised from the maternal skeletal reserves prior to

reaching menopause may be important for bone health in later life. The majority of studies described above recruited women in their 20's – 30's, there are limited data on reproduction and bone health during adolescence and no studies have specifically focused on women who conceive at an advanced maternal age (>35 years). In 2016 54% of all live births in England and Wales were to women aged >30 years. It is likely that the proportion of women having pregnancy later in life will continue to rise in the future. In addition to this age-bias, the majority of studies are in:

a) High income counties (HIC);

b) Women of European ancestry;

c) Populations with habitually high Ca intakes.

Consequently most of the studies cited above are from Europe, North America, and Japan, with few data available are available from LMIC in Africa and Asia. Whatever the barriers to such studies being conducted the result is an almost complete lack of data of bone mineral homeostasis during pregnancy and lactation in populations of different ethnicities and with low habitual Ca intakes. Given our knowledge of ethnic differences in BMD, bone geometry and other bone structural parameters, we cannot confidently apply findings from HIC countries with populations of predominantly European ancestry to other ethnic groups. With the available data also primarily drawn from Ca replete populations, we do not know if fetal demand for Ca places mothers with a habitually low Ca intake under increased pressure during pregnancy.

2.6 Thesis objectives

The overarching aim of my PhD research was to determine whether a maternal skeleton response resulting in the mobilisation of bone mineral occurs during pregnancy, using non-invasive pQCT (single slice- and HRpQCT) techniques. Both studies presented in this thesis are formed around a shared primary research question: is bone mineral mobilised from the maternal skeleton between early- and late pregnancy? Each study also aims to contribute towards bridging some of the major

research gaps highlighted in the above discussion. The ENID Bone Study presented in chapters 3 and 4 is from a Sub-Saharan subsistence farming community in The Gambia, a LMIC resource-poor country with a notably low habitual Ca intake. The Pregnancy and Bone study (PABS) was conducted in Cambridge, UK and focused on women aged 30-45 years to explore mineral adaptations in pregnancy in women with a higher maternal age than found in the majority of the available literature.

2.6.1 Primary thesis objective

This primary objective of this research was to characterise whether changes occur in the maternal appendicular skeleton by measuring total and trabecular vBMD with pQCT (in both the Gambian and Cambridge studies), and HRpQCT (in our Cambridge cohort). HRpQCT also allowed for the investigation of change in trabecular microarchitecture during pregnancy at the distal radius and tibia.

2.6.2 Secondary thesis objective

My secondary objective focused on cortical bone and whether any skeletal adaptions occur during pregnancy at cortical-rich sites of the proximal radius and tibia using pQCT (in the Gambian and Cambridge cohorts) and at distal radius and tibia with HRpQCT (in the Cambridge cohort) to determine if changes in the cortical microarchitecture may occur.

2.6.3 Thesis Aims

My aims were to determine:

1. Bone mobilisation from the trabecular compartment at the distal radius and tibia between early- to late-gestation in:

a. A sub-Saharan African population taking part in a 4-way supplementation trial using pQCT at the distal radius and tibia (total and trabecular vBMD)

b. A Cambridge based cohort using pQCT at the 4% distal radius and tibia (total & trabecular vBMD) and HRpQCT (trabecular vBMD, trabecular microarchitecture: number, thickness, and separation).

2. Whether changes occur in the cortical compartment from early- to late-gestation in:

   a. Both cohorts using pQCT at the proximal radius and tibia with a focus on cortical vBMD and other parameters of bone geometry, mass and distribution (total and cortical cross-sectional area, cortical content (BMC), cortical thickness).

   b. Cambridge using HRpQCT to determine cortical vBMD & cortical microarchitecture (thickness & porosity).

3. To determine any predictors of change in 1. and 2. with a specific focus on:

   a. Supplement, parity, and age effects in the ENID cohort

   b. Maternal age in the PABS cohort

4. To examine the correlation between pQCT and HRpQCT in-vivo measurements in the Cambridge cohort above

3 ENID Bone Study pQCT Subjects and Methods

3.1 Introduction

As outlined in Chapter 2 there are few data from which to quantify a maternal skeletal response to pregnancy. However, there are indications of change in maternal bone turnover from bone turnover markers. The current evidence base is highly skewed towards high-income countries (HIC) with predominantly Caucasian cohorts. No densitometry data have been collected from pregnant Sub-Saharan populations living in low and middle income countries (LMIC). The data presented here were collected as part of a bone-focused add-on study (PI Prof Ann Prentice) to The Early Nutrition and Immune Development (ENID) Trial (PI Dr Sophie Moore, ISRCTN49285450). ENID was a longitudinal randomised controlled trial of nutritional supplementation which ran from April 2010 in the rural West Kiang region of The Gambia to investigate the effects of ante-natal and infant nutritional supplementation on infant immune development. The ENID-Bone add on study was to investigate the effects of nutritional supplementation on the maternal skeleton and on infant bone development The ENID Trial was approved by the joint Gambian Government/MRC The Gambia Ethics Committee (Project number SCC1126v2); ENID Bone Study (L2009.66). Data collection took place in a rural subsistence farming community located along the river Gambia and its tributaries. The Gambia lies just north of the equator at latitude 13°N (Figure 3.1). The study population included all 36 villages currently registered within the West Kiang Demographic Surveillance System (DSS; total resident population approximately 15,000).

Following ethical approval local communities were approached and with permission, all women of reproductive age (18-45 years) were invited to participate in the study; eligible pregnant women received one of 4 antenatal nutritional supplements from <20 weeks of pregnancy until delivery (Table 3.1 & 3.2, Figure 3.2) (Moore et al., 2012). The ENID Trial aimed to recruit up to 1000 pregnant women, to allow for a maximum attrition rate of 20% from withdrawals, out-migrations,

pregnancy losses, and infant deaths. Eight hundred and seventy five women were randomised into the main trial, and there were 799 live births. It was anticipated that not all participants would consent to additional measures and participation in the ENID Bone add-on study, however, the majority did and subsequently entered the ENID Bone study.

The ENID Bone add-on study was powered to detect postpartum changes in maternal DXA bone and body composition outcomes. To calculate the minimum differences between pairs of supplement group that would be detectable, population variance data were used from a previous study of mothers and infants in the same region of The Gambia (Calcium Supplementation in Pregnancy, SCC585; ISCRTN96502494). To detect significant changes at the lumbar spine with p=0.05 and 80% power a sample size of 800 (200 per group) and 400 (100 per group) were required.

pQCT was used to collect densitometry data from the radius (3 sites) and tibia (4 sites) at 3 pregnancy time points: Booking (at approximately 14 weeks of pregnancy; 20 weeks of pregnancy (P20) and 30 weeks of pregnancy (P30). This was both the first application of pQCT in a cohort of Sub-Saharan pregnant women, and one of few studies to investigate the maternal skeletal response to pregnancy in a non-Caucasian population. The collection of pQCT data in the ENID Bone study provided a unique dataset to investigate the potential maternal skeletal response to pregnancy in a Sub-Saharan African population with a habitually low Ca intake. My role in the ENID Bone Study was to a) design specific pQCT pregnancy-related questions, b) analyse and QC all of the 9,159 pQCT scans and c) produce and analyse the subsequent pQCT dataset. The pQCT scans and other data required for my research were provided to me courtesy of Prof Ann Prentice.

3.1.1 Primary hypothesis

My primary hypothesis was that bone mineral mobilisation would occur between mid- to late-pregnancy from trabecular-rich skeletal sites in the maternal appendicular skeleton. This mobilisation would be observable through a decrease in both total and trabecular vBMD at the distal radius and tibia. I also hypothesised a potential supplement modulation of pregnancy-induced

vBMD change in which groups receiving the food supplement that provided additional protein and energy, but contained no Ca (see section 3.2.1.4), would have a greater decrease in vBMD due to the displacement of Ca from the maternal diet.

3.1.2 ENID Bone Study pQCT aims

My aims were to determine in the ENID Bone Study population whether:

1. Maternal bone mineral is mobilised from trabecular-rich appendicular sites (4% distal radius and tibia) between mid- to late-pregnancy (i.e. a decrease in trabecular vBMD)

2. Pregnancy-induced changes occur in other parameters of bone strength between mid- and late-pregnancy, at:

    a. The trabecular-rich distal radius and tibia (total CSA and total vBMD)

    b. The cortical-rich proximal radius and tibia (total CSA, cortical CSA, cortical vBMD, cortical BMC, cortical thickness)

3. Maternal antenatal supplementation or other environmental factors modulate the maternal skeletal response to pregnancy.

3.1.3 ENID Bone Study pQCT primary objective

My primary objective was to analyse the pQCT data from trabecular-rich distal sites at the radius and tibia to determine if a statistically significant decrease in trabecular vBMD could be observed between mid- and late- pregnancy, with any potential supplement modulation of a maternal skeletal response to pregnancy would be explored

3.1.4 ENID Bone Study pQCT secondary objectives

Secondary objectives were to explore:

1. Whether changes occur in the cortical compartment of the maternal skeleton as measured by pQCT at the proximal 33% radius and tibia through decreases in cortical vBMD, total CSA, cortical CSA, cortical BMC, and cortical thickness between Booking and P30.

2. If changes in trabecular and cortical parameters are consistent at;

    a. The distal radius (non-loadbearing) and distal tibia (loadbearing)

    b. The proximal radius (non-loadbearing) and proximal tibia (loadbearing)

3. Whether the following are determinants of the above pQCT bone outcomes:

    a. Age

    b. Parity

    c. Change in maternal weight

Figure 3.1 Map of The Gambia and surrounding Senegal (above), map of West Kiang region (below).

3.2 Study design and data collections

The following sections provide an account of the design of the ENID Trial and the ENID-bone add-on study plus the methods used to collect the data provided to me for my research.

3.2.1 Selection of subjects

3.2.1.1 Recruitment

The main ENID Trial aimed to have complete data for 800 mother-infant pairs, 875 rural Gambian women were randomised into the main trial, and there were 799 live births. Eligibility criteria are outlined in Table 3.1. All women in the main trial were invited before the start of supplementation to participate in the ENID Bone add-on; 811 were recruited to the ENID Bone study.

Table 3.1 Inclusion and exclusion criteria for enrolment into the ENID Trial

Inclusion Criteria	Exclusion Criteria
Aged 18-45 years	Currently pregnant beyond 20 weeks on ultrasound assessment
Living in the West Kiang DSS area	Currently enrolled in another MRC study
	Severe anaemia at booking (haemoglobin (Hb) less than 7 g/dL)
	Onset of menopause

3.2.1.2 Consent

Informed consent was obtained by trained field workers who explained the study to potential participants in their native language, covering all aspects detailed in the participant information sheet (PIS). Questions that arose during the process were answered by the field worker or if necessary referred to the PI for clarification. Participants had the opportunity to speak to a study investigator (PI, study midwife or study clinician) if they wished. Written consent was obtained, through either a signature or a thumb print (illiterate participants).

3.2.1.3 Randomisation

Following enrolment participants were visited monthly by a member of the study team with a short questionnaire on the date of their last menstrual period (LMP). When a menses had been missed,

a urine sample was collected for pregnancy testing using hCG tests (QuickVue™ One-Step hCG Urine Test, bioMérieux, UK). Women who tested positively were invited to MRC Keneba for an ultrasound examination, if confirmed as being between 10-20 weeks pregnant, the participant was randomised into the trial, and arrangements made for the Booking measurements to be conducted. Supplementation commenced the following week. Randomisation into the trial took place in blocks of 8 with the use of an automated system, with the eight groups reflecting the 8 possible combinations of prenatal and infancy supplements, a simplified version of this is shown in Figure 3.2 where only the antenatal groups are shown. The 4 maternal supplements were lipid-based protein-rich energy and protein supplement (PE), a multi-micronutrient supplement (MMN), these two supplements combined (PEMMN), and iron-folic acid (FeFol). More details are given in the next section.

3.2.1.4 Supplement Groups in the ENID Trial

Supplementation began at <20 weeks pregnancy, the week after the Booking measurements were completed. Current national policy in The Gambia recommends a daily supplement of 60 mg iron and 400 μg folate (FeFol) to all pregnant women, with the primary aim of reducing iron deficiency anaemia. At booking eligible women were randomised to one of 4 intervention arms (Table 3.2, Figure 3.2): Iron and Folate (FeFol) as per Gambian Government guidelines; Multiple micronutrients (MMN) a combination of 15 micronutrients specifically designed for use in pregnancy, as formulated by UNICEF/WHO/UNU. MMN were supplemented at twice the recommended daily allowance (RDA) and equal iron and folate to the FeFol arm (Moore et al., 2012); Protein-energy and iron-folate (PE+FeFol) a lipid based nutritional supplement (LNS) providing equal iron and folate to the FeFol arm, with additional energy (746 kcal), protein (20.8 g), and lipids (52.6 g); Protein-energy and multiple micronutrients (PE+MMN) a micronutrient fortified LNS supplement providing the same level of micronutrients as the MMN arm in addition to the energy and protein and lipid content. Ca was not included in any of these supplements.

The antenatal arm of the trial was partly open, it was not possible to blind the field assistants or the women to the supplement type (Tablet vs. LNS, Figure 3.3). All other investigators were blinded to which supplement group the women belonged. The supplements were administered to participants on a weekly basis by field assistants posted to the community. Supplement compliance was assessed by the collection of all unused supplements at the end of each week. Random spot checks to assess used and unused supplements were performed on 10% of participants each week (Moore et al., 2012).

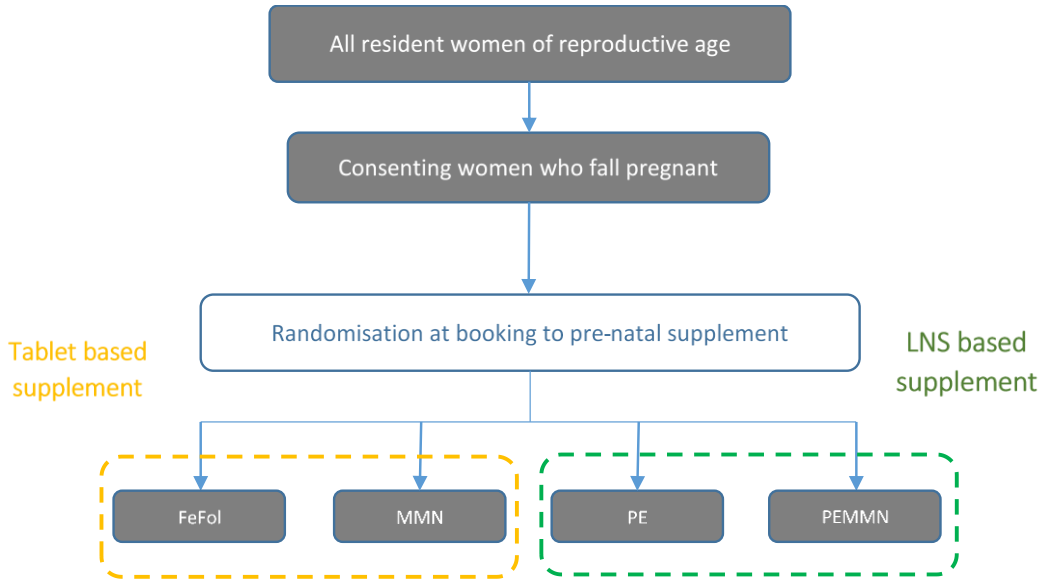

Figure 3.2 Flow diagram of the ENID Trial detailing the antenatal supplement arms.

LNS = lipid nutritional supplement, FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients.

Table 3.2 Nutritional composition of daily intake of pregnancy supplements (Moore et al., 2012).

Nutrients	FeFol	MMN	PE	PEMMN
Iron (mg)	60	60	60	60
Folate (µg)	400	400	400	400
Vitamin A (RE µg)	0	1600	2.85	1600
Vitamin D (IU)	0	400	0	400
Vitamin E (mg)	0	20	4.2	20
Vitamin C (mg)	0	140	2.25	140
Vitamin B1 (mg)	0	2.8	0.3	2.8
Vitamin B2 (mg)	0	2.8	0.45	2.8
Niacin (mg)	0	36	1.35	36
Vitamin B6 (mg)	0	2.8	0.15	2.8
Vitamin B12 (µg)	0	5.2	0.1	5.2
Zinc (mg)	0	30	3.3	30
Copper (mg)	0	4	1.05	4
Selenium (µg)	0	130	6.15	130
Iodine (µg)	0	300	2.6	300
Energy (kcal)	0	0	746	746
Protein (g)	0	0	20.8	20.8
Lipids (g)	0	0	52.6	52.6

All women received 60 mg Iron and 400 µg Folate. All women were randomised to supplementation from booking for antenatal care (<20 weeks gestation) until delivery. Nutrient levels across supplement groups are highlighted by colour (e.g.. all women receive equal iron and folate, highlighted in yellow). FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients

Figure 3.3 Field workers assessing compliance and examples of the tablet and LNS supplements.

Supplement compliance being assessed by the collection of all unused supplements at the end of each week (above), tablet supplements (FeFol and MMN, below left), lipid nutritional supplement (LNS, below right). FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients

3.2.2 Research teams and sites

The collection of all anthropometry and bone densitometry data took place at the MRC Unit The Gambia, Keneba. MRC Keneba is a rural field station situated in the Kiang West region, located in the village of Keneba, the largest village in the DSS. The Bakary Dibba Research Centre hosts the work of the Calcium, Vitamin D and Bone Health (CDBH) group, who conduct all the Gambian projects for Prof Ann Prentice (Figure 3.4). The facility contains pQCT and DXA for assessing bone density, geometry and distribution, infant and adult body composition and there are fully trained, experienced Gambian staff who perform the scanning, under the guidance of Dr Kate Ward and Prof Ann Prentice.

Figure 3.4 Women attending MRC Keneba for procedures during the ENID Bone Study. All densitometry data were collected onsite in a dedicated scanning suite in the Bakary Dibba Research Centre.

Table 3.3 Members of the research team involved in ENID Bone Study pQCT component

	Post	Duties
Mícheál Ó Breasail	PhD student MRC EWL	ENID Bone pQCT Study design Research question design Data auditing ENID Bone pQCT Scan grading ENID Bone pQCT Scan analysis ENID Bone pQCT Post analysis processing pQCT Statistical analysis ENID Bone pQCT
Prof Ann Prentice	Director and NBH CDBH Programme Head EWL/MRC Keneba	ENID Bone study design and conduct
Dr Landing Jarjou	Senior scientist, CDBH lead MRC Keneba	ENID Bone study design and conduct
Dr Kate Ward	Associate Professor: bone physiology MRC EWL	ENID Bone study design and conduct
Dr Gail Goldberg	Senior scientist: population aspects EWL/MRC Keneba	ENID Bone study design and conduct
Dr Sophie Moore	Senior Scientist, MRC Keneba, ENID Trial PI	ENID Trial study design and conduct
Yankuba Sawo	Bone Scanning Manager MRC Keneba	Study conduct and scan acquisition
Michael Mendy	Senior imaging and records coordinator MRC Keneba	Scan acquisition
Mustapha Ceesay	Senior imaging and sample coordinator MRC Keneba	Scan acquisition
Mariama Jammeh	Field assistant/imaging team	
Fatou Manneh	Field assistant/imaging team	
Isatou Camara	Field assistant	
Darren Cole	Data Operations Manager MRC EWL	Development of Microsoft Access tool for post analysis scan processing
Aron Sherry	Research assistant EWL	Scan grading ENID Bone pQCT
Lauren Oliver	Research assistant EWL	Scan grading ENID Bone pQCT
Carla Greenwood	Placement student EWL	Scan grading ENID Bone pQCT

3.3 Outcome Measurements relevant to the pQCT study

Anthropometric measurements including height, weight, forearm (ulna) length, lower leg (tibia) length, waist and hip circumference, mid-upper-arm circumference (MUAC), and body mass index (BMI) were obtained at Booking, P20, and P30 during pregnancy. pQCT was used to measure total area, total and trabecular volumetric bone mineral density (vBMD) at the distal radius and tibia, in addition to cortical vBMD and related parameters of cortical BMC, cortical CSA, and cortical thickness, at the proximal radius and tibia. These outcome measures are summarised in Table 3.4 and described in detail in Chapter 1.

Table 3.4 Summary of study outcome measures recorded in the ENID Bone Study and respective units presented in Chapters 3 and 4

Anthropometry	Peripheral QCT
Height (cm)	**Distal radius and tibia**
Weight (kg)	Total bone density (mg/cm^3)
Radius length (mm)	Trabecular density (mg/cm^3)
Tibia Length (mm)	Total CSA (mm^2)
	Proximal radius and tibia
	Cortical BMC (mg/mm)
	Cortical density (mg/cm^3)
	Cortical CSA (mm^2)
	Cortical thickness* (mm)
	Total CSA (mm^2)

* calculated using circular ring model (Chapter 1.4)

3.3.1 Collection of anthropometric data

Standing height was obtained without headwear or shoes under standardized conditions (horizontal Frankfort plane) to the nearest 0.1 cm by stadiometer (Magnimetre stadiometer; CMS Weighing Equipment Ltd) (Figure 3.5). Participants were instructed to remove footwear and to stand erect with feet together and with the posterior aspects of their heels and shoulders against the wall with knees and back straight. They were asked to bring their heads forward into the Frankfort plane (i.e. the line between the left eye and superior border of the external auditory meatus, was horizontal). Weight was measured to the nearest 0.1 kg while the subjects wore light clothing and no shoes using a digital scale (Wylux digital scales; CMS Weighing Equipment Ltd), routinely calibrated to ensure accuracy (Figure 3.5). Participants were weighed without shoes, in light clothing. Body Mass

Index (BMI) was calculated by dividing the participants weight (kg) by their height (m) squared, this was expressed in kg/m^2.

Figure 3.5 The measurement of standing height and weight during the collection of anthropometric data

3.3.2 Bone Densitometry - pQCT scanning

Peripheral pQCT data (Table 3.4) were obtained by trained technicians using an XCT 2000™ and an XCT 2000L™ bone densitometer (Stratec Medizintechnik, Pforzheim, Germany). All data were processed using the manufacturer's software (Stratec XCT version 6.2.) and standard operating procedures (SOP_0373) were followed for all procedures at each time point. The procedure, including the initial scout scan and successive measurements were explained clearly to the participant prior to scanning. Participants were asked not to talk, and to avoid moving during the procedure.

3.3.3 Limb length measurement

Object length (to the nearest mm) measured using a tape measure between prominent bony landmarks. Participants were asked to remove footwear and clothing from the lower non-dominant leg unless impracticable (e.g. injury, metal implant) which was measured from the tibial plateau to the distal end of the medial malleolus in each participant. Tibia length was measured with the

participant seated, feet flat on the floor, and their lower leg at 90° to thigh. The length of the tibia was measured from the medial malleolus to the tibial plateau using a tape measure to record the distance of the straight line between these two points to the nearest 0.5 mm (Figure 3.6). Radial object length, at the non-dominant arm unless impracticable (as above), was obtained with the arm on bent at a right angle, and the distance between the olecranon (near elbow) and the distal edge of the ulnar styloid process (bony prominence at wrist) measured with a tape measure to the nearest 0.5 mm (Figure 3.6). Scan sites were located by the pQCT scout view as a defined percentage of object (limb) length measured from a physiologically defined reference line at the distal end of the radius or tibia.

Figure 3.6 Schematic diagram highlighting the physiological landmarks used to determine ulna and tibia length

The correct measurement of ulna and tibia object length is important to ensure reproducible scans are acquired within participants and to ensure scan slices are obtained at the same percentage sites for all study participants. At the forearm the distance between A: the ulnar styloid process, and B: the olecranon was measured with a tape measure to provide the radius object length. At the lower leg the tibia object length was measured by tape measure from C: tibial plateau, to D: medial malleolus. Adapted from Madden et al. (2011)

3.3.4 pQCT scan acquisition

Scan sites and parameters are defined by the pQCT measurement mask, this was selected by the operator prior to scanning, entry of the object length in mm allowed the automatic calculation of appropriate scan sites. Scanning could begin once the operator ensured the limb was positioned securely and centrally in the gentry with the laser light distal to the medial malleolus/ulnar styloid

process of the tibia or ulna respectively (Figure 3.7). First a scout view (SV) was obtained, this is a 3 cm coronal view, at the lower leg the distal joint surface of the tibia, fibula, and talus are visible; while at the forearm the distal surfaces of the radius, ulna, and the start of the ossa carpi can be seen (Figure 3.7). An automatically placed reference line was visually assessed by the operator and correctly repositioned as appropriate (Figure 3.7). If there was difficulty identifying the correct region the participant was repositioned and the scout scan was repeated.

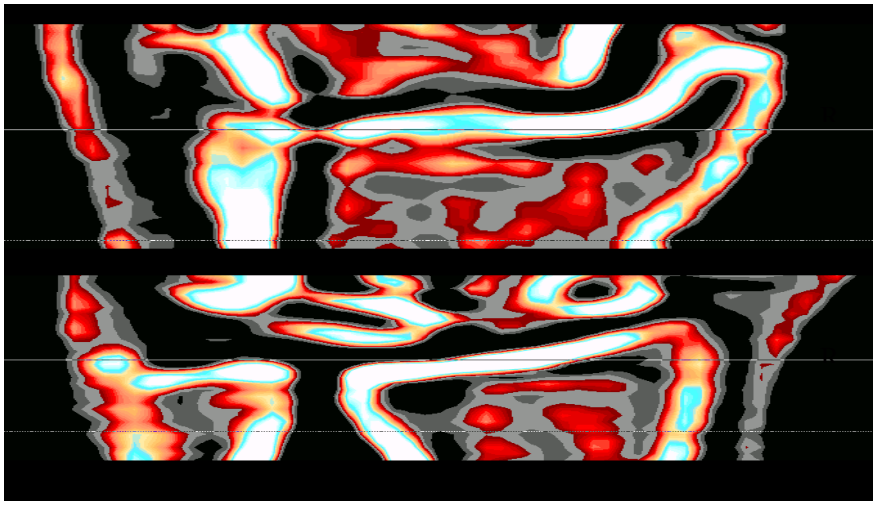

Figure 3.7 Scanograms or scout views at the forearm and lower leg as obtained with pQCT

Scout views of the tibia (above) and the radius (below), "R" indicates the reference line in both scans. The tibia reference line should be placed in the middle of the distal end of the tibia as above. The cortical endplate of the radius is used as an anatomical landmark.

Once the reference line was correctly positioned, the scan began (Figure 3.7). Scan sites (i.e. preselected percentages of the object length) were determined by the pQCT measurement mask selected by the operator prior to scanning. All scans were obtained using a voxel size of 0.5 mm and slice thickness of 2 mm. A CT scan speed of 30 mm/s was used for all slices, and a scout view speed of 40 mm/s was used for all scout views. Due to the resolution of XCT scanners specific sites are selected for the analysis of trabecular either or cortical bone. In the ENID Bone study scans were acquired at 4%, 33%, and 66% radius, and at 4%, 38%, 50%, and 66% tibia (Figure 3.9). Scan images were visually inspected, as pQCT is very sensitive to movement resulting in scan distortion and movement artefacts (Figure 3.8), if a scan slice was of poor quality the participant was asked if they would allow a rescan (Figure 3.11). Participants were exposed to extremely low

doses of ionising radiation during scanning, a set of radius/tibia scans had an effective dose equivalent of <3.2 μSv per visit, the total exposure of 9.6 μSv from being scanned at all 3 time points, is the equivalent of a day and half of background radiation in Cambridge and considered very low. pQCT scanners have negligible scatter however, the fetus will be exposed to a very small amount of radiation because X-rays pass through the mother's body – the calculation of fetal dose is discussed in detail in Chapter 6.

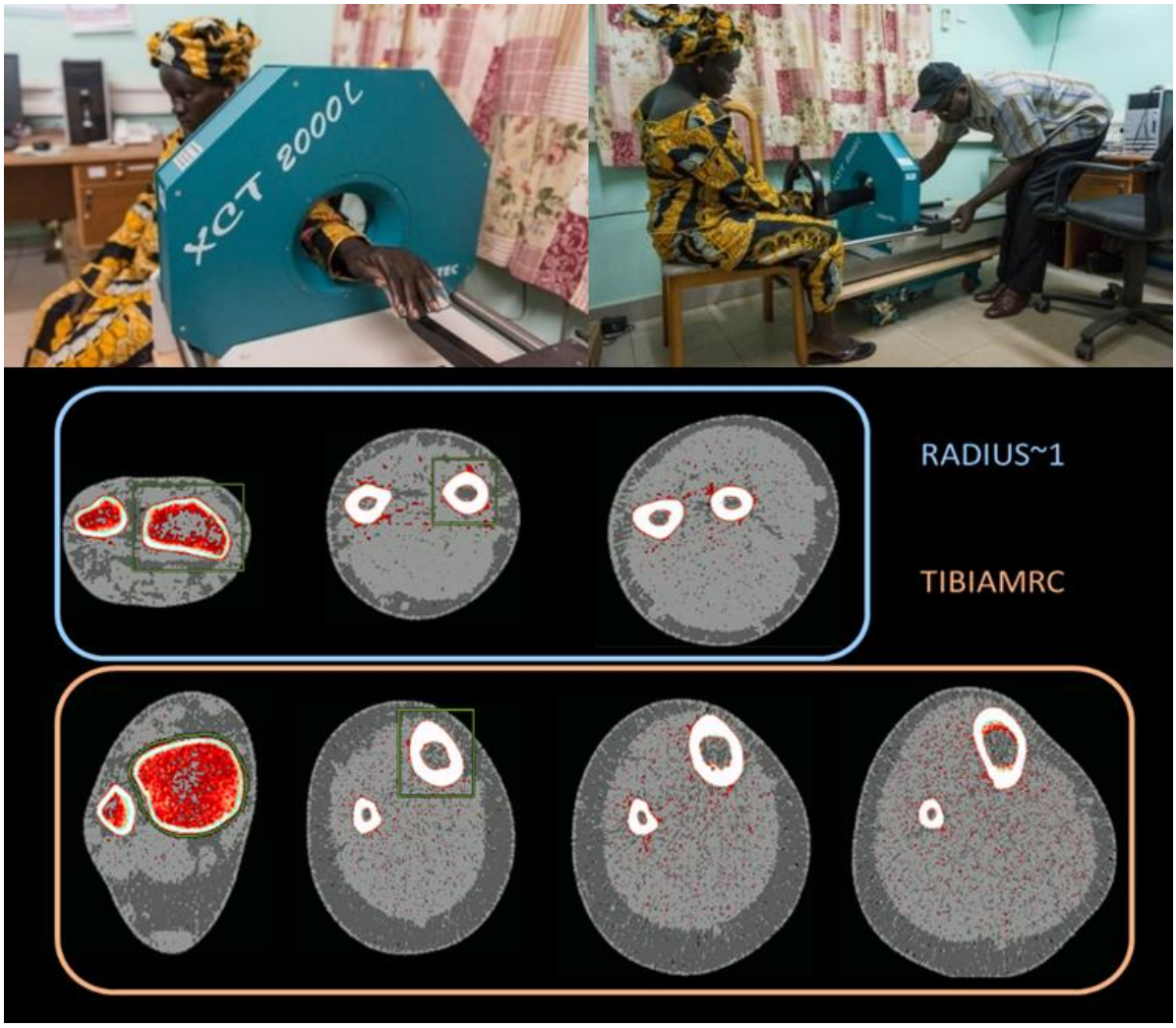

Figure 3.8 Sites at which pQCT scans were obtained – with relevant cross-section:

Above, Participant being scanned at the forearm with pQCT, in ENID Bone the RADIUS~1 mask was used and examples of scan images from the 4%, 33%, and 66% radius are displayed (right). Below, the same participant is being positioned for a lower leg scan, data from the lower leg were acquired using the TIBIAMRC mask: example scan slices from the 4%, 38%, 50%, and 66% tibia are shown (right)

3.3.5 pQCT Quality Assurance (QA)

Daily QA scans were performed with a specifically designed phantom throughout the study period to test scanner performance. Weekly cone phantom scans were also performed on both scanners to ensure the linearity of the results and to confirm the precision of the repositioning of the scanner. During this procedure three different density ranges are measured (Figure 3.9). Inter-operator coefficients of variation are tabulated below (Table 3.5).

Table 3.5 Coefficients of variation for pQCT bone outcome measures

Radius		Tibia	
Bone outcome	CV	Bone outcome	CV
Total vBMD	2.1%	Total vBMD	0.9%
Trabecular vBMD	2.6%	Trabecular vBMD	1.2%
Total CSA	3.4%	Total CSA	1.3%
Cortical BMC	3.4%	Cortical BMC	0.4%
Cortical vBMD	1.1%	Cortical vBMD	0.3%
Cortical CSA	3.0%	Cortical CSA	0.6%
Cortical thickness	5.6%	Cortical thickness	1.8%
Total CSA	6.4%	Total CSA	1.5%

These were measured by performing repeated measures on 31 adult Gambian subjects, vBMD = volumetric bone mineral density, BMC = bone mineral content, CSA = cross-sectional area

Figure 3.9 Standard QA scan (left), cone QA scan (right).

QA scans were performed daily during the study period, while QC scans were acquired weekly. Any inconsistencies were reported to senior scientists at MRC EWL.

3.3.6 Post-hoc powering for ENID Bone pQCT data analysis

The ENID Bone Study design was constrained by that of the main trial and was powered to detect changes in DXA-measured aBMD and body composition during lactation (see above). At that time, there were no suitable published pQCT data from pregnancy to base a formal sample size calculation on. Therefore, to establish whether meaningful changes in maternal bone during pregnancy would be detectable from the pQCT data, I calculated a post-hoc sample size using previous DXA data from our group. Those data showed significant changes in maternal BMC, measured by DXA, from a pre-pregnancy baseline measurement to shortly postpartum (Olausson et al., 2008). I used PS: Power and Sample Size Calculation version 3.1.2 (Dupont and Plummer, 1998) to calculate the numbers required to reproduce the effect sizes seen by Olausson et al. (2008), a change in DXA lumbar spine size adjusted-(SA)BMC from pre-pregnancy to 2 weeks postpartum of -2.6% (±4.2). 188 per group would be needed to detect between-group differences with a power of 0.9, and alpha of 0.05, while for within-group differences with a power of 0.9, and alpha of 0.5, 35 participants per group were required. This was a pragmatic approach in the absence of suitable pQCT data on which to calculate the sample-size for my analysis to detect meaningful change within-groups.

3.3.7 Scan grading and processing of pQCT scans

3.3.7.1 Post-processing - MRC Elsie Widdowson Laboratory (EWL)

Cleaning and processing of the pQCT data was conducted at the MRC Elsie Widdowson Laboratory (EWL), Cambridge. This formed a central aspect of my training and involved the auditing and inspection of all collected ENID Bone Study pQCT data to ensure it was suitable for longitudinal analysis. This is a time intensive process where each scan is individually scrutinised but ensures that only data of the highest quality are included in the final dataset for analyses and involved: 1) auditing the scan record book against the scans on the pQCT software to identify inconsistencies in scan numbering or missing scans; 2) Grading of the scout views for usability

longitudinally (Table 3.7); 3) Grading of each pQCT scan at each site of interest (Figure 3.11); 4) queries, i.e. following up missing scans, flagging errors in data entry, cross checking against data on MRC Keneba computers.

This was performed for all pQCT scans collected in the ENID Bone study (n=9,159) and their scout views to assess their suitability for inclusion in the subsequent data analysis using an SOP (SOP_0373, SOP_0499) designed in-house by imaging experts at MRC EWL. Qualitative analysis of the scans through visual inspection defined whether a scan was suitable for inclusion or not (Table 3.6, Figure 3.12). Scans were examined for movement or other artefacts, scout views were scrutinised to ensure scans were obtained from the correct sites at each time point. Scout views were compared longitudinally to ensure scans could be used for analysis of change. Attention was also paid to each participant's recorded object length as such errors may have led to the incorrect scan sites being scanned (Figure 3.11), in which case the bone outcome data were unusable and excluded from analysis.

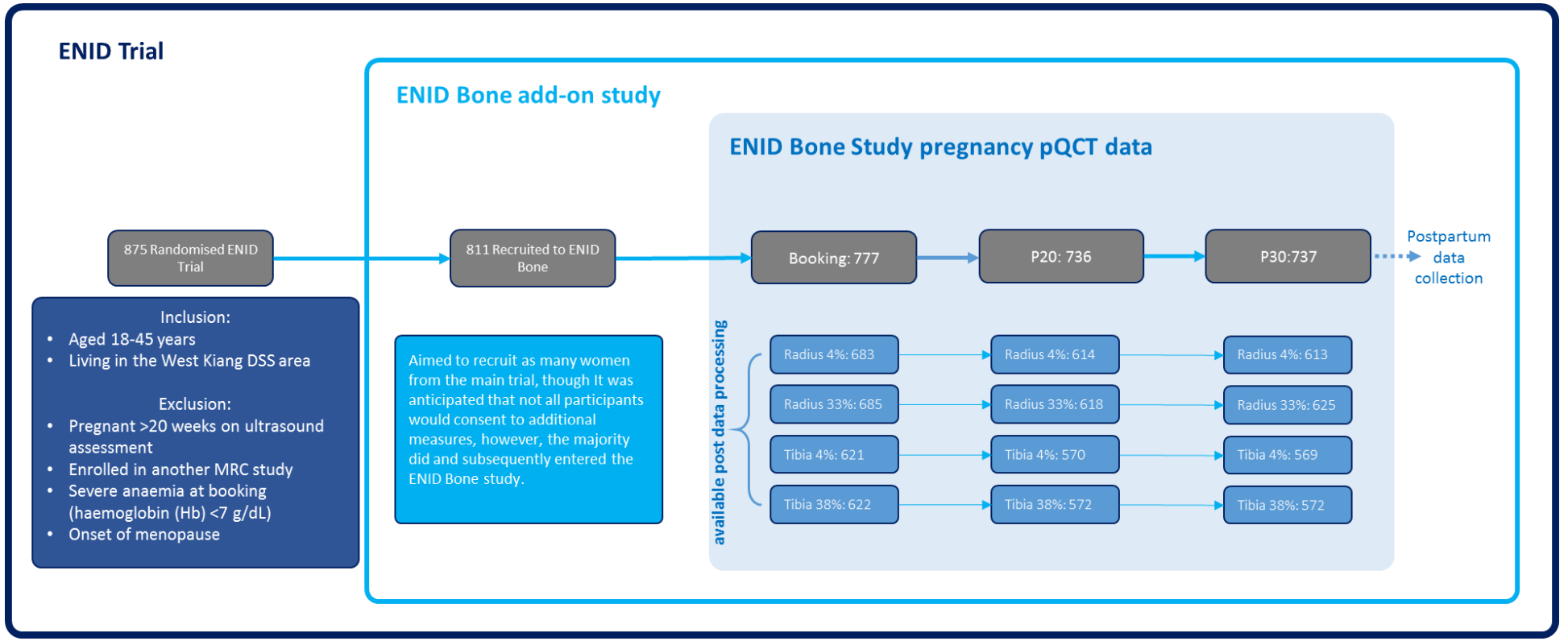

Figure 3.10 Flow diagram explaining the pregnancy pQCT data within the context of the ENID Trial and the ENID Bone add-on study

Number of women who were recruited into the ENID Trial, who were subsequently entered the ENID Bone Study, and who had data collected during pregnancy are highlighted above. Loss to follow-up was generally due to self-withdrawal, out-migration, sickness, miscarriage. A breakdown of pQCT data suitable for longitudinal analysis at each time point at each skeletal sites is also provided.

From the spreadsheet I maintained during the audit of participant CT (and scout views) numbers and scan grades I was able to write pQCT jobfiles to extract all useable scan data. This requires the definition of the appropriate macros and loops required to extract the data. All scans were processed using the manufacturer's software (Stratec XCT version 6.2) which determines the standard analysis parameters, and is capable of segregating cortical and trabecular bone by using a number of predefined modes (Ferretti, 1999), Stratec XCT 2000L manual, Table 3.6).

Table 3.6 pQCT analysis modes for standard analysis parameters using Stratec XCT software

Mode	Function
ContMode	Separates the surrounding soft tissue from the outer bone edge by applying a predefined threshold to eliminate voxels with an attenuation value below the threshold.
PeelMode	Separates the trabecular region from the cortical shell by stripping a selected proportion of the bone from the outer bone edge inwards assigning this as cortical-subcortical while the remaining inner portion is classed as trabecular bone.
CortMode	Separates the cortical bone from the subcortical bone by applying a threshold to peel away voxels with an attenuation value below the defined threshold, this obtains a cortical bone value.

These modes are used to define the trabecular and cortical regions of interest (ROI) and to separate bone from soft tissue. The functions CALCD and CORTBD are applied to these regions to estimate total, trabecular, cortical-subcortical, and cortical vBMD, and the bone's CSA (Ferretti, 1999). The thresholds (Section 1.5.3), analysis modes (above) were defined, and ROIs required for running the loop analysis to extract the pQCT bone parameters. At the distal sites CALCBD analysis using contour mode 1, peel mode 1 at a threshold of 180 mg/cm^3 was used, trabecular bone was defined as the inner area of 45% of the total CSA. At cortical sites a threshold of 710 mg/cm^3 was selected in conjunction with CORTBD separation mode 1. Total CSA was defined at proximal sites at a threshold of 280 mg/cm^3. To streamline the process of selecting and filtering the appropriate data from the raw pQCT output I assisted the MRC EWL Data Manager in the development of an in-house tool (Mr Darren Cole, MRC-EWL 2016) Microsoft Access (Microsoft Corporation, Redmond, Washington, U.S.) based tool.

Table 3.7 Grading of pQCT scout views and scan slice quality to determine if suitable for analysis.

Grading of pQCT scout view examples in Figure 3.11	
0	Perfect: The reference line for the radius goes along the flattest part of the bone, the reference line for the tibia goes through the middle of the bone.
1	Too distal/proximal: The reference is slightly higher/lower than it should be.
2	Out of range: The reference line is not in the correct position at all.
Grading of pQCT slices examples in Figure 3.11	
0	Perfect: No red streaks on the scan and the bone is in perfect shape (not distorted)
1	Slight movement: Small amounts of red patches/steaks
2*	Medium "streaky" movement: Large "streaky" red movement patches, the shape of the bone is distorted due to movement and there may be small "breaks" in the scan image.
3	Unusable: Red streaky patches covering most of the scan picture, the bone doesn't look circular in shape- pulled by the movement, with large "breaks" in the scan image.

* some grade 2 scans were later excluded due to implausible values obtained from these scans

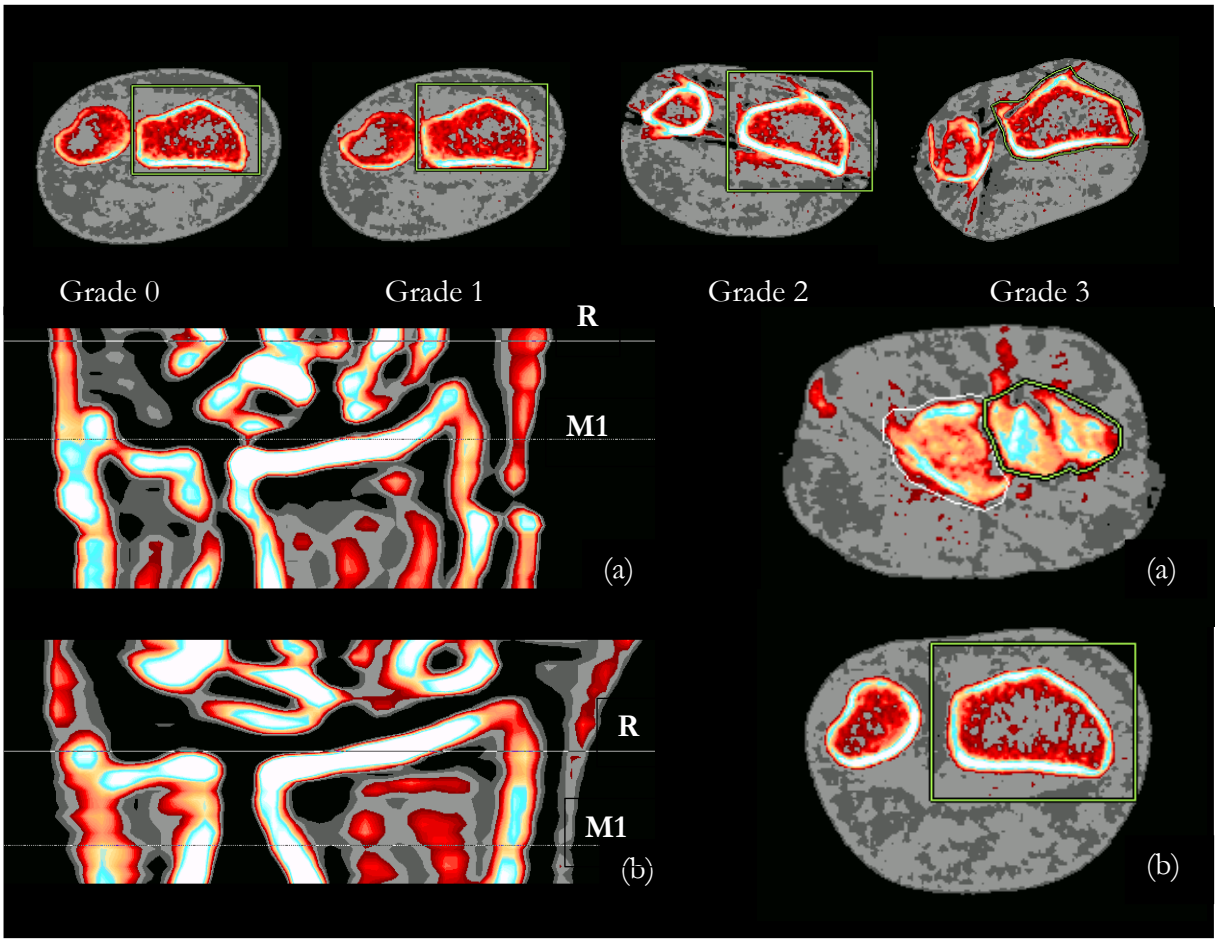

Figure 3.11 Scan grading explained with examples from the forearm.

Above: examples of graded scans at the 4% radius based on the criteria cited in Table 3.7. Middle and bottom left: scout views of the distal radius, "R" indicates the reference line and "M1" where the first measurement slice would be taken based on the reference line. Middle and bottom right scan slices from the distal radius that correspond to the scout views on the left. In (a) the reference line has been positioned too distally, grade "2", with the corresponding slice being unusable. In scout view (b) the reference line is correctly positioned - grade "0".

3.3.7.2 Differences between XCT pQCT scanners

During the initial exploratory analysis of the pQCT data I observed a noticeable difference in bone outcome measures obtained from the XCT 2000 and XCT 2000L, with a tendency for data from the XCT 2000 to be higher regardless of time point or supplement group. Baseline scans were acquired at Booking prior to supplementation, and no between-group differences in any population baseline characteristics were found. The raw between-scanner percentage differences at Booking are summarised in Table 3.8, consistent differences were seen at all time points. While the study protocol had stipulated participants should be scanned consistently at each visit on the same scanner that was used for their scan at Booking. 158 participants were scanned on both scanners during the study partially due to downtime on the XCT 2000L. The proportions of participants with scans on both machines versus those with all their scans on either the XCT 2000 or XCT 2000L were similarly distributed within the supplement groups at each visit (Table 3.9). To adjust for potential confounding due to scanner differences and to ensure that these between- scanner differences did not attenuate any potential pregnancy-induced effects a number of options were available. These options fell into two categories:

1. Cross-calibrating the two scanners by collecting new data :
    a. Using an appropriate phantom such as the European Forearm Phantom (EFP)
    b. Performing in-vivo measures on a cohort of healthy adults on both scanners
2. Adopting a statistical approach to adjust for the between-machine difference without the need to collect new scan data:
    a. Pooling historical data from previous studies using these two devices to determine an appropriate adjustment by which to calibrate the data.
    b. A study-specific approach by which data from one scanner would be calibrated to the other by calculating an appropriate conversion factor.
    c. Adjusting within the modelling of the ENID Bone pQCT data for the effects of changing between machines.

The option of collecting further data (EFP or in-vivo) would be time consuming, requiring the acquisition of an appropriate phantom or in the case of scanning new participants for cross-calibration obtaining ethical approval to collect the data. This was beyond the timeframe of this study. The benefits of adopting a statistical approach included already having access to the necessary data, but also the flexibility in choosing how to approach the problem. Initially, it was decided to perform a study-specific adjustment using the ENID Bone data rather than pooling these data with those from previous studies. While in other studies participants had been scanned on different scanners cross-sectionally, the ENID participants had been scanned longitudinally. Initially I treated the XCT 2000L scanner as the reference machine and used this for the purposes of plotting the data. However, for the final analysis of the dataset I adopted a more flexible approach by treating switching from one machine to the other as I would any other categorical variable. This approach appeared to be satisfactory in making allowance for potential confounding caused by the use of different scanners, however, as a precaution models were also fitted for the subset who did not change scanner (Appendix C).

Table 3.8 Mean differences (%) at Booking between pQCT parameters obtained on XCT 2000 vs XCT 2000L at the forearm and lower leg.

Bone outcomes	Radius	Tibia
Total vBMD	4.9%	5.0%
Trabecular vBMD	4.8%	5.0%
Total CSA	-1.1%	-1.1%
Total CSA proximal	-0.6%	1.1%
Cortical vBMD	3.8%	4.2%
Cortical BMC	4.6%	5.6%
Cortical CSA	0.8%	1.4%
Cortical thickness	1.5%	1.1%

vBMD = volumetric bone mineral density, CSA = cross sectional area

Table 3.9 Participants scanned in each supplement group at each visit scanned on the XCT2000 and XCT2000L

	FeFol		MMN		PE		PEMMN	
Visit	2000	2000L	2000	2000L	2000	2000L	2000	2000L
Booking	75	101	77	98	78	96	78	96
P20	68	91	72	92	64	91	59	92
P30	63	96	73	97	69	90	63	93

FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients

3.4 Data cleaning and initial data exploration

All data exploration, cleaning and manipulation to create a final dataset containing anthropometry and scans of suitable quality for longitudinal analysis were conducted in R version 3.3.1 primarily with the use of the following packages: dplyr version 0.5.0 (Wickham and Francois, 2016) and tidyverse version 1.1.1 (Wickham, 2017) for general data manipulation, and ggplot2 version 2.2.1 for plotting (Wickham 2009).

3.4.1 Data consolidation

Of the total data recorded in the initial check sheet a total of 2,232 and 2,131 radius and tibia scans were respectively available for this study. Of these, 1,933 radius and 1,994 tibia scans could be accounted on the scanners and were of a suitable grade for analysis. These were extracted using the manufacturer's software (Stratec XCT version 6.2) following the relevant SOP (SOP_0499). These longitudinal pQCT data were combined with subject level data including supplement arm, parity, and anthropometry to create a master dataset for analysis. During data consolidation I found some inconsistencies between measurement dates for the anthropometry data and those for pQCT. The anthropometry dates were more accurate because they are entered manually into the pQCT from the anthropometry forms and treated as reference dates for all participants in the dataset. However, care was taken when combining the datasets to ensure that the dates of scanning were closely matched to their corresponding pQCT data. The approach applied allowed the dates of pQCT scans to vary by a defined margin either side of the reference anthropometry date. Observations for 20 participant time points were not satisfactorily matched to the reference dates and were excluded from analysis.

Further to the grading process above the data were also scrutinised through the use of box and whisker plots to identify implausible extreme outliers (defined as 1.5 times the interquartile range or IQR). This was conducted for all subsets at each time point and crucially conducted on the

calculated within-subject change in all pQCT bone outcomes between visits. These were investigated further by visual inspection of the scans and scout views, of which those of clearly poor scan quality, inaccurate positioning, or ill-defined ROI were removed (Table 3.10) from the dataset. At this stage I identified several participants where object length differed between time points, these individuals were systemically identified (Table 3.11), and if object length differed by ≥5 mm from that individuals other scans the data were excluded, anthropometric and subject level data were retained in the dataset and can be found in the full population baseline descriptive statistics in Appendix C.

Table 3.10 Number of pQCT scans excluded from the dataset after being identified as extreme outliers at the distal and proximal radius and tibia at Booking, P20, and P30 from box and whisker plots.

	Radius 4%	Radius 33%	Tibia 4%	Tibia 38%
Booking	7	9	3	2
P20	8	10	3	1
P30	10	10	3	0
Total	25	29	9	3

Table 3.11 Number of pQCT scans excluded from the dataset due to object length differences >5 mm at the radius and tibia at Booking, P20, and P30.

	Radius	Tibia
Booking	1	4
P20	0	1
P30	2	1
Total	3	6

Table 3.12 Number of scan slices analysed at the distal and proximal radius and tibia in the master dataset at Booking, P20, and P30.

	Radius 4%	Radius 33%	Tibia 4%	Tibia 38%
Booking	683	685	621	622
P20	614	618	570	572
P30	613	625	569	572
Total	1910	1928	1760	1766
% of collected	85%	86%	83%	83%

Note that although both distal and proximal slices are obtained during the same scan if movement artefacts were present at one slice but not the other only the affected slice would be excluded, this explains the inconsonant number of data points form distal and proximal slices within the same limb.

Object length discrepancies can be attributed to data collection on two scanners as object was re-measured each time a new participant was enter on a scanner, where the same scanner is used throughout object length remains linked to the participant ID on that scanner. A 5 mm difference is small and within the bounds of inter-operator error even when optimal positioning is used and as such the data useable. The number of data at the distal and proximal sites of interest are summarised in Table 3.12.

3.4.2 Absolute within-group changes between baseline and follow up

For all pQCT data absolute between-visit change was calculated by subtracting the baseline bone outcome measure values from their respective follow-up values. Subsequently I used single-sided t-tests to determine if the absolute within-group change in all variables differed significantly from zero.

3.4.3 Modelling of longitudinal pQCT data

Model and covariate selection was determined by my primary and secondary objectives of exploring potential pregnancy-induced changes in the trabecular and cortical compartments of the maternal skeleton and any potential supplement modulation. However, as between-scanner effects emerged during my initial exploratory analysis, a categorical variable indicating scanner model was also included (Section 3.3.5). Although there were several approaches that could be applied to meet these requirements, I initially planned to use multilevel models (MLM), which were conducted using the LME4 package (Bates et al., 2015). However, as I will discuss in detail below, following my initial use of MLM, I eventually decided to use multiple regression analysis in base R, which allowed me to control for scanner differences while investigating whether any pregnancy-induced bone changes were modulated by supplementation.

3.4.4 Model selection

3.4.4.1 Multilevel models (MLM)

MLM are statistical models of parameters that vary at more than one level (i.e. nested). These models allow for variation in the outcome measures from two sources, between individuals (fixed) and within individuals (random), to be considered separately. The within-individual variation represents changes in the outcome over time. Random effects allow for the variation in unobserved measures in the individual to be considered. For example, an individual whose measures show a smaller change than an individual whose covariates are otherwise identical. Fixed effects in general can be thought of as measures that are fixed or unlikely to vary widely over the course of the study, such as the number of children or age. Random effects are allowed to vary between individuals while fixed effects apply to all individuals. The benefits of using MLM over a repeated-measures ANOVA include: the flexibility of not being limited to considering time as a categorical variable; the ability to deal with unequal time intervals between observations; the ability to handle unbalanced data (i.e. missing data for subjects across time points).

Table 3.13 Overview of multilevel models tested on the ENID Bone pQCT data

Model	Random intercept	Time effect (weeks from P20)	Random Slope	Additional Random Slope
1	Yes	No	No	No
2	Yes	Linear	No	No
3	Yes	Quadratic	No	No
4	Yes	No	Non correlated slope	No
5	Yes	Linear	Non correlated slope	No
6	Yes	Quadratic	Non correlated slope	No
7	Yes	No	Yes	Yes
8	Yes	Linear	Yes	Yes
9	Yes	Quadratic	Yes	Yes

My initial MLM (Table 3.13) centred time (i.e. gestation stage) on P20 due to the wider variation in time at Booking, time was expressed in week from P20, and explored as linear and quadratic terms. However, when these models were built up to explore variation in the ENID Bone pQCT bone outcomes the baseline results were not plausible, making it increasingly complex to model pregnancy-related change with this statistical method. From this point forward I decided to model change from Booking to P30 without the intermediate P20 time point for the following reasons:

1) more data points were available at Booking vs P20; 2) this ensured the maximum period over which to explore changes in maternal bone; 3) no significant between-group differences at Booking, as would be expected from the randomisation process prior to supplementation.

3.4.4.2 Multiple regression analysis

Multivariate linear regression models allowed me to explore differences between Booking and P30 while adjusting for machine bias and my a priori covariates. This allowed me the flexibility to determine any supplement effects, which were coded as a binary dummy variable on the basis of whether they contained PE or MMN (Table 3.14). A similar approach was applied to the scanner differences. Finally, for ease of interpretation, I centred maternal age (subtracting the mean age of women in the ENID-bone cohort from each participant's baseline age). Since the intercept represents an estimate of the mean when all predictors are at their reference level (i.e. 0 for continuous variables), the intercept (or reference group) in these models can be interpreted as representing women: 1) in the FeFol supplement arm (i.e. control); 2) that were scanned on the same scanner at Booking and P30; 3) not change scanner during the study; 3) of mean age (29.6 years). The intercept is expressed in the original units of each respective variable.

The β coefficients of the other independent variables indicate the difference in change over time from the reference/intercept. By adding (or subtracting) these to the reference we can determine the change in the different supplement groups (i.e. the mean differences between Booking and P30). For example to determine the effect size of supplementation in the MMN group for a given pQCT outcome measure the β coefficient for MMN is added to that of the intercept as all women in the MMN group also received FeFol.

3.4.5 Covariate selection

The selection of co-variates in the modelling of change in pQCT bone outcomes between visits was driven by the design of the ENID Bone Study and my primary objective of determining

whether a statistically significant decrease in trabecular vBMD occurs between early- and late-pregnancy at the distal radius and tibia. The selection of confounders for the base models was partly due to participants being scanned across the two pQCT machines (see Section 3.3.5). Participants fell into four different scenarios, i.e. being scanned on XCT 2000 (Scanner 1)/XCT 2000L (Scanner 2) twice, or starting on the XCT 2000/XCT 2000L and being scanned at P30 on the other machine (Chapter 4 Tables 4.10 & 4.11). A categorical variable "machine_cat" was used to control for participants who switched from the XCT2000L to the XCT2000, or from the XCT2000 to XCT2000L between Booking and P30. No major differences were found when models were run with this categorical machine variable compared to models performed on the subset of women who did not change scanner (Appendix C). As the ENID Bone Study had recruited women across a large age range (18-45 years) I also adjusted all models by maternal age. Following on from my base models I subsequently added supplement group to enable me to detect within-group changes between Booking and P30.

3.4.5.1 Maternal age and parity

As outlined previously as women age, natural age-related changes occur and the study population's age range was wide. Women in rural Gambia often have many children and the mean parity tends to be high and strongly correlated with maternal age. This was also the case for women in this study (R^2 = 0.73, Figure 3.12). While in the literature associations have reported between parity and maternal bone outcomes independent of age (Specker and Binkley, 2005), in previous studies at MRC Keneba it has been shown repeatedly that while age and parity may each be predictive of a particular measure, this is often lost for both of them when included together in regression models. It was not, therefore, possible to include both age and parity in the same model so I took the decision to include only maternal age (years) as it had less clustering around discrete values. Thus, in the following models maternal age is a used proxy of parity.

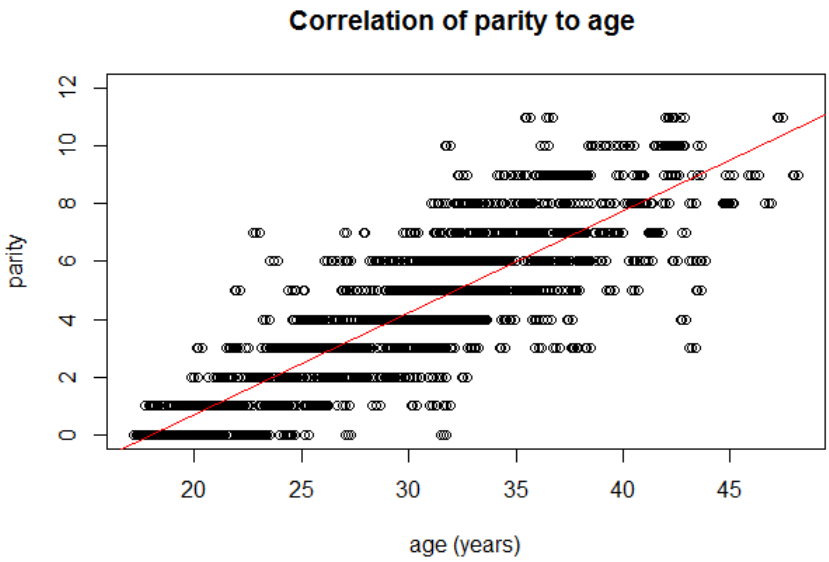

Figure 3.12 Correlation of parity and age in the ENID Bone study (adjusted R^2 = 0.73), regression line in red.

3.4.5.2 Maternal antenatal supplementation

Additionally I built up the initial models outlined below in Tables 5.4 and 5.5 to include supplement group, this was necessary due to the ENID Trial design, supplementation was coded into four categories based on whether participants received MMN or PE as part of their intervention (TRUE) or whether they did not (FALSE). This allowed for intercept to represent the FEFOL group (i.e. FALSE, FALSE).

Table 3.14 Coding of supplement in ENID Bone pQCT models

	Supplement contains MMN	Supplement contains PE
FEFOL	FALSE	FALSE
MMN	TRUE	FALSE
PE	FALSE	TRUE
PEMMN	TRUE	TRUE

FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients

3.4.5.3 Weight change

It was hypothesised that the effects of supplementation could modulate maternal weight gain and body composition. At baseline, there were no significant differences in weight between the 4 supplement groups. When the weight change between Booking and P30 is visualised (Figure 3.13) it can be seen to be fairly normally distributed in all arms. I further tested this through regression

analysis and found no significant supplement modulation of maternal weight gain between Booking and P30. Weight gain did not appear to differ between the groups nor was it predicated by supplement arm. In consequence, weight change was not included in the final models.

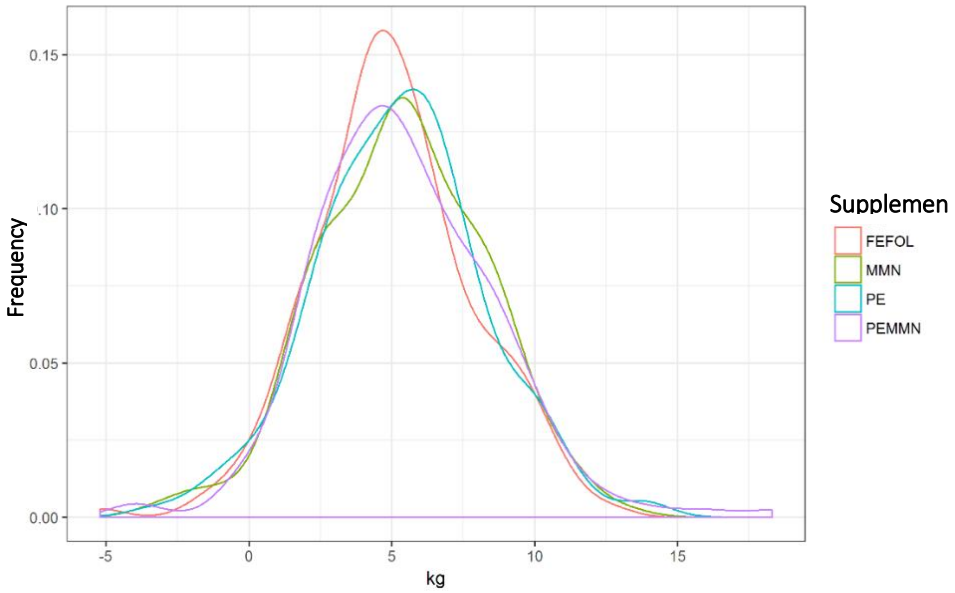

Figure 3.13 Distribution of weight change between Booking to P30 weight in the supplement groups

No baseline weight differences between supplement groups were reported. Linear regression to explore the relationship between supplement groups and maternal weight gain found no significant supplement effects on maternal weight gain between visits. FEFOL = iron folate, MMN = multiple micronutrients, PE = protein Energy, PEMNN = protein energy + multiple micronutrients.

3.4.6 Final model selection

Ultimately two models were applied to all pQCT data Model 1 (Figure 3.14) adjusted for the machine bias in addition to maternal age **(see section 3.4.5**):

$$Model\ 1: Y_i = \beta_o + \beta_1 machine\ + \beta_2 age + \epsilon$$

Model 2 added supplement effects (as binary dummy variables, Figure 3.15) to Model 1 to allow the effect of MMN, PE, and a PE x MMN interaction term to be explored.

$$Model\ 2: Y_i = \beta_o + \beta_1 machine\ + \beta_2 age + \beta_3 PE * MMN + \epsilon$$

In all models Yi is the absolute change in bone outcome as measured by pQCT for participant *I*.

A woman of mean age, who did not change pQCT scanner is estimated to have had a statistically significant increase in trabecular vBMD of 1.52 (0.44) mg/cm³ between visits

	Total vBMD (mg/cm³)	Trabecular vBMD (mg/cm³)	Total CSA (mm²)
XCT 2000L to XCT 2000	**17.3 (3.1) *****	**13.7 (1.6) *****	7.35 (3.89)
XCT 2000 to XCT 2000L	**-17.9 (2.5) *****	**-16.4 (1.3) *****	**-10.3 (3.2) ****
Age	-0.0779 (0.1147)	0.0230 (0.0604)	0.0548 (0.1450)
Constant	0.905 (0.828)	**1.52 (0.44) *****	1.90 (1.04)
Observations	521	521	521
R^2	0.148	0.320	0.0290

vBMD = volumetric bone mineral density, CSA = cross sectional area, BMC = bone mineral content, *p<0.05, **p<0.01, ***p<0.001

Figure 3.14 Example of Model 1 applied to trabecular vBMD the 4% distal tibia.

In the above, taking trabecular vBMD as an example the intercept represents the absolute change between visits in mg/cm³ for a participant of mean age who was scanned on the same scanner at Booking and P30. This example highlights the need to control for changing scanner showing as evidenced by the opposite direction and similar magnitude of the beta-coefficients for changing scanner in either way.

Women in the FEFOL group had an increase of 8.40 mg/cm³ in cortical vBMD

Women in the MNN group (also received FEFOL) had an increase of 3.01 mg/cm³ in cortical vBMD

Women in the PE group (also received FEFOL) had a 0.39 mg/cm³ increase in cortical vBMD

Women in the PEMNN group (received FEFOL, PE, and MMN) had an increase of 8.90 mg/cm³ in cortical vBMD

	Cortical vBMD (mg/cm³)	Cortical vBMD (mg/cm³)	Cortical vBMD (mg/cm³)	Cortical vBMD (mg/cm³)
Protein Energy (PE)	-8.01 (2.71) **	-8.01 (2.71) **	-8.01 (2.71) **	-8.01 (2.71) **
Multiple Micronutrients (MMN)	-5.39 (2.67) *	-5.39 (2.67) *	-5.39 (2.67) *	-5.39 (2.67) *
XCT 2000L to XCT 2000	63.6 (3.7) ***	63.6 (3.7) ***	63.6 (3.7) ***	63.6 (3.7) ***
XCT 2000 to XCT 2000L	-71.2 (3.3) ***	-71.2 (3.3) ***	-71.2 (3.3) ***	-71.2 (3.3) ***
Age	-0.397 (0.147) **	-0.397 (0.147) **	-0.397 (0.147) **	-0.397 (0.147) **
PExMMN	13.9 (3.9) ***	13.9 (3.9) ***	13.9 (3.9) ***	13.9 (3.9) ***
Constant	8.40 (1.94) ***	8.40 (1.94) ***	8.40 (1.94) ***	8.40 (1.94) ***
Observations	531	531	531	531
R^2	0.622	0.622	0.622	0.622

Figure 3.15 Example of Model 2 applied to the cortical vBMD at the 33% proximal radius

In the above, taking cortical vBMD as an example the intercept represents the absolute change between visits in mg/cm³ for a participant receiving FEFOL, of mean age who was scanned on the same scanner at Booking and P30. Supplement effects can be interpreted as highlighted in the text boxes above. FEFOL is highlighted in green where the intercept can be taken as is, however, to interpret the change in a woman who received MMN the effect of MMN must be added to that of the intercept (as all women will have received FEFOL, yellow). This also holds true for those who received the PE supplement (blue, intercept + PE). For women who received PEMNN it is necessary to add the effect of MMN, PE, and the PExMMN interaction term to the intercept (red).

4 ENID Bone Study Results

4.1 Introduction

My analysis explored whether there was change in the entire cohort but also whether ante-natal supplementation modulated any pregnancy-induced changes in the trabecular compartment. In the same manner I explored my secondary objective which focused on the cortical-rich proximal sites of the radius and tibia to determine if any pregnancy-induced adaptations occurred in cortical bone density, mass, distribution, or strength. As at trabecular-rich sites I needed to consider whether supplementation modulated change in cortical bone outcomes during gestation. Finally my models explored the influence of potential determinants of change such as maternal age.

4.2 Baseline descriptive characteristics

Complete case analysis for participants at Booking and P30 (i.e. included in my final models) was carried out and showed no difference to the entire ENID bone pQCT cohort (Appendix C), these data are presented in Table 4.1. No statistically significant between-group differences were found between supplement arms as examined by ANOVA with a post-hoc Tukey test (Table 4.1) this was expected because of randomisation. The distribution of age, height, weight, and weight change are shown in Figure 4.1, while Figure 4.1 displays the parity within the four supplement groups.

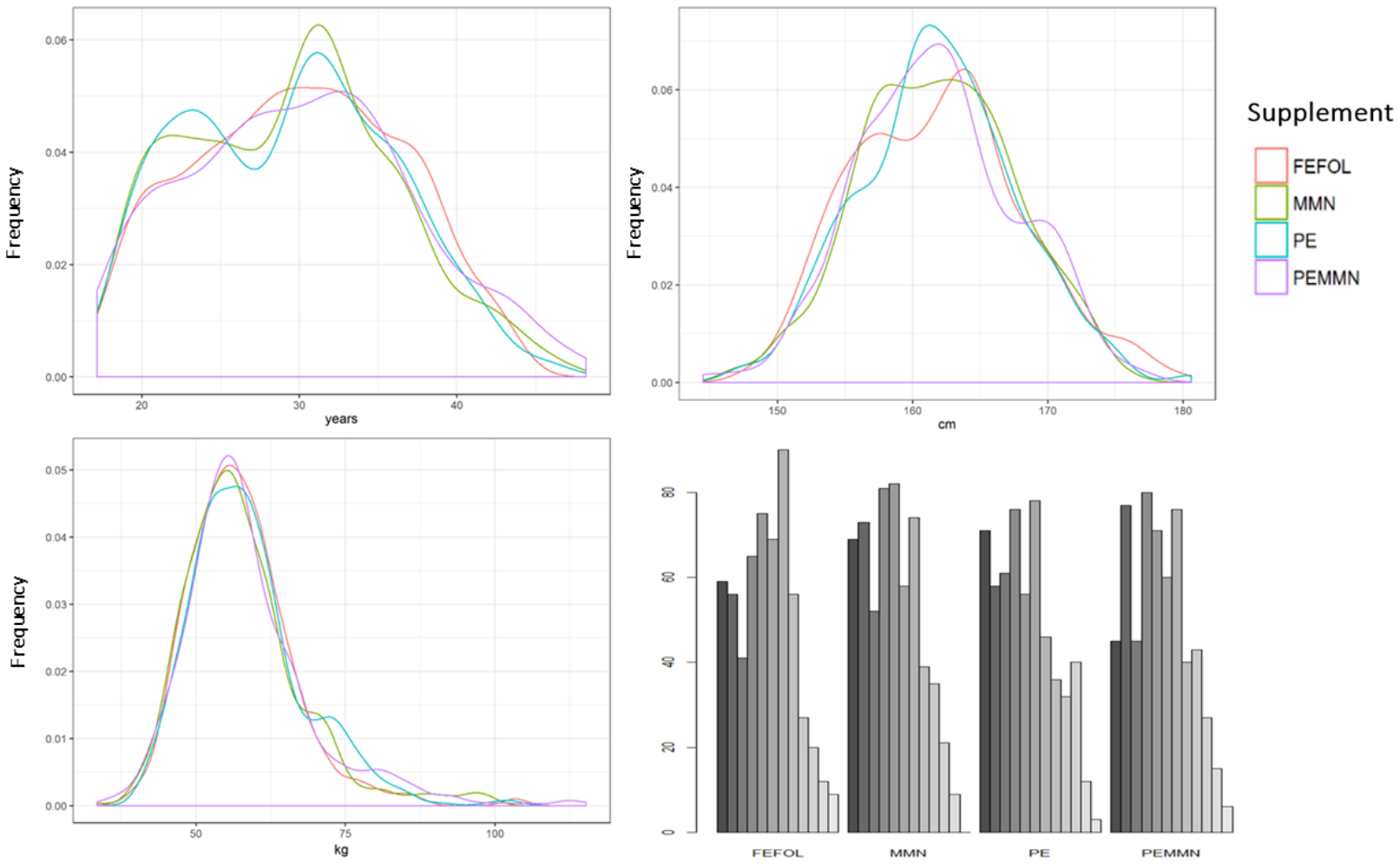

Figure 4.1 Distribution of maternal age (years), height (cm), weight (kg), and parity by supplement group at Booking.

No statistically significant between-group differences were observed at Booking. Parity was calculated as the sum of living children, children who died, and stillbirths, minus the number of abortions. FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients.

Table 4.1 Baseline descriptive statistics for participants with complete data at Booking and P30 for the 4 supplement groups in the ENID Bone Study, mean (SD)

	FeFol	MMN	PE	PEMMN
	n=143	n=151	n=141	n=129
Age (years)	29.73 (6.52)	29.33 (6.60)	29.19 (6.57)	30.05 (7.19)
Weight (kg)	55.18 (8.76)	55.19 (10.06)	55.95 (9.39)	56.23 (10.88)
Height (cm)	161.86 (6.25)	161.89 (5.68)	161.94 (5.69)	161.65 (5.90)
Sitting height (cm)	80.24 (3.04)	80.31 (2.99)	80.08 (3.12)	80.02 (4.09)
Parity, median [IQR]	5 [2, 6]	4 [2, 6]	4 [2, 6]	4 [2, 6]
4% Radius	n=136	n=136	n=135	n=123
Total vBMD (mg/cm^3)	318.53 (42.05)	321.12 (46.86)	321.58 (42.95)	320.46 (44.96)
Trabecular vBMD (mg/cm^3)	159.49 (34.30)	162.79 (36.02)	163.51 (35.03)	158.85 (37.04)
Total CSA (mm^2)	337.06 (41.67)	334.06 (38.45)	339.95 (42.17)	333.41 (46.68)
33% Radius	n=137	n=145	n=136	n=122
Total CSA (mm^2)	119.70 (16.51)	119.32 (15.76)	122.41 (17.66)	119.54 (16.93)
Cortical vBMD (mg/cm^3)	1239.93 (39.05)	1245.71 (37.98)	1243.53 (38.47)	1241.60 (35.76)
Cortical BMC (mg/mm)	93.50 (11.76)	94.46 (10.60)	95.72 (11.32)	93.52 (10.41)
Cortical CSA (mm^2)	75.38 (8.84)	75.80 (7.99)	76.93 (8.45)	75.34 (8.22)
Cortical Thickness (mm)	2.43 (0.24)	2.45 (0.21)	2.46 (0.25)	2.44 (0.23)
4% Tibia	n=120	n=125	n=114	n=118
Total vBMD (mg/cm^3)	282.82 (39.21)	285.26 (37.10)	288.09 (35.29)	280.52 (35.45)
Trabecular vBMD (mg/cm^3)	191.02 (34.92)	192.69 (30.00)	194.14 (30.73)	189.27 (29.98)
Total CSA (mm^2)	949.27 (112.26)	935.17 (108.58)	941.56 (110.92)	945.26 (116.34)
38% Tibia	n=119	n=129	n=114	n=119
Total CSA (mm^2)	382.03 (54.33)	377.37 (52.24)	379.55 (42.04)	375.50 (46.12)
Cortical vBMD (mg/cm^3)	1232.43 (36.32)	1233.61 (39.40)	1232.32 (40.06)	1233.11 (36.53)
Cortical BMC (mg/mm)	280.75 (37.92)	280.74 (38.81)	285.15 (33.20)	277.32 (31.84)
Cortical CSA (mm^2)	227.76 (29.71)	227.44 (29.46)	231.18 (23.91)	224.75 (23.47)
Cortical Thickness (mm)	4.04 (0.43)	4.07 (0.46)	4.14 (0.44)	4.03 (0.39)

Between-group baseline differences were tested by ANOVA with posthoc Tukey Test. FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients, vBMD = volumetric bone mineral density, CSA = cross sectional area

4.3 Unadjusted analyses of change between Booking and P30

Change in all bone outcomes between Booking and P30 was computed, and independent t-tests were used to determine if the change was significantly different to zero (i.e. $p<0.05$). No significant differences were found in trabecular vBMD or in total vBMD at either the distal radius or tibia. Small but statistically significant differences were found at the cortical-rich proximal radius and tibia for several changes in bone outcomes between visits these data are tabulated in Table 4.2 (all $p<0.05$). Data for a reduced subset who did not change scanner can be found in Appendix C. Following stratification by supplement group, I repeated this process and found statistically significant within-group differences between Booking and P30 in parameters of bone geometry cortical CSA (proximal radius and tibia) in the MMN group only (Table 4.4, all $p<0.05$).

Table 4.2 ENID Bone Study pQCT bone parameters of interest at Booking and absolute change by P30, mean (SD) for participants who had data at both time points.

	Booking	Change by P30
4% Radius	n=530	
Total vBMD (mg/cm^3)	320.42 (44.12)	0.35 (18.65)
Trabecular vBMD (mg/cm^3)	161.21 (35.53)	0.83 (11.00)
Total CSA (mm)	336.18 (42.19)	1.32 (22.18)
33% Radius	n=540	
Total CSA (mm^2)	120.24 (16.71)	-0.25 (11.14)
Cortical vBMD (mg/cm^3)	1242.77 (37.85)	2.86 (36.12)
Cortical BMC (mg/mm)	94.32 (11.05)	**0.34 (3.49)***
Cortical CSA (mm^2)	75.87 (8.38)	0.09 (1.72)
Cortical Thickness (mm)	2.45 (0.23)	0.01 (0.17)
4% Tibia	n=477	
Total vBMD (mg/cm^3)	284.15 (36.81)	0.27 (10.61)
Trabecular vBMD (mg/cm^3)	191.77 (31.43)	0.19 (7.39)
Total CSA (mm^2)	942.74 (111.79)	-1.41 (27.22)
38% Tibia	n=481	
Total CSA (mm^2)	378.57 (48.97)	0.59 (14.82)
Cortical vBMD (mg/cm^3)	1232.89 (38.00)	2.26 (29.34)
Cortical BMC (mg/mm)	280.94 (35.66)	**0.85 (9.41)***
Cortical CSA (mm^2)	227.74 (26.90)	**0.31 (3.12)***
Cortical Thickness (mm)	4.07 (0.43)	0.00 (0.12)

*p<0.05 Independent T-test change vs zero, vBMD = volumetric bone mineral density, CSA = cross sectional area

Table 4.3 ENID Bone Study pQCT bone parameters of interest in their respective units of measurement at Booking with the absolute change by P30 mean (SD) for participants who had data at both time points following stratification by supplement group

	FeFol		MMN		PE		PEMMN	
	Booking	Change by P30	Booking	Change by P30	Booking	Change by P30	Booking	Change by P30
4% Radius	n = 136		n = 136		n = 135		n = 123	
Total vBMD (mg/cm^3)	318.53 (42.05)	-0.43 (19.07)	321.12 (46.86)	2.29 (21.07)	321.58 (42.95)	-0.71 (18.90)	320.46 (44.96)	0.22 (14.72)
Trabecular vBMD (mg/cm^3)	159.49 (34.30)	0.53 (11.02)	162.79 (36.02)	1.21 (12.05)	163.51 (35.03)	0.42 (11.24)	158.85 (37.04)	1.20 (9.51)
Total CSA (mm^2)	337.06 (41.67)	1.45 (23.02)	334.06 (38.45)	-0.80 (22.71)	339.95 (42.17)	3.25 (23.19)	333.41 (46.68)	1.42 (19.41)
33% Radius	n = 137		n = 145		n = 136		n = 122	
Total CSA (mm^2)	119.70 (16.51)	-1.52 (11.26)	119.32 (15.76)	0.19 (9.72)	122.41 (17.66)	0.54 (12.46)	119.54 (16.93)	-0.20 (11.03)
Cortical vBMD (mg/cm^3)	1239.93 (39.05)	5.25 (34.80)	1245.71 (37.98)	1.59 (36.96)	1243.53 (38.47)	-0.23 (39.36)	1241.60 (35.76)	5.11 (32.73)
Cortical BMC (mg/mm)	93.50 (11.76)	0.34 (3.47)	94.46 (10.60)	0.64 (3.92)	95.72 (11.32)	-0.02 (3.45)	93.52 (10.41)	0.38 (3.01)
Cortical CSA (mm^2)	75.38 (8.84)	-0.05 (1.67)	75.80 (7.99)	**0.42 (1.90)***	76.93 (8.45)	0.00 (1.60)	75.34 (8.22)	-0.04 (1.63)
Cortical Thickness (mm)	2.43 (0.24)	0.02 (0.18)	2.45 (0.21)	0.02 (0.16)	2.46 (0.25)	-0.01 (0.19)	2.44 (0.23)	0.00 (0.17)
4% Tibia	n=120		n=125		n=114		n=118	
Total vBMD (mg/cm^3)	282.82 (39.21)	-0.64 (8.73)	285.26 (37.10)	0.39 (11.52)	288.09 (35.29)	-0.08 (12.31)	280.52 (35.45)	1.42 (9.54)
Trabecular vBMD (mg/cm^3)	191.02 (34.92)	-0.07 (7.17)	192.69 (30.00)	-0.01 (7.66)	194.14 (30.73)	0.36 (7.87)	189.27 (29.98)	0.50 (6.89)
Total CSA (mm^2)	949.27 (112.26)	0.04 (21.98)	935.17 (108.58)	-3.15 (26.09)	941.56 (110.92)	1.95 (26.85)	945.26 (116.34)	-4.29 (32.85)
38% Tibia	n = 119		n = 129		n = 114		n = 119	
Total CSA (mm^2)	382.03 (54.33)	-1.63 (15.01)	377.37 (52.24)	1.79 (17.09)	379.55 (42.04)	1.08 (10.91)	375.50 (46.12)	1.03 (15.19)
Cortical vBMD (mg/cm^3)	1232.43 (36.32)	0.82 (25.67)	1233.61 (39.40)	3.69 (32.20)	1232.32 (40.06)	0.63 (32.21)	1233.11 (36.53)	3.69 (26.77)
Cortical BMC (mg/mm)	280.75 (37.92)	0.46 (7.76)	280.74 (38.81)	1.50 (11.02)	285.15 (33.20)	0.26 (10.36)	277.32 (31.84)	1.09 (8.03)
Cortical CSA (mm^2)	227.76 (29.71)	0.20 (2.64)	227.44 (29.46)	**0.63 (3.96)***	231.18 (23.91)	0.13 (3.00)	224.75 (23.47)	0.22 (2.61)
Cortical Thickness (mm)	4.04 (0.43)	0.02 (0.13)	4.07 (0.46)	0.00 (0.14)	4.14 (0.44)	0.00 (0.09)	4.03 (0.39)	0.00 (0.11)

*p<0.05 Independent T-test change vs zero, FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients, vBMD = volumetric bone mineral density, CSA = cross sectional area

4.4 Adjusted analyses of change between Booking and P30

4.4.1 Distal 4% radius

The results of the [1]Model 1 at the distal tibia are presented in Table 4.4. In women of an age at the mean age of the cohort who did not change scanner, a small but statistically significant increase in trabecular vBMD (<1%) was found ($p<0.001$). With the addition of supplement group in [2]Model 2 the increase in trabecular vBMD at the distal radius was no longer statistically significant (Table 4.5). No effect was observed for maternal age (also a proxy for parity) at the 4% distal radius in either model.

4.4.2 Proximal 33% radius

Small (<1%) but statistically significant increases were found in the reference group for both cortical vBMD and cortical BMC (Table 4.4, both $p<0.05$). Maternal age had a small but statistically significant age effect on cortical vBMD and cortical BMC, indicating that the increases in these parameters were lower the older the participants were (Table 4.4, both $p<0.05$).

Controlling for supplement group increased the effect-size of the increase in cortical vBMD ($p<0.001$) and cortical BMC ($p<0.01$) in the reference group. However, the increase remained small in magnitude representing <1% of a change (Table 4.5). The modest age effect remained significant for cortical vBMD and cortical BMC (both $p<0.05$). In Model 2, small but statistically significant supplement effects on the change in cortical vBMD and BMC were detected between Booking and P30 (all $p<0.05$). PE was found to be a significant negative predictor of cortical vBMD ($p<0.01$) i.e. there was less of an increase, almost attenuating the effects of FeFol.

Model 1 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30
Model 2 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30, and were in the FeFol arm (i.e. control group)

Cortical BMC also decreased in the PE arm compared to the reference group, however although statistically significant, the effect size was negligible ($p<0.05$).

The MMN only supplement, was also found to attenuate the significant increase in cortical vBMD, which increased to a lesser extent in women in this arm than the reference. In the MMN arm a small significant increase in cortical CSA was found compared to the reference group ($p<0.05$).

The combined PE and MMN supplement behaved differently to PE and MMN alone: where alone the increases in cortical vBMD were below that of the reference group; combined in the PEMMN arm there was an increase in cortical vBMD that exceeded that of the reference group (8.90 mg/cm^3 compared to 8.40 mg/cm^3) (Table 4.5).

4.4.3 Distal 4% tibia

At the distal tibia there were small but statistically significant increases in the reference group for both total and trabecular vBMD using [1]Model 1 (both $p<0.01$). As seen at the distal radius these small increases in total and trabecular vBMD were attenuated in [2]Model 2, and no supplement effects were found at the 4% distal tibia (Table 4.7). Maternal age was not a significant predictor of any parameter at the distal tibia in either model.

4.4.4 Proximal 38% tibia

There were small but statistically significant increases in several parameters at the cortical-rich proximal tibia from Model 1: cortical vBMD ($p<0.001$), BMC ($p<0.001$), and cortical CSA ($p<0.01$) increased between Booking and P30. Maternal age did not significantly influence these increases. Model 2 attenuated the significant increase in cortical CSA.

Model 1 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30
Model 2 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30, and were in the FeFol arm (i.e. control group)

However, significant increases in cortical vBMD ($p<0.001$) and BMC ($p<0.01$) remained at the 38% proximal tibia in the reference group. No additional supplementation effect was found for MMN, PE, or PEMMN (Table 4.7), nor was maternal age a significant predictor.

Model 1 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30
Model 2 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30, and were in the FeFol arm (i.e. control group)

Table 4.4 Summary of Model 1 for change in bone outcome measures at the 4% distal and 33% proximal radius between Booking and P30.

	Total vBMD (mg/cm^3)	Trabecular vBMD (mg/cm^3)	Total CSA 4% (mm^2)	Total CSA 33% (mm^2)	Cortical vBMD (mg/cm^3)	Cortical BMC (mg/mm)	Cortical CSA (mm^2)	Cortical thickness (mm)
XCT2000L to XCT2000	**17.31 (3.08)[c]**	**13.70 (1.62)[c]**	7.35 (3.89)	1.70 (1.86)	**62.82 (3.76)[c]**	**6.77 (0.36)[c]**	**1.50 (0.28)[c]**	0.03 (0.03)
XCT2000 to XCT2000L	**-17.95 (2.53)[c]**	**-16.41 (1.33)[c]**	**-10.35 (3.20)[b]**	-0.88 (1.64)	**-71.40 (3.30)[c]**	**-6.53 (0.32)[c]**	**-0.92 (0.24)[c]**	-0.03 (0.03)
Age	-0.08 (0.11)	0.02 (0.06)	0.05 (0.15)	0.10 (0.07)	**-0.38 (0.15)[a]**	**-0.03 (0.01)[a]**	-0.005 (0.01)	-0.002 (0.001)
Constant	0.91 (0.83)	**1.52 (0.44)[c]**	1.90 (1.05)	-0.34 (0.53)	**5.14 (1.07)[c]**	**0.47 (0.10)[c]**	0.07 (0.08)	0.01 (0.01)
Observations	521	521	521	531	531	531	531	531
R^2	0.15	0.32	0.03	0.01	0.61	0.62	0.08	0.01

vBMD = volumetric bone mineral density, CSA = cross sectional area, BMC = bone mineral content, [a]= p<0.05, [b]= p<0.01, [c]=p<0.001

Model 1 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30
Model 2 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30, and were in the FeFol arm (i.e. control group)

Table 4.5 Summary of Model 2 for change bone outcome measures at the 4% distal and 33% proximal radius between Booking and P30.

	Total vBMD (mg/cm^3)	Trabecular vBMD (mg/cm^3)	Total CSA 4% (mm^2)	Total CSA 33% (mm^2)	Cortical vBMD (mg/cm^3)	Cortical BMC (mg/mm)	Cortical CSA (mm^2)	Cortical thickness (mm)
PE	-0.58 (2.12)	-0.28 (1.11)	1.75 (2.67)	2.00 (1.36)	**-8.01 (2.71)[b]**	**-0.60 (0.26)[a]**	0.01(0.20)	-0.03 (0.02)
MMN	2.19 (2.12)	0.70 (1.12)	-1.82 (2.68)	1.70 (1.34)	**-5.39 (2.67)[a]**	0.10 (0.26)	**0.42 (0.20)[a]**	-0.01 (0.02)
XCT2000L to XCT2000	**17.20 (3.09)[c]**	**13.70 (1.63)[c]**	7.41 (3.91)	1.56 (1.87)	**63.56 (3.73)[c]**	**6.79 (0.36)[c]**	**1.47 (0.28)[c]**	0.03 (0.03)
XCT2000 to XCT2000L	**-17.98 (2.54)[c]**	**-16.39 (1.34)[c]**	**-10.35 (3.21)[b]**	-0.91 (1.64)	**-71.18 (3.27)[c]**	**-6.53 (0.31)[c]**	**-0.93 (0.24)[c]**	-0.03 (0.03)
Age	-0.07 (0.12)	0.02 (0.06)	0.05 (0.15)	0.10 (0.07)	**-0.40 (0.15)[b]**	**-0.04 (0.01)[a]**	-0.004 (0.01)	-0.002 (0.001)
PExMMN	-0.79 (3.05)	0.26 (1.61)	-0.10 (3.85)	-2.50 (1.94)	**13.89 (3.88)[c]**	0.60(0.37)	-0.40 (0.29)	0.02 (0.03)
Constant	0.31 (1.51)	1.25 (0.80)	1.95 (1.91)	-1.55 (0.97)	**8.40 (1.94)[c]**	**0.57 (0.19)[b]**	-0.05 (0.14)	0.02 (0.02)
Observations	521	521	521	531	531	531	531	531
R^2	0.15	0.32	0.03	0.01	0.62	0.63	0.09	0.02

vBMD = volumetric bone mineral density, CSA = cross sectional area, BMC = bone mineral content, a= p<0.05, b= p<0.01, c=p<0.001

Model 1 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30
Model 2 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30, and were in the FeFol arm (i.e. control group)

Table 4.6 Summary of Model 1 for change in bone outcome measures at the 4% distal and 38% proximal tibia between Booking and P30.

	Total vBMD (mg/cm^3)	Trabecular vBMD (mg/cm^3)	Total CSA 4% (mm^2)	Total CSA 33% (mm^2)	Cortical vBMD (mg/cm^3)	Cortical BMC (mg/mm)	Cortical CSA (mm^2)	Cortical thickness (mm)
XCT2000L to XCT2000	**21.21 (1.27)[c]**	**10.51 (1.11)[c]**	**-13.90 (5.37)[b]**	**19.81 (2.55)[c]**	**64.42 (2.31)[c]**	**19.65 (0.88)[c]**	**4.07 (0.51)[c]**	**-0.06 (0.02)[b]**
XCT2000 to XCT2000L	**-22.83 (1.06)[c]**	**-14.47 (0.92)[c]**	7.00 (4.48)	**-15.97 (2.20)[c]**	**-74.32 (1.99)[c]**	**-22.97 (0.76)[c]**	**-4.54 (0.44)[c]**	0.03 (0.02)
Age	0.03 (0.04)	0.02 (0.04)	0.07 (0.19)	0.05 (0.09)	-0.10 (0.08)	-0.02 (0.03)	-0.004 (0.02)	-0.0004 (0.001)
Constant	**0.91 (0.32)[b]**	**0.77 (0.28)[b]**	-1.20 (1.34)	0.68 (0.66)	**4.39 (0.59)[c]**	**1.52 (0.23)[c]**	**0.42 (0.13)[b]**	0.005 (0.01)
Observations	474	474	474	478	478	478	478	478
R^2	0.63	0.44	0.02	0.21	0.83	0.76	0.28	0.02

vBMD = volumetric bone mineral density, CSA = cross sectional area, BMC = bone mineral content, a= p<0.05, b= p<0.01, c=p<0.001

Model 1 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30
Model 2 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30, and were in the FeFol arm (i.e. control group)

Table 4.7 Summary of Model 2 for change in bone outcome measures at the 4% distal and 38% proximal tibia between Booking and P30.

	Total vBMD (mg/cm^3)	Trabecular vBMD (mg/cm^3)	Total CSA 4% (mm^2)	Total CSA 33% (mm^2)	Cortical vBMD (mg/cm^3)	Cortical BMC (mg/mm)	Cortical CSA (mm^2)	Cortical thickness (mm)
PE	0.34 (0.84)	0.52 (0.73)	2.62 (3.54)	2.55 (1.75)	-0.60 (1.58)	-0.31 (0.60)	-0.09 (0.35)	-0.02 (0.02)
MMN	0.51 (0.82)	-0.24 (0.72)	-3.04 (3.46)	2.76 (1.70)	0.65 (1.54)	0.36 (0.58)	0.28 (0.34)	-0.02 (0.02)
XCT2000L to XCT2000	**21.30 (1.27)[c]**	**10.53 (1.11)[c]**	**-14.17 (5.38)[b]**	**19.57 (2.56)[c]**	**64.52 (2.31)[c]**	**19.65 (0.88)[c]**	**4.05 (0.51)[c]**	**-0.06 (0.02)a**
XCT2000 to XCT2000L	**-22.79 (1.06)[c]**	**-14.49 (0.93)[c]**	6.69 (4.48)	**-16.05 (2.20)[c]**	**-74.21 (1.99)[c]**	**-22.93 (0.76)[c]**	**-4.53 (0.44)[c]**	0.03 (0.02)
Age	0.03 (0.04)	0.02 (0.04)	0.09 (0.19)	0.06 (0.09)	-0.11 (0.08)	-0.03 (0.03)	-0.004 (0.02)	-0.0004 (0.001)
PExMMN	1.22 (1.17)	0.30 (1.03)	-3.93 (4.98)	-2.84 (2.44)	2.30 (2.21)	0.42 (0.84)	-0.20 (0.49)	0.02 (0.02)
Constant	0.17 (0.59)	0.57 (0.52)	0.09 (2.50)	-1.25 (1.23)	**3.76 (1.12)[c]**	**1.38 (0.43)[b]**	0.37 (0.25)	0.02 (0.01)
Observations	474	474	474	478	478	478	478	478
R^2	0.64	0.44	0.03	0.21	0.83	0.76	0.28	0.02

vBMD = volumetric bone mineral density, CSA = cross sectional area, BMC = bone mineral content, a= p<0.05, b= p<0.01, c=p<0.001

Model 1 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30
Model 2 reference group: women of mean age at Booking, who were scanned on the same pQCT scanner at Booking and P30, and were in the FeFol arm (i.e. control group)

4.5 Discussion and conclusions from ENID Bone Study

These pQCT data from the ENID Bone Study indicate that significant mobilisation of bone mineral from the maternal skeleton does not occur in rural Gambian women during pregnancy. I had hypothesised that a maternal response to pregnancy would be most plausible at trabecular-rich sites. However, to the contrary, Model 1 found statistically significant increases in total (tibia) and trabecular vBMD (radius and tibia) although these were no longer significant after adjusting for supplement group (Model 2). Most interestingly, I found strong evidence of change in the cortical compartments of both radius and tibia during pregnancy, with small but statistically significant increases in cortical vBMD and BMC at both sites (Model 1). These increases remained following adjustments for supplement group, though supplement modulation of these increases was only observed at the radius (Model 2). This was the only notable difference between loadbearing and non-loadbearing sites. These data suggest statistically significant pregnancy-induced adaptations in the cortical compartment of the maternal appendicular skeleton between mid- to late-gestation, however, any clinical or physiological significance of these increases would be negligible as all were <1%, however, such changes may reflect changes in the Ca homeostasis.

While the potential for supplement effects was hypothesised at both distal and proximal sites, supplement modulation of change was only significant at the proximal radius. Possible mechanisms for between-group differences had been theorised as the displacement of dietary Ca from the maternal diet in these mothers with a habitually low Ca intake, or a possible effect of supplementation on maternal weight gain during gestation. In the case of Ca displacement, I would have expected to see greater mobilisation of bone mineral in the PE and PEMMN arms of the study, which was not the found. If maternal weight gain had been influenced we could have expected increased weight gain in women receiving PE and PEMMN compared to the MMN and FeFol arms. However, weight gain did not differ markedly in the supplement groups between Booking and P30. The observed supplement effects support neither hypothesis and are difficult to explain.

In the PE only arm the increase in cortical vBMD at the radius was almost fully attenuated, while less of a change compared to FeFol (although clinically negligible in magnitude) was found in cortical BMC. Similarly supplementation with MMN partially attenuated the increase in cortical vBMD compared to FeFol at the radius. In women receiving MMN only a significant increase was found in cortical CSA compared to the FeFol women. Most curiously the effect of the PExMMN interaction term acted in the opposite direction of PE or MMN alone and women in the PEMMN arm had an increase in cortical vBMD greater than the reference group. It is difficult to determine through what mechanism these subtle differences may be acting, however, a potential reason for these unusual finding may relate to the particularly poor compliance of participants in the LNS arms of the ENID Trial (Moore, personal communication 2018). While these supplement-effects are statistically significant, they are extremely small in magnitude. The effects of maternal age and parity were also explored as determinates of change in bone outcomes during pregnancy, while significant age effects were found for cortical vBMD and cortical BMC, older women had a lower increase in these parameters vs the reference group, the magnitude of these however was very small and unlikely to be of any clinical or physiological significance at this stage of the lifecourse.

The lack of mineral mobilisation from trabecular-rich distal sites is in keeping with some evidence from studies using DXA and SPA during pregnancy where few changes have been reported at the distal forearm (Christiansen et al., 1976, Cross et al., 1995, Kolthoff et al., 1998, Black et al., 2000, More et al., 2001). Our findings at the distal radius are in contrast to Wisser et al. (2005) where decreases in trabecular vBMD were observed in pregnant Swiss women using a different pQCT technique. However, the slight increases seen in cortical bone in the Gambian study may be in-keeping with the conservation of cortical bone reported by Wisser et al. (2005) at the forearm. The lack of mineral mobilisation from the cortical compartment of the forearm is also line with DXA data during pregnancy (Cross et al., 1995, Kolthoff et al., 1998, Black et al., 2000, More et al., 2001, Olausson et al., 2008). Furthermore, studies of bone changes in lactation have previously suggested

a possible conservation of bone mineral at cortical-rich sites of the appendicular skeleton, which could perhaps also be extended to pregnancy (Laskey et al., 1998, Olausson et al., 2008).

There are several limitations to this work that must be considered. Although, dietary data were collected in ENID Bone these were not allocated to me for analysis as part of my PhD. This was a pragmatic decision as although diet plays a crucial in maintaining bone health, it would have changed the scope of my project and would not have been feasible within the time constraints of the project. An important aspect that cannot be accounted for is that mineral mobilisation may have occurred prior to Booking. Limited data (Purdie et al., 1988) suggest the potential for trabecular mobilisation in early-pregnancy which are reversed near term (Shahtaheri et al., 1999) although this seems somewhat unlikely as fetal demand is low in the first trimester and greatest in late gestation. Although the BTM evidence mostly supports a resorptive state in late gestation through increases in resorption markers rising from the first trimester (Cross et al., 1995, Black et al., 2000, Yoon et al., 2000, Moller et al., 2013), some formation markers have been reported to increase also: increases in P1NP (Naylor et al., 2000, Kaur et al., 2003a); P1CP (Black et al., 2000, Naylor et al., 2000); and BAP (Black et al., 2000, Naylor et al., 2000, Yoneyama and Ikeda, 2010) have been found into the third trimester. These bone formation markers must be cautiously interpreted due to potential fetal or placental origin. On balance it is unlikely that pregnancy-induced mobilisation would have been initiated and reversed within the course of several months, prior to lactation-induced mobilisation. Given the evidence for late-pregnancy being a resorptive state, it may be that to detect potential decreases in the maternal appendicular skeleton with pQCT data may need to be collected closer to term.

It is well documented in the literature that during lactation maternal bone mineral is mobilised preferentially from trabecular-rich sites. In contrast I found no evidence of mobilisation until 30 weeks of pregnancy for either the distal radius or tibia, it is possible that this may represent the conservation of maternal trabecular bone during pregnancy. Whether there is mobilisation from these sites during lactation remains to be determined in this cohort.

Despite finding no evidence of bone mineral mobilisation during pregnancy in this study, mobilisation may occur in pregnancy through other mechanisms which are not detectable with pQCT, such as changes in maternal trabecular (i.e. trabecular thickness, trabecular number, trabecular separation) or cortical (thickness and porosity) microarchitecture. These would require a higher resolution technique to be used. To further describe changes in the trabecular compartment of the maternal appendicular skeleton beyond trabecular vBMD as measured with our pQCT XCT 2000/2000L, more advanced modalities such as high- resolution pQCT, which have a much greater resolution (i.e. voxel size of 82 μm vs 0.5 mm of the XCT2000/2000L) may be required.

In summary these data provide a unique insight into maternal bone mineral homeostasis during pregnancy in rural Gambian women. As these are the first such densitometry data collected during gestation in either a Sub-Saharan African population or a population with a habitually low Ca intake during pregnancy, it is difficult to compare these findings to the literature which has mostly accumulated from white women in HIC. In contrast to my hypotheses of bone mineral mobilisation between Booking and P30 in this population, it may be that conservation of bone mineral is an adaptation to a habitually low Ca intake and is normal in this population. These data highlight that although there is some evidence to support bone mineral mobilisation in women with high Ca intakes and high quality diets in HIC, such assumptions may not hold true in all populations especially where dietary quality is lower and Ca intake habitually low. My findings from the ENID bone data may be summarised as a lack of pregnancy-induced bone mineral mobilisation between Booking and P30 in the maternal appendicular skeleton with:

1. Conservation or small increases of bone mineral in the trabecular compartment (radius and tibia)
2. Small but significant increases cortical compartment in vBMD and BMC at:
    a. The loadbearing 38% proximal tibia
    b. Non-loadbearing 33% proximal radius

3. Significant supplement effects of negligible magnitude found at the proximal radius
4. Significant but extremely small maternal age effects confined to the proximal radius, increases lower in older mothers.

In conclusion although the magnitude of change seen in the ENID Bone Study pQCT analysis was small and increases were observed in cortical vBMD and BMC contrary to my initial hypothesis of bone mineral mobilisation, there may lasting consequences of these small changes if they prove to be cumulative across multiple pregnancies. Previous research has shown that repletion of lactation-induced mineral mobilisation occurs post-lactation in this population. It is possible that the physiological response to pregnancy in women with habitually low Ca intakes is such that bone mineral is not mobilised and skeletal reserves are preserved.

5 Pregnancy and Bone Study Subjects and Methods

5.1 Introduction

The Pregnancy and Bone Study (PABS) was a longitudinal observational study designed to investigate whether bone mineral changes occur in the maternal appendicular skeleton during pregnancy. I was responsible for the design, ethical approval and management of the study together with all data analysis and interpretation. Ethical approval was obtained from the East of England – Essex Research Ethics Committee (REC) (Project ID 199857) to recruit up to 100 women aged 30 to 45 years from the Cambridge area to participate in the study. Pregnant women (n=53) were recruited from within the catchment area of the Rosie Cambridge University Hospital Trust (CUH), and healthy non-pregnant non-lactating (NPNL, n=37) women were recruited from the community. Data collection occurred between March 2017 and April 2018, bone densitometry data from participants at two time points in mid- and late-pregnancy using peripheral pQCT and HRpQCT. NPNL participants were scanned contemporaneously to account for age-associated changes (Uusi-Rasi et al., 2007, Burt et al., 2017).

5.1.1 PABS hypothesis

My primary hypothesis was that maternal trabecular volumetric BMD (pQCT and HRpQCT) in the appendicular skeleton would decrease from mid- to late-pregnancy due to maternal bone mineral mobilisation from trabecular-rich skeletal sites, and that changes in trabecular microarchitecture would also be observed (HRpQCT).

5.1.2 PABS Aims

My aims were to determine whether:

1. Maternal bone mineral is mobilised between mid- to late-pregnancy from trabecular-rich sites in the maternal appendicular skeleton measured as decreases in trabecular vBMD (pQCT and HRpQCT) and changes in trabecular microarchitecture compared to NPNL controls.

2. Adaptations occur in the cortical compartment between mid- and late-pregnancy:

    a. Distal sites: cortical vBMD and microarchitecture (HRpQCT).

    b. Diaphyseal sites: cortical vBMD, BMC, CSA, and thickness (pQCT)

5.1.3 PABS objectives

The primary objective of this study was to determine whether pregnancy-induced bone mineral mobilisation occurs between 14-16 weeks and 34-36 weeks of pregnancy in my primary outcome measures of trabecular vBMD (pQCT and HRpQCT) and trabecular microarchitecture (HRpQCT: trabecular number, separation, and thickness).

Secondary objectives were to explore:

1. Whether changes occur in the cortical compartment between early- to late-gestation:
    a. At proximal radius and tibia with a focus on cortical vBMD, cortical BMC, cortical CSA, cortical thickness, and total CSA (pQCT)
    b. At the distal radius and tibia to determine cortical vBMD, cortical thickness, and cortical porosity (HRpQCT)
2. To determine any predictors of change in 1. and 2. with a specific focus on:
    a. Maternal age
    b. Weight change
    c. Parity
3. Correlation between pQCT and HRpQCT in-vivo measurements for trabecular vBMD, cortical vBMD, and cortical thickness.

Figure 5.1 Catchment area of Cambridge University Hospitals Trust of which the Rosie Hospital is a member shown in context of the East of England Nomenclature of Territorial Units for Statistics (NUTS) region. Source https://www.cuh.nhs.uk/sites/default/files/misc/Trust-Catchment-Area-Map-A4-NEW.PDF

5.2 Pre-study Work-Up

Study design was undertaken with the support and supervision of Ass. Prof. Kate Ward and Prof. Ann Prentice of the Nutrition and Bone Health group (NBH) at Medical Research Council Elsie Widdowson Laboratory (MRC EWL) Practical advice was received from my thesis advisory committee, Dr. Sophie Moore (King's College London) and Dr. Catherine Aiken (University of Cambridge).

Measurement timings: I planned to include at least two pregnancy time points to detect mid- to late-pregnancy changes in maternal bone outcomes. For logistical and ethical reasons the first visit could not be prior to each participant's 12 week antenatal ultrasound scan when due date is estimated and potential fetal abnormalities may be detected. Recruitment took place at this ultrasound scan appointment, as few data support early pregnancy mineral mobilisation, allowing visit 1 to be booked between 14-16 weeks gestation. This was as close to the first trimester as possible, while allowing for: a) for appropriate screening; b) adequate time to reflect on participation; c) flexible scheduling of visits around each participant's other appointments. When deciding on an appropriate follow-up time point, I balanced: a) allowing sufficient time for any

changes to be detected in late pregnancy; b) minimising participant burden; c) reducing loss to follow up from premature deliveries. Thirty four to 36 weeks gestation met these criteria, and was similar to the timing of the late-pregnancy time points in other densitometry studies (Wisser et al., 2005, Moller et al., 2012) and is 4-6 weeks later in the third trimester than the final ENID Bone visit. I considered including an interim visit. However, this would increase participant burden and was not central to my primary hypothesis, I therefore did not include this extra visit. I also considered including a potential postpartum visit for a DXA scan but because this additional data would: a) be confounded by the initiation of lactation; b) significantly increase the timeframe of the study; c) DXA data would be cross-sectional only and d) was not central to my research question, I decided not to include this measurement.

Diet: I considered the collection of dietary intake data during pregnancy using either 4 day weighed intake diaries or potentially food frequency questionnaires (FFQ). 4 day weighed intake diaries would greatly increase participant burden and for this reason were not collected. The use of an FFQ was considered further and advice was received from Dr. Gail Goldberg (MRC EWL) and Prof. Sian Robinson (MRC LEU) about the practicalities and efficacy of using an FFQ in my study population. Using or adapting an FFQ would present several challenges: the use of CALQUEST would not be appropriate as it has not validated for use in pregnancy, while the use of an FFQ validated in pregnancy such as that used in SWS may not be appropriate given the socio-demographic differences between Cambridge and Southampton. Also Olausson et al. (2008) found no association between maternal Ca intake and the bone outcomes on which PABS was powered. Reluctantly, I took the pragmatic decision against collecting dietary data to reduce burden of the study interventions.

Recruitment site: A meeting with Dr. Hanna Missfelder-Lobos (Consultant and Specialty Lead in Obstetrics and Fetal Medicine), at the Rosie Maternity Hospital, Cambridge University Hospitals Trust (CUH) was convened to discuss the feasibility and logistics organising a study at the Rosie. The outcome of the meeting was favourable and following this I began to prepare to apply for

ethical approval. Descriptive statistics from a six-year period (2008-2013) were provided by Dr. Catherine Aiken (Consultant in Obstetrics and Fetal Medicine) and helped demonstrate the feasibility of recruiting the necessary number of women in the age range of 30-45 years which formed my primary inclusion criterion (Tables 5.1 and 5.2, Figure 5.1). Setting a lower age limit at 30 years would allow the study to have a higher mean age than most studies discussed in Chapter 2 and above the national mean age of pregnancy in England and Wales (30.5 years, ONS 2017). An upper age limit of 45 years was established as very few pregnancies reported across the 6 year period to women aged ≥45 years (Figure 5.1, Table 5.2). As discussed in chapter 2 women having later pregnancies may be more vulnerable to potential bone mineral mobilisation during pregnancy as: a) mobilisation may take place against background of gradual age related mineral loss, b) there may be less time to replenish any mobilised mineral before menopause. I considered recruiting only primiparous women in this age range to reduce possible confounding from previous pregnancies and periods of lactation, though this would have been unfeasible as it would greatly reduce the proportion of potential participants from 52% of all pregnancies (aged 30-45 years) to 14% of all pregnancies (primiparous & aged 30-45 years) at the Rosie Hospital (Table 5.2).

Table 5.1 Maternal age (years) distribution from Jan 2008-Dec 2013 – 32,079 deliveries at Addenbrooke's.

1st Quartile	Median	Mean	3rd Quartile
27.00	31.00	30.73	35.00

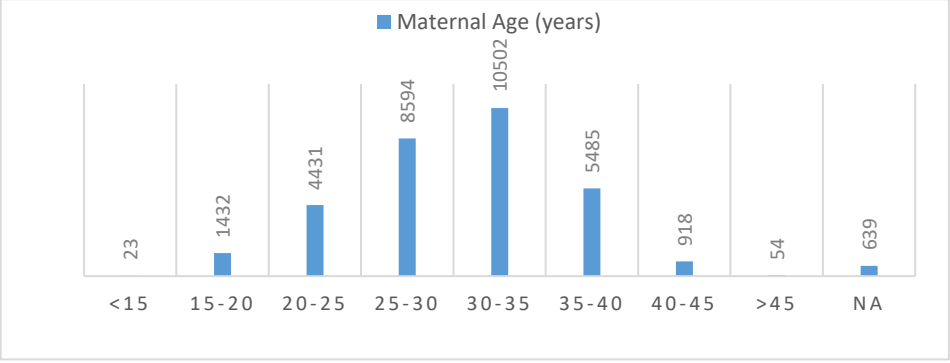

Figure 5.2 Distribution of pregnant women giving birth at the Rosie Hospital CUH (2008 - 2013) in 5 year age bands (n = 32,079).

Table 5.2 Numbers of deliveries by parity and maternal age (total from 6 years) n = 32,079

	Parity											
Maternal age (years)	0	1	2	3	4	5	6	7	8	9	10	11
<15	17	3	3	0	0	0	0	0	0	0	0	0
15-20	701	533	164	22	10	3	0	0	0	0	0	0
20-25	1604	1868	686	197	51	15	4	0	2	0	0	0
25-30	2851	3928	1249	359	123	51	16	9	1	0	0	0
30-35	2910	5183	1722	406	165	63	30	9	4	2	0	0
35-40	1306	2470	1120	338	155	54	26	6	2	1	1	2
40-45	250	327	199	82	32	15	8	1	1	1	0	0
>45	28	12	7	3	2	2	0	0	0	0	0	0

To reduce potential confounding in the pregnant arm, I screened out potential study volunteers who had conceived using IVF or had a twin/multiple pregnancy. The need for IVF may indicate a possible underlying medical condition and without a detailed medical history it would not be possible to know whether this may have affected their BMD prior to pregnancy. Additionally, if bone mineral is mobilised during pregnancy it is plausible that multiple fetuses would require a greater mobilisation of maternal bone mineral; biochemistry and bone turnover markers in twin pregnancies have been reported to differ to singleton pregnancies (Nakayama et al., 2011, Goswami et al., 2016). To reduce this potential confounding, women with twin pregnancies were not recruited. Additionally a number of more general exclusion criteria were applied to both groups (see Section 5.4.1, Table 5.3).

5.2.1 Sample size calculation

Prior to the initiation of this study there were no suitable published pQCT data from pregnancy on which to base a formal sample size calculation. This study was therefore powered to be able to reproduce the effect sizes seen by Olausson et al. (2008) who observed a change between baseline and postpartum measurements of 2.6% (±4.2) in DXA-measured size-adjusted BMC at the lumber spine. It was calculated to have a power of 0.9, and alpha of 0.05, that a sample size of 35 participants would be required to detect within group change in BMD from baseline to the 3rd trimester of pregnancy similar to Olausson et al. (2008).

5.3 Final Study Design

The PABS was designed to detect within group change in pregnant women aged 30-45 years and in NPNL women in the same age category. As described above an early pregnancy time point of 14 – 16 weeks gestation was selected for the baseline visit, with follow-up data collected twenty weeks later (34-36 weeks). The NPNL group were scanned with a similar length of time between visits. Ethical approval for this study was sought on the 07/07/2016 from the East of England – Essex Research Ethics committee and was obtained on the 08/09/2016. The study protocol and all supporting documents were submitted and approved by the R&D department at Cambridge University Hospitals Trust with Dr Jeremy Brockelsby (Consultant in Obstetrics and Fetal Medicine and Clinical Director in Obstetrics and Gynaecology) acting as our local collaborator providing clinical oversight at the Rosie Hospital.

Figure 5.3 Schematic overview of the Pregnancy and Bone Study (PABS)

The above figure highlights recruitment, screening, and procedures performed at each visit. NPNL = non-pregnant non-lactating. The two sites involved in the Pregnancy and Bone Study (PABS), above the Elsie Widdowson Laboratory (MRC EWL) where all procedures took place. Below, the Rosie hospital which was our recruitment site for women in the pregnant arm of the study.

5.4 Selection of subjects

5.4.1 Eligibility criteria

Criteria for the inclusion and exclusion of potential participants were selected to ensure that we recruited women within our age range of interest, with no previous history of medical conditions or significant use of medications affecting bone health as summarised in table 5.3.

Table 5.3 Eligibility criteria used during recruitment of pregnant and non-pregnant non-lactating (NPNL) participants in the pregnancy and bone study (PABS)

Pregnant Women	Healthy NPNL Women
Inclusion Criteria	
Aged 30-45 years	Aged 30-45 years
Singleton pregnancy	Living within travelling distance of the Rosie
Natural conception	>3 months after the cessation of lactation from previous child
Living within travelling distance of the Rosie	Ability to give written, informed consent
>3 months after the cessation of lactation from previous child prior to this pregnancy	
Ability to give written, informed consent	
Exclusion Criteria	
Known condition affecting bone/calcium metabolism	Pregnant at screening/trying to conceive
Use of medication affecting bone/calcium metabolism	Known condition affecting bone/calcium metabolism
Immobilisation	Use of medication affecting bone/calcium metabolism
Conception by IVF	Immobilisation
Prolonged periods of amenorrhea	Prolonged periods of amenorrhea
Multiple pregnancy	

5.4.2 Recruitment

Recruitment of pregnant participants, between late March and October 2017, occurred while women attended their 12 week ultrasound scans and was led by research midwives not directly involved in the women's current clinical care. Potential participants were informed about the study, and had an opportunity to discuss any concerns. Contact details were obtained if women were interested in taking part and, if unsure, they were given a copy of the PIS to take away to consider. In addition to the active recruitment of pregnant women from the Rosie, posters and leaflets were also placed in the ultrasound department with the contact details of the research team. Posters were also distributed locally, in shops, gyms, and educational institutions. Gumtree was used to advertise the study in the local Cambridge area. NPNL from the community were recruited using several

methods. An email was circulated to staff within MRC EWL seeking potential recruits. Emails were also sent to individuals from the MRC EWL Volunteer database, which contained the details of former volunteers who had consented to being contacted for future research, and were in the required age range. As above, posters and Gumtree were used to advertise locally so that if interested potential participants could contact the research team for further information.

5.4.2.1 Telephone Screening

Potential participants were screened with a telephone-based screening questionnaire to assess eligibility (Appendix E). Those found eligible received an appointment date and time for their first visit. If there was uncertainty whether a potential participant was eligible, Dr. Kate Ward was consulted.

5.4.2.2 Obtaining informed written consent

All participants were given a minimum of 24 hours to consider participation in the study prior to written consent being obtained. During screening, potential participants had an opportunity to ask questions relating to the study. All potential participants who had given their contact details to the study midwives received a copy of the PIS. Potential pregnant participants who had seen the adverts elsewhere were sent a copy of the PIS via email following the screening process, as were all NPNL participants. At visit one, informed written consent was obtained from all participants in a private room by trained members of the research team, who explained the study in full as detailed in the PIS. Questions that arose during the process were answered by the team member acquiring consent or if necessary referred to the principal investigator for further clarification. Written consent was obtained on two copies of the PIS (signed and dated), and the consent forms (signed and initialled). Participants were made aware that they could freely withdraw consent at any point during the study. Permission was also obtained to inform their GP of their participation in the study. Participants received a copy of all signed documentation.

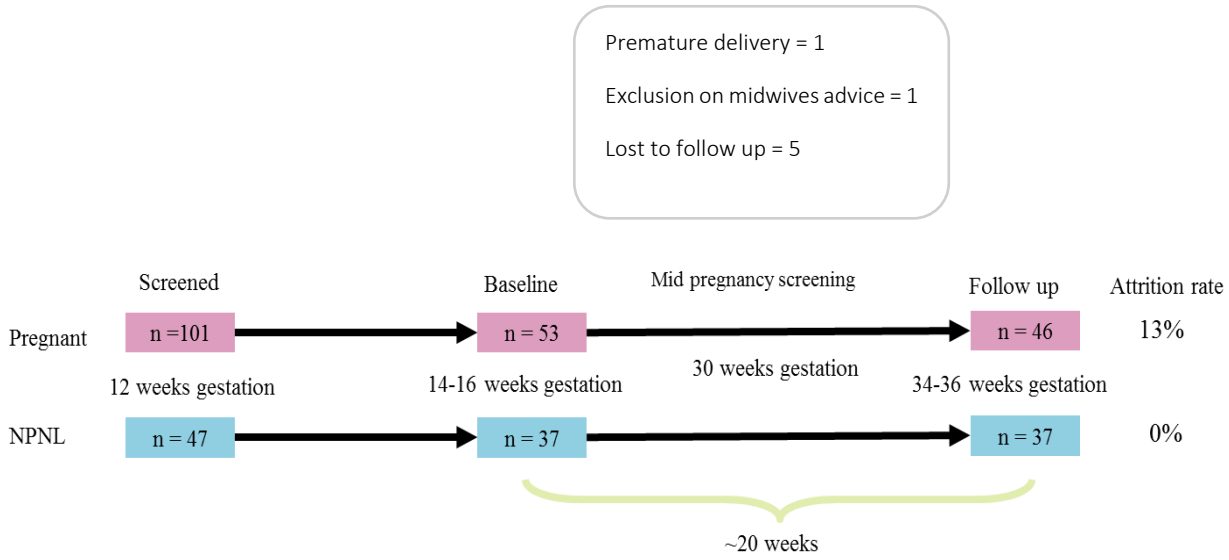

Figure 5.4 Diagram of number of participants screened, and then subsequently scanned at each time point.

A comprehensive break-down is provided in Tables 6.10 and 6.11 as not all participants have complete data at each time point.

5.4.2.3 Screening between study visits

The eligibility of women in the pregnancy arm of the study was reassessed from their medical notes by a research midwife at week 30 of pregnancy (Figure 5.3). This also ensured that participants who had unfortunate pregnancy outcomes, would not be contacted directly by the research team to avoid causing any additional distress. Additionally this mid-pregnancy screening would identify participants who had moved out of area or lost the ability to give informed consent.

5.4.3 Research Team and Research Sites

Our primary recruitment site was the Ultrasound Department of the Rosie Hospital, while all investigative procedures took place at the MRC Elsie Widdowson Laboratory, Cambridge (Figure 5.4). The research team and those who supported the study are listed in Table 5.4. Scanning was primarily performed by me but support was provided by a number of additional trained technologists when required.

Table 5.4 Members of the research team in the Pregnancy and Bone Study (PABS)

Individual	Role	Duties
Mícheál Ó Breasail	Principal Investigator (PI)	Study design Screening Recruitment Data collection Data entry Scan grading pQCT/HRpQCT Scan analysis HRpQCT Data processing Data analysis
Ass. Prof. Kate Ward	PhD supervisor	Study design Oversight
Prof. Ann Prentice	Group Leader/Director MRC-EWL	Study design
Jennifer Woolston	Study set up	Screening Recruitment Data Collection Data entry
Dr Sophie Moore	Thesis Advisory Committee	Study design
Dr Catherine Aiken	Thesis Advisory Committee	Study design
Julie Holgate Sue Tiplady	Research Midwives	Recruitment Mid-pregnancy screening
Rebecca McGrath	Research Assistant	Screening Data Collection Data entry
Dr Sumantra Ray	Study Clinician	Clinical support
Dr Jeremy Brockelsby	Collaborator/Local	Clinical oversight
Darren Cole	Head of Data Operations at MRC-EWL	Database design pQCT Access tool design
Sophie Yelland	Placement student	Data entry Scan analysis HRpQCT

5.5 Selection of Outcome Measures

Anthropometric measures of height (cm), weight (kg), forearm length (mm), and lower leg length (mm) were obtained at visit 1 (Table 5.5), at visit 2 only weight was repeated. Bone densitometry was conducted at both visits and included scans using a Stratec 2000L™ pQCT (Stratec Medizintechnik, Pforzheim, Germany) and HRpQCT (XtremeCT, Scanco Medical AG, Brüttisellen, Switzerland) at the forearm and lower leg to measure volumetric bone mineral density (vBMD) and related parameters of bone geometry and structure, which effect overall bone strength.

Table 5.5 Summary of investigations conducted at each study time point

Outcome	14-16 weeks	34-36 weeks
Anthropometric Measurements		
Height (cm)	✓	
Weight (kg)	✓	✓
Forearm length (mm)	✓	
Lower leg length (mm)	✓	
Questionnaire Measurements		
Musculoskeletal Questionnaire	✓	✓
pQCT		
Radius	✓	✓
Tibia	✓	✓
HRpQCT		
Radius	✓	✓
Tibia	✓	✓

5.5.1 Collection of anthropometric measures

Height was obtained without footwear using standard operating procedures to the nearest 0.1cm using a stadiometer (Seca GmbH, Hamburg, Germany). Weight was measured to the nearest 0.1 kg while the subjects wore light clothing without footwear using a digital scale (Seca GmbH, Hamburg, Germany), which were routinely calibrated to ensure accuracy. Body Mass Index (BMI) was calculated by dividing the participants weight (kg) by their height (m) squared, this was expressed in kg/m^2.

5.5.1.1 Musculoskeletal Questionnaire

A musculoskeletal questionnaire was administered to all participants at each visit to collect relevant information relating to the participant's musculoskeletal health including family history of musculoskeletal disease, previous fractures, conditions that may affect bone or Ca homeostasis, use of medications that may affect bone or Ca homeostasis, contraceptive use, and the use of vitamin and mineral supplements. Lifestyle factors such as smoking, alcohol consumption, and physical activity were also assessed. Details of their reproductive history such as gravidity, parity, and whether they had previously breastfed were also collected in the questionnaire. These data were entered by members of the research team.

5.5.2 Bone Imaging

Volumetric BMD and related parameters of bone strength were measured (pQCT and HRpQCT) twice during the study period at the forearm and lower leg. All procedures were conducted at MRC EWL in a dedicated scanning room located within the purpose built volunteer suite. All scans were performed by trained investigators in compliance with local radiation use guidelines (IRMER, IRR 2017). Due to ethical considerations and to put pregnant participants at ease the option of wearing a lead apron as used clinically (Lead equivalence 0.35 mm) was offered to pregnant participants. Prior to the submission of the study for ethical review the dose and associated risk of the bone imaging procedures were quantified and approved by the East Anglian Regional Radiation Protection Service and were considered to be low (Table 5.6). Dosimeters present in the scanning rooms monitor the level of X-ray scatter from the devices and the EWL records indicate that no scatter is detectable within the scan rooms. The total effective dose to the participant was estimated to be <18.4 μSv (Table 5.6). The effective fetal dose was calculated to at <10 μSv. This effective dose presents no risk of any deterministic (acute) effects. Any future risk was quantified using the Health Protection Agency's "Protection of Pregnant Patients during Diagnostic Medical Exposures to Ionising Radiation" (Health Protection Agency, 2009). The increased risk of cancer occurring in the infant up to the age of 15 due to the exposure (10 μSv) will be ~1 in 1,300,000, much lower than the natural risk of childhood cancer in the UK (1 in 500). Wearing the lead apron over the abdomen reduced the risk by a factor of approximately 10.

Cambridgeshire is considered to be a low background radiation area in the UK (~7 μSv/day) meaning that the exposure to a woman in this study was equivalent of less than three days of background radiation in Cambridgeshire, or less than a quarter of the exposure from a return transatlantic flight (~80 μSv). MRC EWL has strict policies for the use of ionising radiation and adheres to IRR 2017 (Department of Health, 2017).

Table 5.6 Maternal effective dose in microSieverts (µSv) per scan, estimates provided by the East Anglian Regional Radiation Protection Service (EARRPS)

Procedure	Estimated visit dose	Total Estimated study dose
pQCT scan of the tibia	<1.6 µSv	<3.2 µSv
pQCT scan of the radius	<1.6 µSv	<3.2 µSv
HRpQCT scan of the tibia	<3 µSv	<6 µSv
HRpQCT scan of the radius	<3 µSv	<6 µSv
Estimated Total	<9.2 µSv	<18.4 µSv

All acquired scans were reviewed by me, any unusual or abnormal scans were brought to the attention of Dr Kate Ward and the study clinician, Dr Sumantra Ray. If required, scans could be sent for review by musculoskeletal health experts at Cambridge University Hospitals who would decide if clinical follow up was required via the participant's GP.

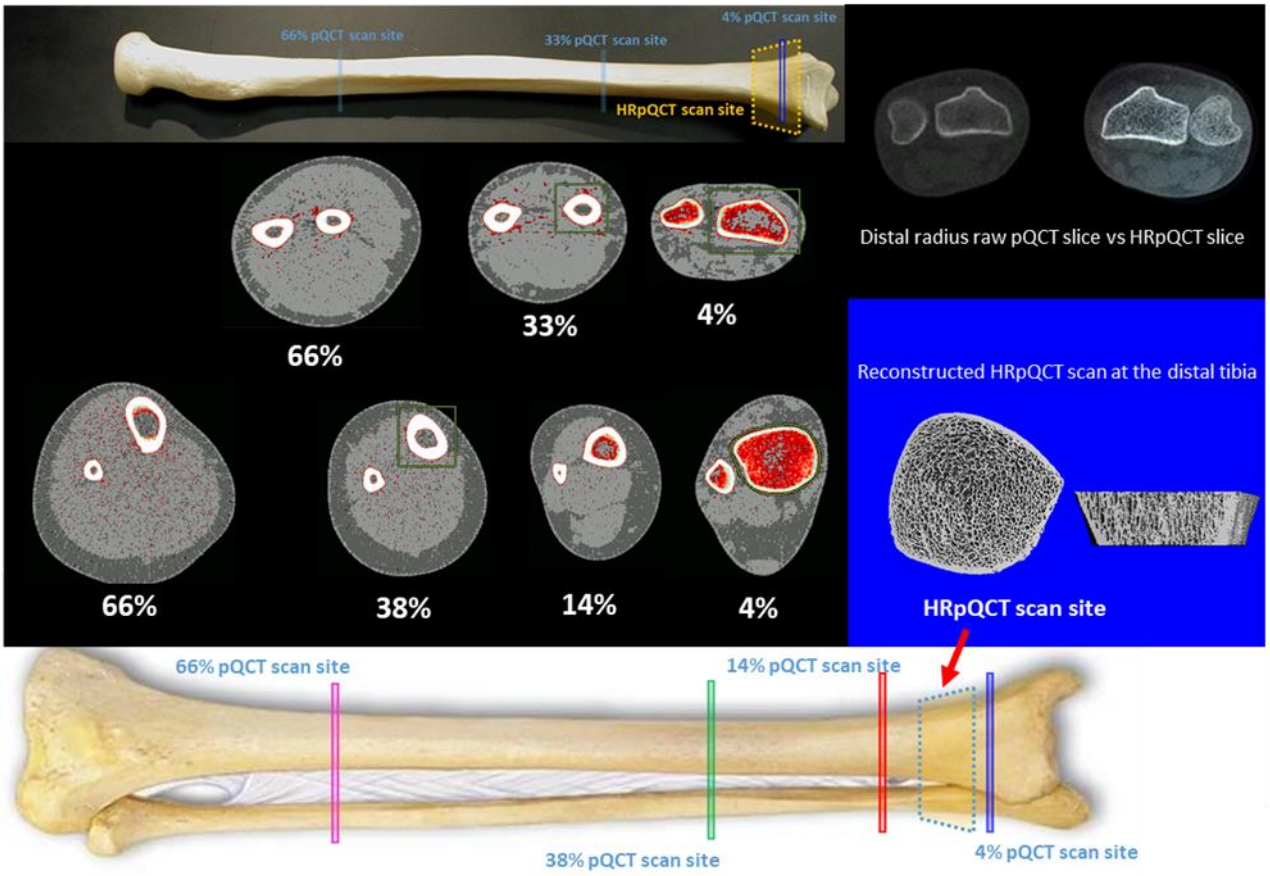

Figure 5.5 Location of pQCT regions of interest (ROI) and HRpQCT volume of interest (VOI) at the radius and tibia

Location of pQCT and HRpQCT scan sites at the radius (top) and tibia (bottom), shown with corresponding pQCT scan images (centre left) An example of HRpQCT 3D reconstruction of the distal tibia is displayed centre right. For contrast radius scan slices(top right) from pQCT (voxel size 0.5 mm) and HRpQCT (voxel size 82 µm) are shown from almost equivalent sites note the difference in scan detail as a result of the discrepancy in resolution

5.5.3 High Resolution peripheral-QCT

HRpQCT scanning was performed using a first generation XtremeCT (Scanco Medical AG, Brüttisellen, Switzerland) and all scans were assessed using the manufacturer's image processing language software (μCT Evaluation Program v6.0; Scanco Medical AG, Brüttisellen, Switzerland). The following scan settings were used for all scans: an X-ray potential of 60 kVp, X-ray tube current of 900 μA, integration time of 100 mins, matrix size of 1536 x 1536 and voxel size of 82 μm. This system enables the simultaneous acquisition of a stack of 110 CT slices, which correspond to a 9 mm section along the axial direction; the result is a volume of interest of the scanned distal radius and tibia. Prior to scanning, the procedure, including the initial scout scan and measurements, was explained clearly to the participant. The participant was seated in an adjustable chair and the same arm/leg scanned as with pQCT. The limb was immobilised in a carbon fibre cast (Figure 5.7).

Figure 5.6 Carbon fibre casts allow for the limb to be immobilised during scanning.
Left, tibia cast with adjustable insoles and calf pads. Right, radius cast with pads of different sizes.

At baseline if the participant's non-dominant limb had previously been fractured the manufacturer's instructions to select the dominant side were followed. At follow-up if an injury had occurred to the previously scanned radius or tibia, the unaffected limb on the opposite side was scanned. The cast maintained the position of the participant's forearm or lower leg in the correct position within the gantry to reduce participant movement. In the case of the tibia it was

necessary to select the proper rubber foot-well fittings (Figure 5.7). For the radius it was necessary to rotate the joint so that the dorsal side of the limb touched the cast; foam was then used on the other side and the straps were attached. The height of the chair was adjusted as required, particularly during the leg measurement, to ensure that the participant's leg was in a horizontal position within the gantry.

5.5.3.1 HRpQCT Scan Acquisition

Prior to scanning pre-calibration was required, it was necessary to first specify which mask to apply (i.e. left/right and radius/tibia). An incorrect mask will scan the wrong region (incorrect limb) and/or at the wrong angle (incorrect side). Pre-calibration was performed without the participant in the scanner so as not to expose them to unnecessary ionising radiation. In-keeping with the manufacturer's guidelines and as described in detail by Boutroy et al. (2005) antero-posterior 2D scout views were performed to determine the region to be scanned (Figure 5.8).

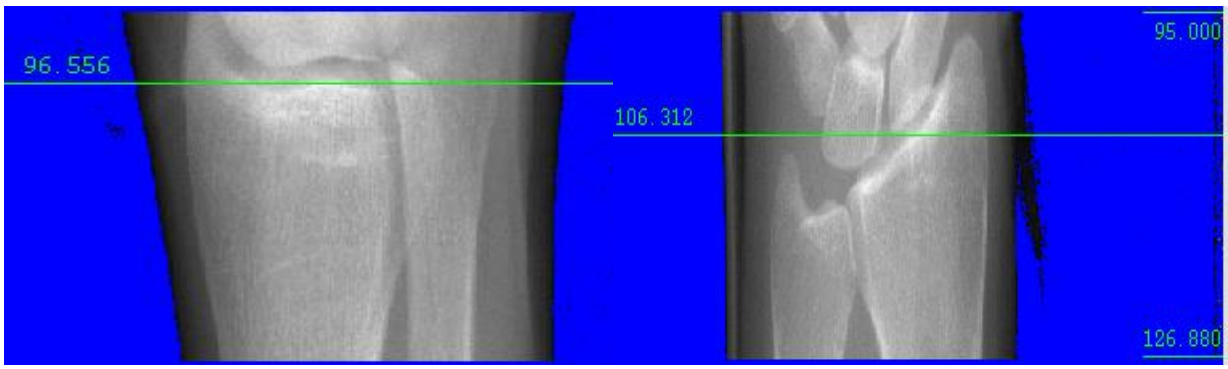

Figure 5.7 Examples of the correct positioning of reference line at the distal radius and tibia

A reference line was manually placed at the endplate of the radius and tibia (Figure 5.6), with the first CT slice 9.5 mm and 22.5 mm proximal to the reference line for the distal radius and distal tibia, respectively. Following this, a stack of parallel CT slices were acquired using a 2D detector array. The entire volume of interest was automatically separated into a cortical and a trabecular region. All scans were acquired by trained technicians using the standard positioning techniques as described by Boutroy et al. (2005). All scans were assessed for motion artefact following acquisition

(see below), if present the participant was asked if they would consent to a second scan being performed.

5.5.3.2 HRpQCT Image Grading

The quality of the HRpQCT measurements of the distal radius and tibia was assessed using a five-grade scale as outlined Pauchard et al. (2012) and recommended by the manufacturer (1, excellent; 2, good; 3, acceptable; 4, poor; 5, unacceptable) (Pauchard et al., 2012). Scans graded 1 to 4 were included in the analysis, grade 5 images with significant motion artefacts causing inblurring and discontinuities in the cortical shell were excluded (Figure 5.9).

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Streaks	None	Small	Medium-Large	Medium-Large	Medium-Large
Discontinuities	None	None	None	Small	Medium-Large

Figure 5.8 HRpQCT scan grading for inclusion in data analysis.

Grade 5 scans are excluded from the final dataset (red box). Scans of grade 4 (green solid box) and below are acceptable for inclusion in analysis. I tested grade 4 against grade 3 (dashed green box) scans and found little difference in output. Figure adapted from Pauchard et al. (2012)

5.5.3.3 HRpQCT Scan Analysis

All analyses were performed using the manufacturer's software installed on a HP AlphaStation DS25 operating in a VMS environment (Hewlett-Packard, Palo Alto, CA, USA). The first stage of the standard analysis began using a semi-automated, hand-drawn contouring system to delineate the periosteal surface. This contour line was applied around the periosteal boundary of the cortex in the first (most distal) CT slice to separate the bone from the surrounding soft tissue. The semi-automatic contour script applied a contour line to all CT slices within the stack. Manual checking of all slices allowed for the amendment of the contour line if required (Figure 5.10). Following this a threshold-based algorithm was used to separate cortical from trabecular compartments (Laib et al., 1998). This threshold was based on the assumption that trabecular bone is one-third of the apparent cortical vBMD (Burrows et al., 2010). The CT slices were binarised (i.e. converted into grey scale images) and subsequently the cortex was segmented from the trabecular compartment using a Gaussian filter and threshold which removed thin trabeculae and noise from the images, allowing for the separate analysis of the cortical and trabecular compartments (Laib and Ruegsegger, 1999). Following this, the linear (X-ray coefficient) attenuation values of the CT slices were converted into hydroxyapatite (HA) mineral densities using a calibration phantom and a beam-hardening correction (see below).

This process allowed for the direct estimation of whole, cortical, and trabecular vBMD. Total bone area was calculated by averaging the total area of each CT slice. In the standard analysis cortical thickness was calculated as mean cortical volume divided by the periosteal surface. On average the thickness of the trabecular rod and plate structures fall between 100 to 150 μm which is the equivalent of 1-2 voxels. As a result of this several XtremeCT trabecular structural parameters (i.e. trabecular thickness and trabecular separation) are derived rather than directly measured (Figure 5.10).

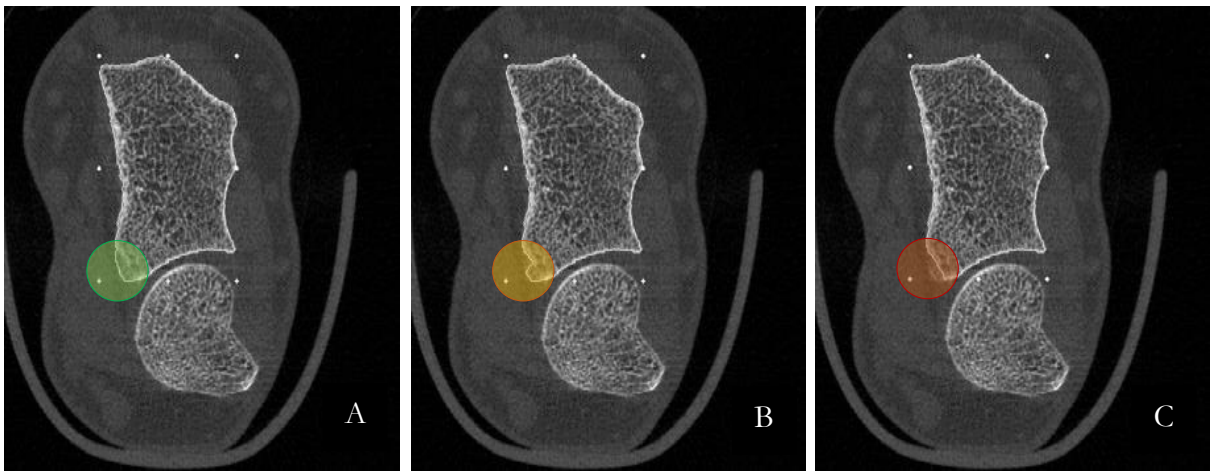

Figure 5.9 Contouring the periosteal perimeter examples of appropriate, adequate, inadequate contouring at the distal radius

The above examples show the difference between appropriate, adequate, inadequate contouring of the periosteal perimeter at the distal radius. Correct region of interest select involves the use of an automated contouring algorithm but requires the subjective assessment of each slice by the operator to correct any errors or suboptimal contouring: A (above left) shows correct contouring no action necessary; B above centre is adequate but would benefit from adjustment; C (above right) highlights incorrect and unusable contouring. Of the 3 examples C would require correction prior to the evaluation of the scan as a section of bone tissue is not included in the volume of interest (VOI) and thus the outcome measures will be affected if not manually corrected by the operator.

As outlined in Chapter 1 PVEs can occur which reduces precision (Laib et al., 1998, Laib and Ruegsegger, 1999, Cheung et al., 2013). With the exception of Tb.N all trabecular parameters are derived. Tb.N is calculated by using a 3D ridge extraction algorithm, which measures inter-trabecular distances between the "centre points of the trabeculae" (i.e. ridges), by placing a sphere between the ridges (Figure 5.11) (Laib et al., 1997, Laib et al., 1998, Laib and Ruegsegger, 1999). Tb.N is defined as the inverse of the mean spacing of the mid-axes and does not depend on the assumptions of the plate-and-rod model (Boutroy et al., 2005). Longitudinal HRpQCT data were selected for analysis if a suitable common region was present in baseline and follow-up scans; only scans with ≥80% matched region were included. This was to ensure that the mean values obtained from the VOI were not disproportionately affected by scanning physiologically dissimilar regions at the two visits. This approach is in-keeping with published longitudinal data (Burt et al., 2017).

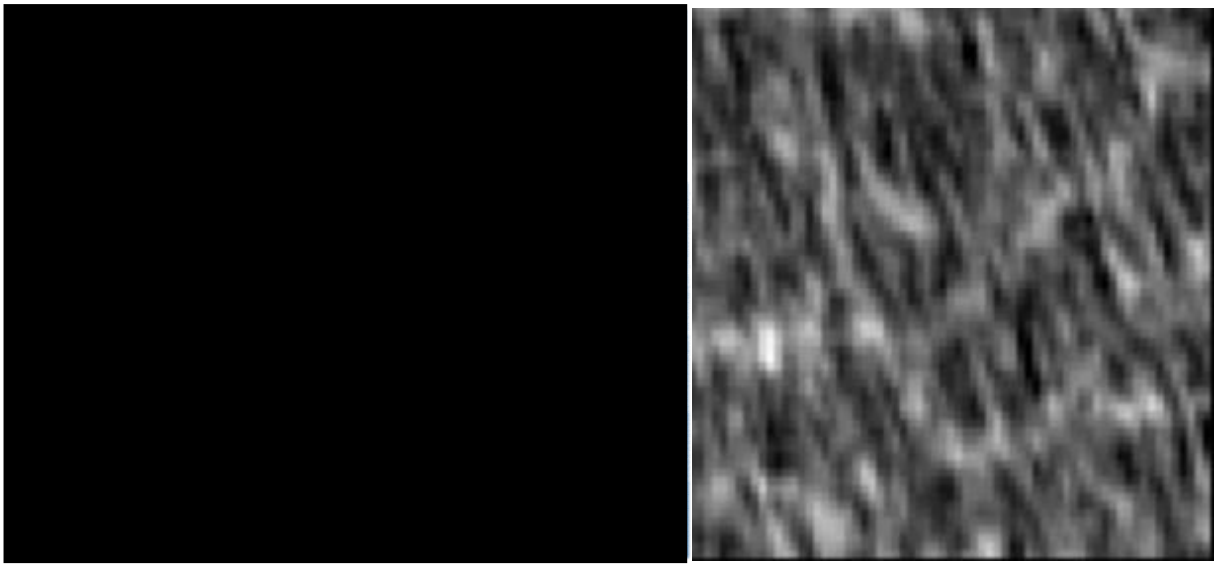

Figure 5.10 Schematic diagram of HR-pQCT trabecular microarchitectural parameters.

Trabecular number (Tb.N) is calculated by placing a sphere between two trabecular ridges and measures the inter-trabecular distance. Right: trabecular compartment as measured with HRpQCT with an integration time of 100 ms (the same as used in PABS). Adapted from Laib and Ruegsegger (1999).

5.5.3.4 HRpQCT Quality Assurance (QA)

Quality Quality control testing was performed on a weekly basis and quality assurance on a daily basis using a calibration phantom (Scanco Medical AG, Bruttisellen, Switzerland) to monitor the performance of the scanner and to ensure there was no drift in the measurements. In addition to the standard phantom, the scanner was pre-calibrated before every subject was scanned. The manufacturer's phantom is composed of 5 cylinders containing a mixture of hydroxyapatite and resin (Figure 5.12). The mineral concentrations of these cylinders were 0, 100, 200, 400 and 800 mgHA/cm^3. The value of 0 mgHA/cm^3 equates to that of a soft tissue background devoid of mineral. Short term precision values for cortical and trabecular BMD have been shown to range from 0.3% to 1.2% (Table 5.8) (Paggiosi et al., 2014).

Figure 5.11 HR-pQCT calibration phantom scanned daily during the study period.

The mineral concentrations of these cylinders were 0, 100, 200, 400 and 800 mgHA/cm^3. QC1 (daily) and QC2 (weekly) shown far left and far right. Images obtained from Scanco Medical

Table 5.7 Coefficients of variation for HRpQCT parameters from the standard analysis Pagiossi et al (2014).

Parameter	Radius (%)	Tibia (%)
Total vBMD	1.1	0.2
Trabecular vBMD	1.1	0.3
Cortical vBMD	4.8	0.1
Trabecular number	4.3	3.0
Trabecular thickness	3.8	2.9
Trabecular separation	4.4	3.0
BV/TV	1.2	0.3
Cortical thickness	2.7	0.4
Cortical porosity	12.7	5.2

vBMD = volumetric bone mineral density, BV/TV = Trabecular bone volume/bone volume

5.5.4 Peripheral QCT Scanning

Standard operating procedures (SOP_0373, SOP_0499) were followed for all procedures to obtain the outcome measures listed in Table 5.6 at each time point. These are consistent with those used in the ENID Bone Study (Section 3.3.2). The scanning procedure including the initial scout scan and successive measurements were explained to participants prior to scanning. The participants were made comfortable, asked not to talk, and instructed to keep perfectly still during the procedure to minimise movement.

5.5.4.1 Limb length measurement

Scan sites were located by the pQCT scout view as a set percentage of object (limb) length measured from a physiologically defined reference line at the distal end of the long bone. Object length was measured using a metal ruler between prominent bony landmarks. Participants were asked to remove footwear and clothing from the lower non-dominant leg (unless impracticable) which was measured from the tibial plateau to the medial malleolus (ankle) with a metal ruler to record the distance, in a straight line, between these two points to the nearest 0.5 mm. For radius length, as above the non-dominant side was always used. Radial object length was obtained with the arm on the non-dominant side bent at a right angle, and the distance between the olecranon (near elbow) and the distal edge of the ulnar styloid process (bony prominence at wrist) measured with a metal ruler to the nearest 0.5 mm.

5.5.4.2 pQCT Scan Acquisition

Scan parameters were specified in the measurement mask (Figure 5.6), the object length is entered to automatically calculate the scan site. The operator ensured that the limb was centrally positioned before scanning, with the limb positioned so that the laser light was distal to the medial malleolus/ulnar styloid process of the tibia or ulna respectively. Next, a scanogram was obtained to allow visual assessment in the positioning of the reference line which the software automatically places; if incorrect this line was repositioned manually as appropriate. The scout scan was repeated if the operator failed to identify the correct region due to poor positioning or participant movement. Once the reference line was in the correct region, the acquisition of scan images began. The scan sites were determined by the mask selected by the operator prior to scanning. All pQCT scans were obtained using a voxel size of 0.5 mm and slice thickness of 2 mm. A CT scan speed of 30mm/s was used for all slices, and a scout view speed of 40 mm/s was used for all scanograms.

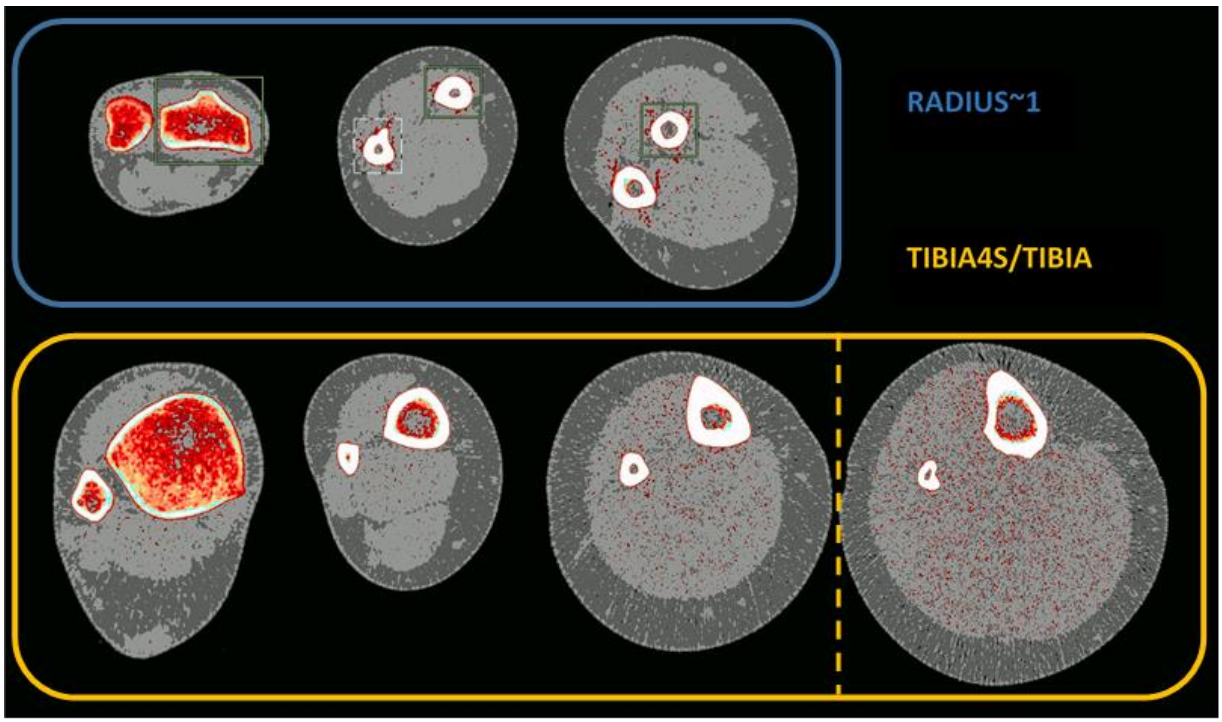

Figure 5.12 Masks used in PABS and sites selected by the mask

Scan slices at the distal and proximal radius were obtained using the RADIUS~1 mask in all participants, scans at 4%,33%, and 66% of the radius length are shown above. At the distal and proximal tibia the TIBIA4S mark (below) was used to obtain scan data at 4%, 14%, 38%, and 66% of the tibia length. Note that when a subject's leg was too wide to obtain data comfortably at the 66% tibia an alternative mask, called TIBIA was used to obtain data at the 3 other tibial sites.

Scans were acquired at 4%, 33%, and 66% radius sites (Radius~1 mask), and at 4%, 14%, 38%, and 66% tibia sites (Tibia4S mask). The only deviation from this protocol was the use of the TIBIA mask of 4%, 14%, and 38% for participants whose lower leg was too wide to fit through the gantry at 66% of the tibia. All scan images were visually inspected by me directly following scan completion. The pQCT scanner is very sensitive to movement which can result in scan distortion. If a scan slice was particularly poor the participant was asked if they would consent to a rescan. Scan grading and data processing are consistent with the methods outlined for the ENID Bone pQCT data in Chapter 3 but briefly, at distal sites CALCBD analysis using contour mode 1, peel mode 1 at a threshold of 180 mg/cm^3 was used with trabecular bone defined as the inner area of 45% of the total CSA. At cortical sites the threshold of 710 mg/cm^3 was selected for cortical bone parameters and analysed using CORTBD separation mode 1. Total CSA at proximal sites was determined using a threshold of 280 mg/cm^3. I used an in-house Microsoft Access tool, designed

by Darren Cole (Manager of Data Operations at MRC EWL) with my technical input and testing, to select and filter the appropriate data for our outcome measures of interest prior to statistical analysis and data modelling in R.

5.5.4.3 pQCT Quality Assurance (QA)

Calibration of the XCT 2000L™ system was performed on a routine basis with a specifically designed phantom. Daily QA scans were performed throughout the study period to test scanner performance. Weekly QC scans were also performed by the research team as required. The inter-operator precision for each scan site is summarised in Table 5.8. These were measured by performing two repeat scans on 30 participants at MRC EWL, Cambridge.

Table 5.8 Coefficients of variation (CVs) for pQCT parameters as measured by Stratec XCT 2000L

Parameter	Radius	Tibia
Total vBMD	4.28%	1.10%
Trabecular vBMD	2.27%	0.94%
Total CSA (distal)	4.03%	1.61%
Total CSA (proximal)	3.71%	1.01%
Cortical CSA	1.97%	0.89%
Cortical vBMD	0.89%	0.41%
Cortical BMC	1.78%	0.59%
Cortical thickness	4.25%	1.49%

vBMD = volumetric bone mineral density, CSA = cross sectional area, BMC = bone mineral content

5.6 PABS data management

All data from screening questionnaires, measurement visits and the midwife screening was entered into the study database by the research team. The PABS database was developed on Microsoft Access by Darren Cole (MRC EWL Head of Data Management). Researchers were able to read, create and edit data generated and the database was backed up on a daily basis. Participant ID numbers were assigned in the database at the point of telephone screening. These were automatically generated when a new record was created. Once an ID number was assigned, it remained linked to that participant for the remainder of the study.

Table 5.9 Documentation entered into the database from each of the study time points

Study form	Visit 1	Visit 2
Telephone screening	✓ Ahead of visit 1	X
Consent	✓	X
CRF visit 1	✓	X
Musculoskeletal questionnaire visit 1	✓	X
CRF visit 2	X	✓
Musculoskeletal questionnaire visit 2	X	✓
Mid-pregnancy midwife screening form	X	✓ Ahead of visit 2

CRF= case report form

All data collected at the visits e.g. CRF and musculoskeletal questionnaire, were subsequently entered into this database by members of the research team (Table 5.9). Following 10% checks of the entered data, the relevant data were selected and extracted to create a database containing all subject level anonymised data. As described above bone densitometry data were extracted from both the pQCT and HRpQCT following the auditing and grading of all scans. pQCT, anthropometry and questionnaire data were combined in preparation for analysis.

5.6.1 Data cleaning and preparation for analysis

Further to the grading processes outlined in detail in Chapter 4 for pQCT data and above for HRpQCT, the extracted data were also scrutinised in R through the use of box and whisker plots to identify extreme outliers (>1.5 times the IQR), the relevant scans and scout views were visually inspected and scans of poor quality or inaccurate positioning were subsequently removed from the analysis dataset. Incorrect ROIs were amended and then the data were re-extracted. In addition to examining outliers in the absolute values from both scanners, I also explored the change in the outcome measures of interest to detect any change that may have been the result of poor/incorrect positioning (i.e. seem exceedingly large). Again these data were plotted with box and whisker plots and outliers in between time point change scrutinised.

Table 5.10 HRpQCT scans at the distal tibia, number acquired and number subsequently used in longitudinal analyses of scan grade ≤4 and with ≥80% common region.

	Pregnant (n = 53)		NPNL (n = 37)	
	Tibia	Radius	Tibia	Radius
Acquired visit 1	50*#	49*#	37	36
Acquired visit 2	40#	37#	34	31
Longitudinal analysis	35	33	33	27

*machine down time resulted in 3 participants not having baseline scans

Table 5.11 Number of pQCT scans excluded from the dataset after being identified as extreme outliers at the distal and proximal tibia at visit 1, visit 2, or the between visit change using box and whisker plots.*

	Tibia						Radius			
	4%		14%		38%		4%		33%	
	P	N	P	N	P	N	P	N	P	N
Acquired visit 1	53	37	53	37	53	37	53	37	53	37
Acquired visit 2	46	37	46	37	46	37	46	37	46	37
Longitudinal analysis*	46	34	46	35	46	35	42	35	45	37

*scan images were visually inspected before any data were omitted from subsequent analyses

5.7 Statistical methods

All statistical analyses presented below were conducted in R version 3.3.1 primarily using dplyr (version 0.5.0, Wickham and Francois, 2016) and tidyverse (version 1.1.1, Wickham, 2017) for general data manipulation, and ggplot2 (version 2.2.1, Wickham 2009) for graphics in addition to ggfortify (version 0.4.5, Horikoshi and Tang, 2016). MASS (version 7.3-51, Ripley et al, 2018) was used to assist the generation of linear regression models. Data presented from regression models were tabulated using stargazer (Hlavac, 2018).

5.7.1 Initial analysis and testing for normalcy after initial cleaning of the data

Histograms were used to explore whether the data were normally distributed. Between-group differences at baseline were explored by independent t-tests where the data were normally distributed; an appropriate non-parametric test (Mann-Whitney U test) was used where the data were skewed.

5.7.1.1 Absolute within-group changes between baseline and follow up

For all HRpQCT and pQCT data absolute between-visit change was calculated by subtracting the baseline bone outcome measure values from their respective follow-up values. Subsequently I used single-sided t-tests to determine if the absolute change variables in the Pregnant and NPNL groups differed significantly from zero.

5.7.2 Modelling of the longitudinal HRpQCT and pQCT data

5.7.2.1 Computation of standardised residuals

Baseline and follow up measures were very tightly correlated so I chose to use a measure of conditional change i.e. standardised residuals. Standardised residuals were calculated for all pQCT and HRpQCT bone outcome measures using simple linear regression. Follow up outcome measures were regressed by their respective baseline values with standardised residuals extracted from the models. The use of standardised residuals would allow for the simple comparison of change across scanners and the various units and scales of my outcome measures. The use of SD scores (Z scores) is routinely used in clinical practice and is a convenient means of visualising change e.g. from aging studies it is known that a decrease of 1SD in aBMD at the femoral neck doubles the risk of fracture (Marshall et al., 1996). The examination of the standardised residuals for all outcome measures of interest through the use of diagnostic plots also allowed for the identification of scans that needed further scrutiny with all data and the original scans visually inspected (Figure 5.13). If a participant was identified as an outlier on these plots I selected all of their scan data for inspection, however, the decision to remove data from analysis was taken carefully as several "fast losers" of bone mineral were identified where a) the positioning of the scans were almost identical, b) with no change in bone CSA, c) high quality of scan grade/matched scan region. Any scans with all of a-c mean that the large between-visit changes would be unlikely to be due to poor measurement technique and for this reason I applied the following approach to scan exclusion:

For pQCT I used diagnostic plots for all parameters to identify scans that required further scrutiny. I tabulated the residuals for all parameters for these individuals and to avoid over-cleaning the data I based my inclusion criterion on total CSA. The rationale for this was simple, in adults a small to moderate change in total CSA over the study time frame would be physiologically unlikely. This also avoided the risk of excluding true physiological outliers as seen in Figure 5.13 of total vBMD. The criterion I applied was if the standardised residual for total CSA exceeded 1SD for a participant then their data would be excluded. Following this approach I had usable longitudinal pQCT data: at the 4% distal tibia for 77 subjects; at the 14% tibia for 75 subjects; at the 38% tibia for 77; at the 4% distal radius for 71 subjects; at the 33% radius for 76.

For HRpQCT the same approach was applied to identify participants whose scans required further scrutiny. These data were then examined with a focus on a) the percentage common region between the scans, b) the scan grade at each visit, and c) any implausible changes in parameters of bone microarchitecture. At the tibia all scans identified for scrutiny had a very high common region and the grade quality of the scans were also good, for this reason no data points were excluded. At the radius while the matched common region of scans requiring scrutiny were high, several grade 4 scans were present at one time point or other. I pragmatically decided not to exclude any of the data points from my final models as running the models with these data included/excluded had little to no bearing on the results.

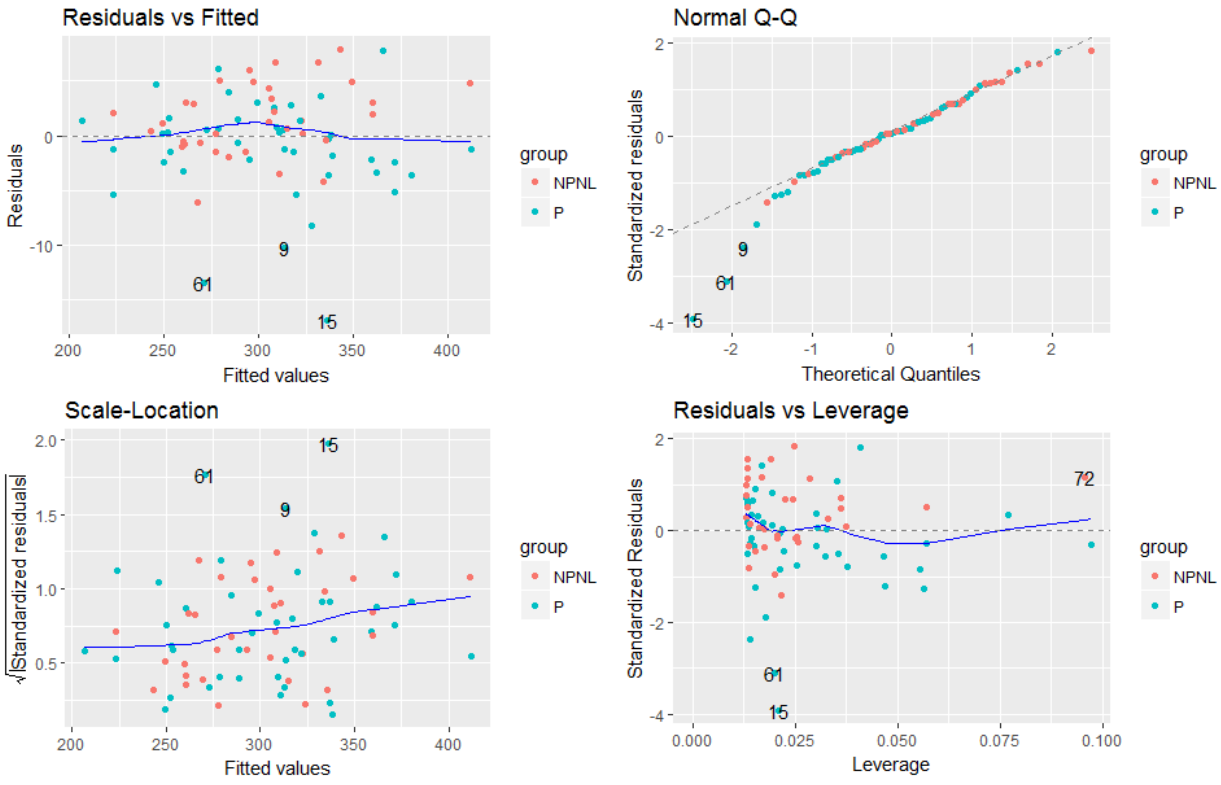

Figure 5.13 Diagnostic plots of the linear regression of pQCT total vBMD at follow up by baseline total vBMD. NPNL = non-pregnant non-lactating controls, P = Pregnant.

5.7.3 Model and covariate selection

Following these steps I performed linear regression adjusting my models as follows to explore between-group differences:

5.7.3.1 Model 1: unadjusted between-group differences

Model 1 simply explores the between-group difference in the standardised residuals generated above where Yi is the standardised residual bone outcome as measured for participant I.

$$Model\ 1: Y_i = \beta_o + \beta_1 Group + \epsilon$$

5.7.3.2 Model 2: between-group differences adjusted for height and weight, and age

If between-group differences emerged in Model 1 it would be important to determine if adjusting for height and weight parameters attenuated any findings. Both height and weight are known to be associated with inter-individual differences in bone outcome measures particularly in the axial and weight-bearing regions of the appendicular skeleton (Christensen et al., 1981). From a physiological

perceptive the relationship between bone and weight is an important one. Greater weight can be associated with higher BMD, while gaining weight can potentially increase strain leading to increased turnover and increases in BMD and BMC. My decision to use baseline weight only in these analyses followed the exploration of weight change as a possible covariate, the use of weight change was rejected as it over fitted the models effectively controlling for group twice. The within group weight changes are shown below (Figure 5.14).

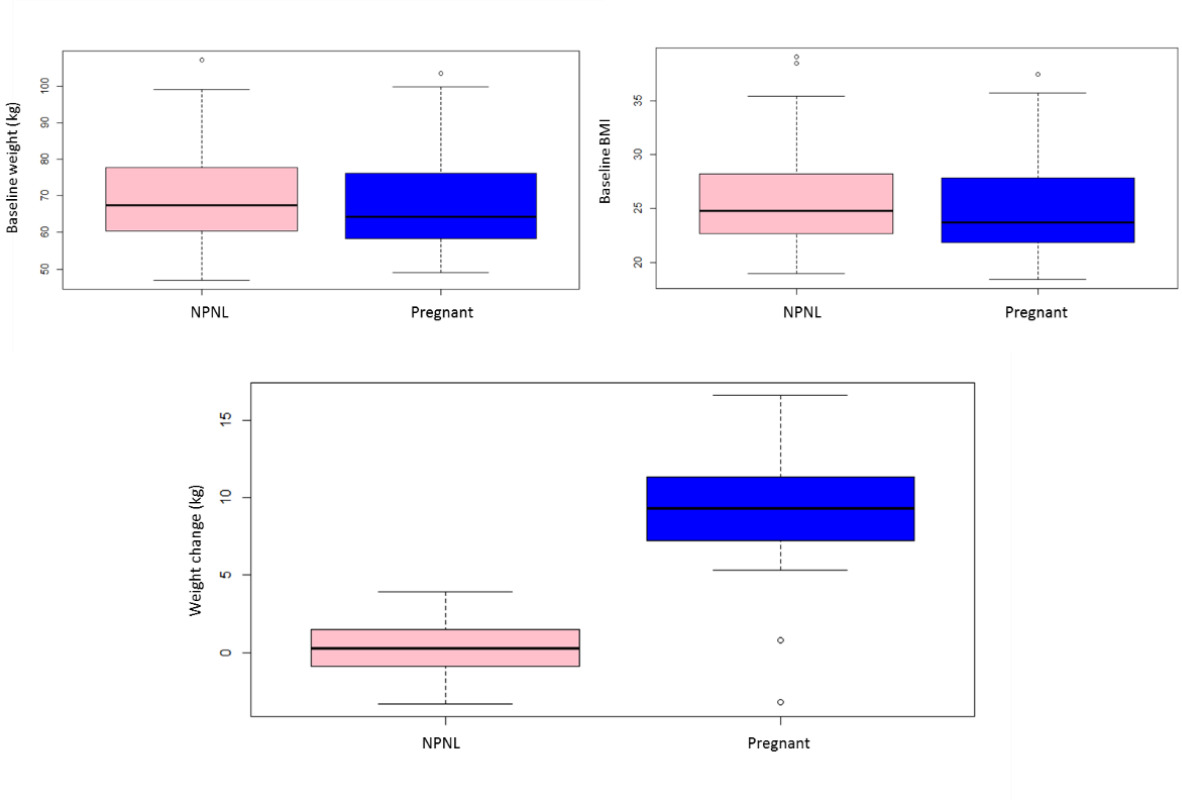

Figure 5.14 Boxplots of baseline weight, BMI, and weight change stratified by group.

At baseline NPNL were heavier and had a higher mean BMI than pregnant women, however, neither of these were statistically significant. Pregnant women gained significantly more weight than NPNL (p<0.05).

Age was also adjusted for as bone changes naturally occur from mid-life onwards and my target population range encompassed women aged 30-45 years (Uusi-Rasi et al., 2007). The models were constructed as follows where Yi is the standardised residual bone outcome as measured by pQCT for participant *I*:

$$Model\ 2: Y_i = \beta_o + \beta_1 Group\ +\ \beta_2 weight + \beta_3 height\ + \beta_4 age + \epsilon$$

5.7.3.3 Model 3: between-group differences from parsimonious models of best fit

Covariate selection was defined by several a priori considerations. In addition to baseline weight, height and age (Model 2) a number of additional factors were considered. In some populations such as The Gambia (see section 3.4.5) parity is highly correlated with maternal age however in PABS there was poor correlation (adj R2 = -0.01) and I chose to include both variables. Parity was recoded into a binary variable for analysis 0 for nulliparous women or 1 for where parity was ≥1 (Olausson et al., 2008). Potential environmental confounders were also included: historical or current use of hormonal contraceptives which may influence bone; history of smoking (i.e. ever smoked) as smoking has been associated with lower BMD and related bone parameters (Eleftheriou et al., 2013, Oyen et al., 2014).

When this initial model was run, with all independent variables included simultaneously, all between-group differences detected in Model 1 and 2 were attenuated due to multicollinearity. Therefore I used the MASS package to perform backwards elimination of non-significant factors, the least significant being removed first, to generate models which could be compared to select a model of best fit for each bone outcome. These were compared by ANOVA and the model of best fit selected as the model with the lowest AIC value. AIC did not appear to favour the selection of an excessive number of covariates as in most models only a handful of covariates were selected in the model of best fit. I included group in all models even if group was not selected in the final model of best fit to allow for the interpretation of any group differences.

For all outcome measures of interest the initial model was as follows where Yi is the standardised residual for bone outcome as measured by pQCT for participant *I*:

$$Model\ 3: Y_i = \beta_o + \beta_1 Group\ + \beta_2 weight + \beta_3 height\ + \beta_4 age + \beta_5 parity$$
$$+\ \beta_6 contraceptives + \beta_7 smoking + \epsilon$$

6 Results from PABS

In total 53 pregnant women (recruited at the Rosie Hospital (CUH) n=50, recruited from the community n=3) met our eligibility criteria and were scanned between 14 – 16 weeks of pregnancy (mean 15.5 (SD 0.9) weeks). A control group of 37 NPNL women was recruited from the community through internal MRC unit emails, the MRC EWL VSCS volunteer database, posters, and local advertisements.

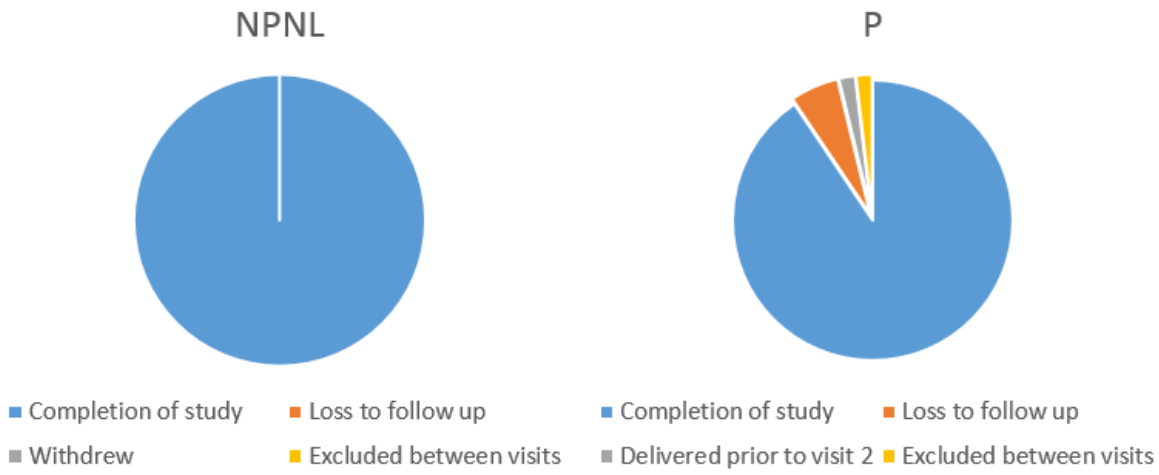

Figure 6.1 Proportion of participants in each group who completed the study or were lost to follow up.

Ninety women, 53 pregnant and 37 non-pregnant non-lactating controls (NPNL) were recruited into the Pregnancy and Bone Study (PABS). All NPNL completed the study, while 86% of the pregnant group (P, n = 46) completed the study. In the pregnant group 5 women were lost to follow up, 1 participant delivered prior to visit 2, and 1 participant was not contacted following mid-pregnancy screening.

6.1 Baseline descriptive statistics

Pregnant women had on average a 19.6 (SD 1.6) week difference between baseline and follow-up. While this was found to be statistically significantly different compared to that of the control arm, 21.6 (SD 1.7) weeks ($p<0.05$), from an aging perceptive 2 weeks can be considered negligible and for pregnancy if time extended likely increase any differences found.

6.1.1 Group differences in age, anthropometry, and parity

Group characteristics are summarised in Table 6.1 with the baseline mean (SD) for all continuous parameters of interest with the exception of parity expressed as median (IQR). The only significant between-group difference at baseline was that the control group had a higher parity.

Table 6.1 Baseline mean (SD) for anthropometry measures at baseline in non-pregnant non-lactating (NPNL) and pregnant groups. Parity is expressed as the median [IQR].

	NPNL (n = 37)	Pregnant (n = 53)
Age (years)	36.04 (3.99)	34.90 (3.58)
Weight (kg)	70.41 (13.70)	68.34 (13.58)
Height (cm)	164.16 (7.19)	165.13 (5.70)
BMI	26.19 (5.22)	25.00 (4.51)
Parity	**2 [0, 4]**	**1 [0, 2]***
Contraceptives (1 = ever , 2 = never)	1.14 (0.35)	1.06 (0.23)
Weeks between visits	**21.7 (1.7)**	**19.8 (1.6)***

*p<0.05, **p<0.01, ***p<0.001 independent t-test between groups at baseline.

6.1.2 Differences at baseline in bone outcomes

Tables 6.2 and 6.3 contain baseline data from participants with complete data at baseline and follow up. Reasons for incomplete data were a) there was a 13% attrition rate (n=7) in the pregnant arm, b) HRpQCT down time resulted in 3 participants not having baseline scans, c) unusable scans were excluded leading to missing data at either time point. The data presented (Tables 6.2 & 6.3) did not differ from that when all baseline data were included nor did the pattern of between-group differences differ (Appendix F). Statistically significant between-group baseline differences were found for a number of HRpQCT bone outcome measures at the distal tibia. In pregnant women at 14-16 weeks gestation, trabecular number (1.98 (0.27) mm^{-1}) was lower compared to NPNL controls (2.13 (0.27) mm^{-1}) ($p<0.05$, Table 6.2). While the mean trabecular thickness of pregnant women (0.073 (0.013) mm) was found to be significantly greater than NPNL controls (0.065 (0.014) mm) ($p<0.05$, Table 6.2). At the distal radius no significant between-group baseline differences in any HRpQCT bone outcome measures were found. Nor were any significant pQCT between-groups differences observed.

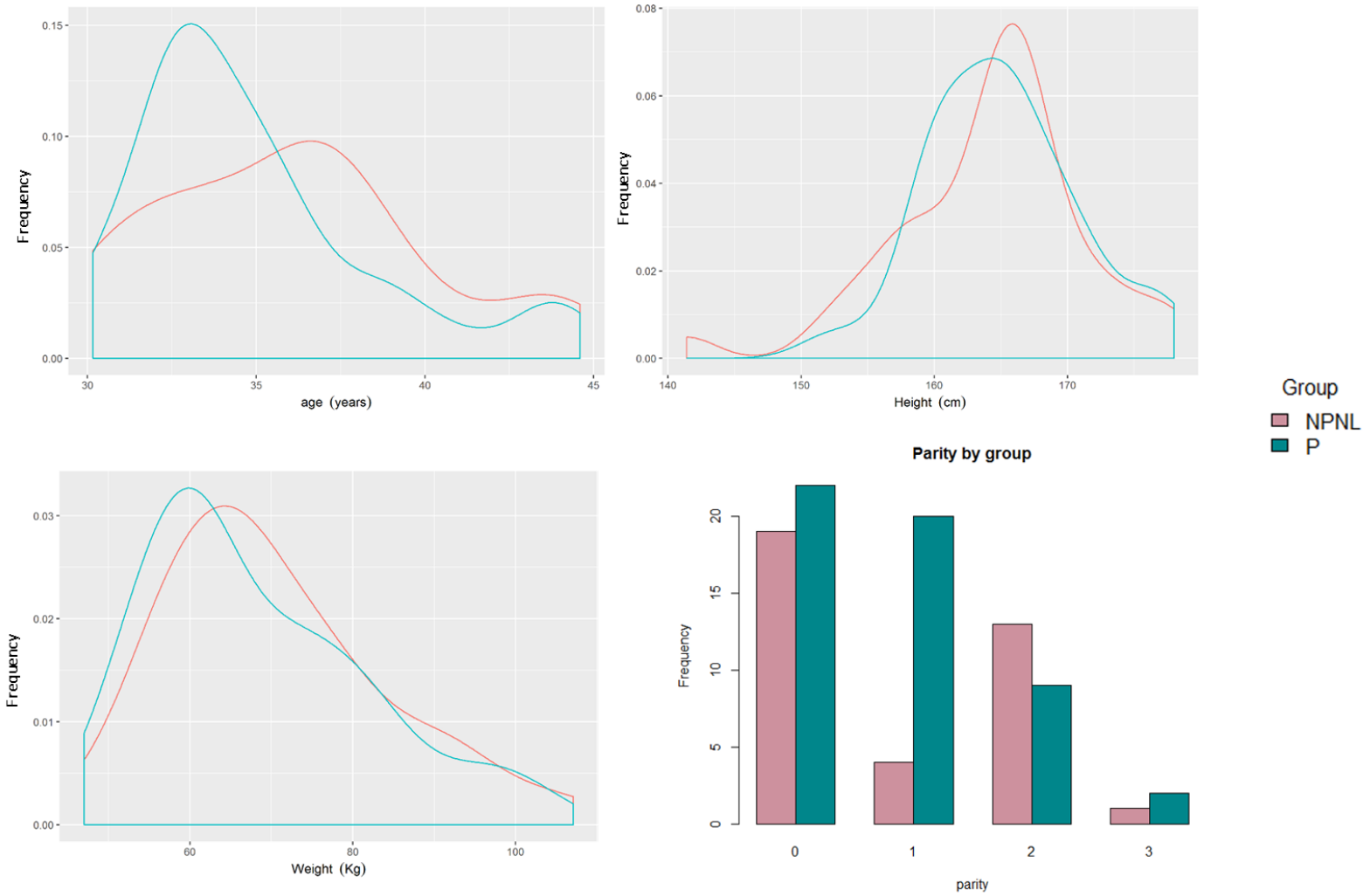

Figure 6.2 Distribution of age (years), height (cm), weight (kg), and parity at visit 1.

Non-pregnant non-lactating (NPNL) women are in pink, pregnant women (P) are represented in blue. When tested, with t-tests for normally distributed data and Mann-Whitney U tests for skewed data, no statistically significant between group differences were found at baseline (Table 6.1).

6.2 Single-sided t-test to assess within-group change

6.2.1 Absolute change within-group change

6.2.1.1 Tibia: Absolute within-group changes between baseline and follow up

In pregnant women all HRpQCT bone outcome variables decreased with the exception of trabecular number and cortical porosity which increased, all changes were statistically significantly different to zero (Table 6.2). While in the control group total vBMD, trabecular vBMD, and BV/TV decreased between visits (all $p<0.05$) cortical thickness increased ($p<0.05$) (Table 6.2). In pregnant women there were significant between visit decreases in all pQCT parameters of total vBMD, trabecular vBMD (both $p<0.001$), total CSA ($p<0.05$) and cortical subcortical vBMD ($p<0.01$) at the 4% distal tibia, whereas in the control group a drop in trabecular vBMD ($p<0.05$) was the only significant change. At the 14% proximal tibia within-group changes were not found in either group. In the pregnant group significant increases in periosteal ($p<0.05$) and endosteal circumferences ($p<0.01$) were found at the 38% tibia. In NPNL a significant increase was found in periosteal and endosteal circumferences ($p<0.05$), with a decrease in cortical thickness ($p<0.05$).

6.2.1.2 Radius: Absolute within-group changes between baseline and follow up

In the pregnant women significant decreases in total vBMD ($p<0.05$), trabecular vBMD ($p<0.001$), BV/TV ($p<0.001$), and cortical vBMD ($p<0.01$) were found (Table 6.3). While in the NPNL group decreases in total vBMD ($p<0.05$), trabecular vBMD ($p<0.001$) and BV/TV ($p<0.05$) were statistically significantly different to 0 (Table 6.3). No statistically significant within-group changes were found in either group with pQCT at the 4% distal radius, however, within-group differences were found at the 33% proximal radius (Table 6.3). In pregnant women the only significant change was an increase in cortical BMC ($p<0.05$), while in the NPNL group significant within-group change was found for cortical thickness which increased ($p<0.01$) and periosteal ($p<0.05$) and endosteal ($p<0.01$) circumferences decreased.

Table 6.2 Baseline characteristics (mean (SD)) and within-group absolute change between visits (mean (SD)) for pQCT and HRpQCT bone outcome measures of interest at the tibia in non-pregnant non-lactating (NPNL) and pregnant groups in PABS Cambridge.

	NPNL		Pregnant	
	Visit 1	Between-visit change	Visit 1	Between-visit change
HRpQCT distal Tibia		n=32		n=33
Total vBMD (mg/cm^3)	315.26 (53.64)	**-1.50 (3.02) ‡‡**	317.43 (68.09)	**-4.69 (6.32) ‡‡‡**
Trabecular vBMD (mg/cm^3)	165.53 (38.02)	**-2.55 (2.36) ‡‡‡**	174.68 (41.33)	**-2.79 (3.59) ‡‡‡**
Cortical vBMD (mg/cm^3)	921.45 (33.66)	-0.85 (6.47)	916.26 (38.81)	**-7.40 (12.91)‡‡**
BV/TV	0.14 (0.03)	**-0.002 (0.002)‡‡‡**	0.15 (0.03)	**-0.002 (0.003) ‡‡‡**
Trabecular number (mm^{-1})	2.13 (0.27)	-0.01 (0.15)	**1.98 (0.27)***	**0.09 (0.15) ‡‡**
Trabecular thickness (mm)	0.065 (0.014)	-0.0007 (0.0043)	**0.073 (0.013) ***	**-0.004 (0.005) ‡‡‡**
Trabecular separation (mm)	0.41 (0.06)	0.00 (0.03)	0.44 (0.08)	**-0.02 (0.03) ‡‡‡**
Cortical thickness (mm)	1.25 (0.22)	**0.01 (0.01) ‡‡‡**	1.21 (0.29)	**-0.01 (0.02)‡‡**
Cortical porosity (%)	99.91 (7.16)	0.03 (0.23)	99.6 (8.3)	**0.25 (0.25) ‡‡‡**
pQCT 4% tibia		n=34		n=43
Total vBMD (mg/cm^3)	301.57 (40.36)	-0.18 (3.12)	306.25 (47.42)	**-3.07 (4.89) ‡‡‡**
Trabecular vBMD (mg/cm^3)	219.59 (39.15)	**-0.89 (2.25) ‡**	227.91 (44.01)	**-2.16 (3.63) ‡‡‡**
Total CSA (mm^2)	989.76 (111.06)	-0.68 (10.75)	980.36 (115.47)	**-4.73 (13.89) ‡**
Cortical subcortical vBMD (mg/cm^3)	368.61 (44.90)	0.39 (5.54)	370.31 (54.56)	**-3.81 (7.74) ‡‡**
pQCT 14% tibia		n=32		n=43
Total CSA (mm^2)	416.34 (57.20)	-0.46 (3.15)	409.23 (62.70)	-0.80 (2.84)
Cortical vBMD (mg/cm^3)	1138.03 (18.84)	-0.01 (5.62)	1141.29 (18.33	-2.15 (8.26)
Cortical BMC (mg/mm)	189.70 (23.34)	0.45 (2.34)	187.06 (25.16)	0.27 (2.56)
Cortical CSA (mm^2)	166.61 (19.83)	0.38 (2.42)	163.83 (21.38)	0.59 (2.04)
Cortical thickness (mm)	2.57 (0.33)	0.01 (0.04)	2.57 (0.41)	0.01 (0.04)
Periosteal circumference (mm)	73.12 (4.90)	-0.01 (0.33)	72.37 (5.51)	-0.02 (0.25)
Endosteal circumference (mm)	56.96 (5.74)	-0.04 (0.45)	56.21 (7.03)	-0.10 (0.41)
pQCT 38% tibia		n=35		n=42
Total CSA (mm^2)	392.90 (45.52)	1.26 (4.26)	378.45 (43.34)	0.92 (3.94)
Cortical vBMD (mg/cm^3)	1169.09 (24.25)	0.15 (4.63)	1172.08 (19.34)	-1.53 (6.05)
Cortical BMC (mg/mm)	324.76 (40.87)	-0.17 (3.47)	315.44 (38.98)	-0.26 (4.21)
Cortical CSA (mm^2)	277.81 (34.70)	-0.21 (3.71)	269.21 (33.59)	0.15 (3.75)
Cortical thickness (mm)	4.91 (0.55)	**-0.03 (0.08) ‡**	4.84 (0.44)	-0.02 (0.08)
Periosteal circumference (mm)	72.09 (4.59)	**0.25 (0.52) ‡‡**	70.75 (4.18)	**0.23 (0.60) ‡**
Endosteal circumference (mm)	41.24 (5.20)	**0.47 (0.81) ‡‡**	40.34 (3.52)	**0.38 (0.87) ‡‡**

*p<0.05, **p<0.01, ***p<0.001 independent t-test between groups at baseline; ‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001 single-sided t-test vs 0. vBMD = volumetric bone mineral density, CSA = cross sectional area, BV/TV = trabecular bone volume/bone volume

Table 6.3 Baseline characteristics (mean (SD)) and within-group absolute change between visits (mean (SD)) for pQCT and HRpQCT bone outcome measures of interest at the radius in non-pregnant non-lactating (NPNL) and pregnant groups in PABS Cambridge.

	NPNL		Pregnant	
	Visit 1	Between-visit change	Visit 1	Between-visit change
HRpQCT distal Radius	n=27		n=33	
Total vBMD (mg/cm^3)	334.75 (63.13)	**-3.15 (7.70) ‡**	334.20 (58.74)	**-2.89 (6.90) ‡**
Trabecular vBMD (mg/cm^3)	155.10 (32.80)	**-2.53 (2.23) ‡‡‡**	153.28 (39.86)	**-2.18 (3.34) ‡‡‡**
Cortical vBMD (mg/cm^3)	916.35 (49.22)	-3.45 (10.17)	920.46 (42.47)	**-4.32 (9.42) ‡**
BV/TV	0.13 (0.03)	**-0.002 (0.002) ‡‡‡**	0.13 (0.03)	**-0.002 (0.003) ‡‡‡**
Trabecular number (mm^{-1})	2.02 (0.23)	0.04 (0.20)	1.95 (0.33)	0.01 (0.19)
Trabecular thickness (mm)	0.06 (0.01)	**-0.002 (0.005) ‡**	0.07 (0.01)	-0.001 (0.007)
Trabecular separation (mm)	0.44 (0.06)	-0.005 (0.0419)	0.46 (0.10)	-0.002 (0.051)
Cortical thickness (mm)	0.81 (0.16)	0.003 (0.0307)	0.82 (0.14)	-0.005 (0.026)
Cortical porosity (%)	66.74 (5.35)	-0.10 (0.36)	66.68 (5.80)	0.08 (0.35)
pQCT 4% Radius	n=31		n=40	
Total vBMD (mg/cm^3)	323.20 (51.77)	2.85 (14.28)	314.64 (48.69)	-1.28 (16.21)
Trabecular vBMD (mg/cm^3)	180.41 (38.03)	0.76 (4.05)	176.45 (32.83)	0.06 (4.95)
Total CSA (mm^2)	328.69 (45.69)	-0.44 (12.90)	335.80 (45.41)	0.34 (14.53)
Cortical subcortical vBMD (mg/cm^3)	439.83 (75.29)	4.56 (24.45)	427.56 (68.27)	-2.39 (27.76)
pQCT 33% Radius	n=35		n=41	
Total CSA (mm^2)	99.43 (12.33)	-0.51 (1.52)	98.05 (12.56)	0.09 (1.32)
Cortical vBMD (mg/cm^3)	1218.13 (17.45)	-1.32 (7.72)	1217.49 (15.81)	2.97 (12.35)
Cortical BMC (mg/mm)	88.88 (9.37)	0.19 (0.99)	87.66 (10.31)	**0.40 (1.05) ‡**
Cortical CSA (mm^2)	72.96 (7.63)	0.24 (1.05)	72.02 (8.55)	0.15 (1.18)
Cortical thickness (mm)	2.54 (0.21)	**0.04 (0.08) ‡‡**	2.55 (0.22)	0.01 (0.07)
Periosteal circumference (mm)	36.68 (2.08)	**-0.23 (0.55) ‡**	36.21 (2.34)	-0.02 (0.55)
Endosteal circumference (mm)	20.71 (2.10)	**-0.48 (0.96) ‡‡**	20.2 (2.3)	-0.08 (0.89)

*p<0.05, **p<0.01, ***p<0.001 independent t-test between groups at baseline; ‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001 single-sided t-test vs 0. vBMD = volumetric bone mineral density, CSA = cross sectional area, BV/TV = trabecular bone volume/bone volume

6.3 Group differences in conditional change

6.3.1 HRpQCT at the distal tibia

6.3.1.1 Model 1: Group only adjustment

At the distal tibia significant between-group differences were found in total vBMD with the pregnant group decreasing by 0.64 (0.24) SD ($p<0.01$) (Table 6.4). While no significant changes were found in either trabecular vBMD or BV/TV several significant between-group differences were found in parameters of trabecular microarchitecture: number (increase by 0.53 (0.24) SD), thickness (-0.51 (0.24) SD), and separation (-0.61 (0.24) SD) (all $p<0.05$). Cortical vBMD was found to decline by -0.64 (0.24) SD between groups in the study period ($p<0.01$). In addition to changes in cortical vBMD, cortical microarchitecture was also significantly different between the groups, cortical thickness decreased (-1.6 (0.22) SD) while porosity increased (0.83 (0.23) SD) (both $p<0.001$).

6.3.1.2 Model 2: Between-group differences adjusted for size and age

Adjusting for size (baseline height and baseline weight) and age at baseline reduced the magnitude of the decrease in total vBMD (-0.56 (0.24) SD), however it remained significant ($p<0.05$). Adjusting for size and age slightly modulated the beta coefficients for parameters of trabecular microarchitecture increasing the between-group differences found in model 1: number 0.59 (0.24) SD ($p<0.05$), thickness -0.53 (0.24) SD ($p<0.05$), and separation -0.66 (0.24) SD ($p<0.01$). Baseline weight significant was a positive predictor of trabecular number ($p<0.05$), while there was a negative trend for both trabecular thickness and number. Model 2 reduced the magnitude of the decrease in cortical vBMD -0.62 (0.23) SD slightly in magnitude although this between-group difference remained significant ($p<0.05$). Between-group differences in cortical microarchitecture parameters remained highly significant (both $p<0.001$) though the magnitude of the difference in cortical thickness decreased slightly (Table 6.4).

6.3.1.3 Model 3: models of best fit

As in models 1 and 2 above, using the parsimonious models outlined in Table 6.4 significant between-group differences were found in total and cortical vBMD, in addition to both trabecular and cortical micro-architecture. Total vBMD was found to decline by -0.49 (0.24) SD in the pregnant group vs NPNL ($p<0.05$), with no statistically significant predictors. No significant differences were found in the parameter of trabecular vBMD or BV/TV. Significant between-group differences in trabecular number (0.47 (0.23) SD) and separation (-0.54 (0.24) SD) remained (both $p<0.05$) although the between-group difference in trabecular thickness was attenuated (-0.47 (0.24) SD, $p=0.06$). Baseline weight (positive) and parity (negative) were significant predictors of trabecular number (both $p<0.05$). Between-group differences remained in both cortical vBMD and cortical micro-architecture, cortical vBMD decreased by 0.67 (0.23) SD vs NPNL ($p<0.01$), while cortical thickness fell by 1.01 (0.21) SD ($p<0.001$). Cortical porosity increased 0.78 (0.23) SD in pregnant vs NPNL ($p<0.01$). Weight was a significant negative predictor of cortical vBMD ($p<0.01$).

Table 6.4 Summarising results of linear regression models testing for group differences in change over time at distal tibia with HRpQCT in PABS Cambridge

	Tibia HRpQCT (n=65)						
Dependant variable	Group β_1	p-value	Group β_2	p-value	Group β_3	p-value	Covariates in parsimonious model
Total vBMD	**-0.64 (0.24)**	**P=0.01**	**-0.56 (0.24)**	**P=0.02**	**-0.49 (0.24)**	**P=0.04**	**age***, contraceptives
Trabecular vBMD	-0.01 (0.25)	P=0.95	0.06 (0.25)	P=0.83	0.04 (0.25)	P=0.9	age
BV/TV	-0.05 (0.25)	P=0.84	0.02 (0.25)	P=0.92	0.004 (0.25)	p=0.9	age
Trab. number	**0.53 (0.24)**	**P=0.03**	**0.59 (0.24)**	**P=0.02**	**0.47 (0.23)**	**P<0.05**	**weight*, parity***
Trab. thickness	**-0.51 (0.24)**	**P=0.04**	**-0.53 (0.24)**	**P=0.03**	-0.47 (0.24)	P=0.06	weight, parity
Trab. separation	**-0.61 (0.24)**	**P=0.02**	**-0.66 (0.24)**	**P<0.01**	**-0.54 (0.24)**	**P=0.02**	weight, parity
Cortical vBMD	**-0.64 (0.24)**	**P=0.01**	**-0.62 (0.23)**	**P=0.01**	**-0.67 (0.23)**	**P<0.01**	**weight***
Cortical thickness	**-1.06 (0.22)**	**P<0.001**	**-1.03 (0.22)**	**P<0.001**	**-1.01 (0.21)**	**P<0.001**	age
Cortical porosity	**0.83 (0.23)**	**P<0.001**	**0.83 (0.24)**	**P<0.001**	**0.78 (0.23)**	**P<0.001**	parity

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values.
β = beta coefficient, vBMD = volumetric bone mineral density, Trab. = trabecular, *p<0.05, **p<0.01, ***p<0.001

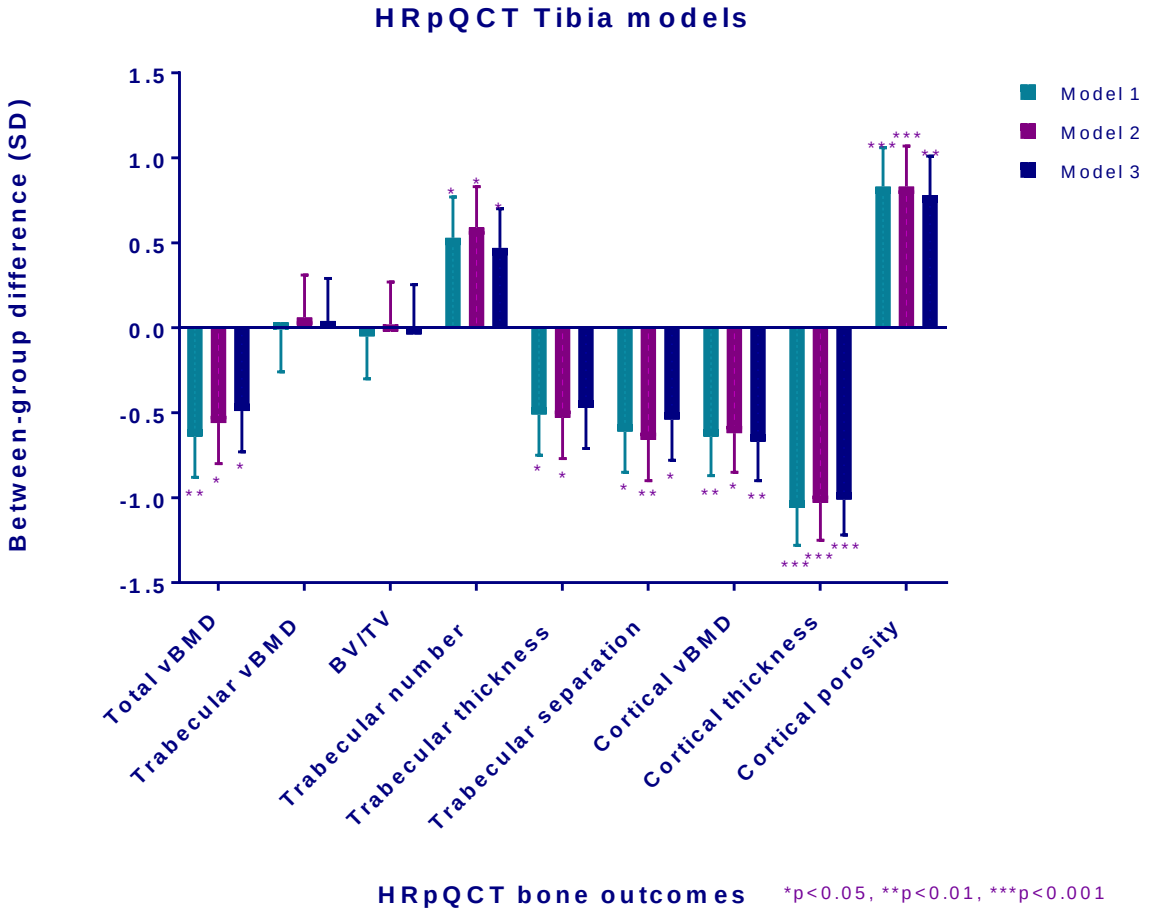

Figure 6.3 Beta-coefficients of between-group differences from models applied to HRpQCT bone outcomes at the distal tibia.

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. In these parsimonious models total vBMD was adjusted for age, contraceptives (ever); trabecular vBMD and BV/TV were adjusted for age; Trabecular number, thickness and separation were adjusted for weight and parity; cortical vBMD was adjusted for baseline weight; cortical thickness was adjusted for age; cortical porosity was adjusted for parity. vBMD = volumetric bone mineral density, BV/TV = trabecular bone volume/total volume.

6.3.2 pQCT Tibia 4%

6.3.2.1 Model 1

Adjusting for group only I found significant between-group differences in total (-0.65 (0.22) SD) and cortical subcortical (-0.60 (0.22) SD) vBMD at the distal 4% tibia (both $p<0.01$). Trabecular vBMD showed a trend towards a decrease -0.40 (0.23) (Table 6.5, $p=0.08$), while no significant changes were found in total CSA.

6.3.2.2 Model 2

Adjusting for size and age increased the reductions in total and cortical subcortical vBMD, respectively -0.68 (0.23) SD and -0.63 (0.22) SD (both $p<0.01$). Weight, height, or age did not predict any of the change in pQCT outcomes at the distal tibia (Table 6.5).

6.3.2.3 Model 3

When selecting a best fit model for total vBMD the group only model (above) was found to have the lowest AIC (Table 6.5). A statistically significant between-group difference of -0.5 (0.23) SD in trabecular vBMD emerged following adjustments for weight, parity, and having ever smoked ($p<0.05$). A -0.60 (0.22) SD difference in cortical subcortical vBMD between-groups, when adjusting for height and ever smoking ($p<0.01$, Table 6.5).

6.3.3 pQCT at the 14% proximal tibia

6.3.3.1 Model 1

When adjusting for group only, no significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 14% proximal tibia (Table 6.5).

6.3.3.2 Model 2

Adjusting for baseline height, weight, and age did not reveal any significant between-group differences in change in any pQCT bone outcome measures at the 14% proximal tibia.

Baseline weight was found to be a negative significant predictor of cortical vBMD ($p<0.01$, Table 6.5).

6.3.3.3 Model 3

As in Model 2 the parsimonious models of best fit found no significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 14% proximal tibia. Baseline weight remained a significant negative predictor of cortical vBMD ($p<0.01$, Table 6.5).

6.3.4 pQCT at the 38% proximal tibia

6.3.4.1 Model 1

No significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 38% proximal tibia when the models were adjusted for group only (Table 6.5).

6.3.4.2 Model 2

No significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 38% proximal tibia when the models were adjusted for size and age. Baseline height was found to be a significant positive predictor of change in cortical BMC, cortical CSA, and cortical thickness (all $p<0.05$, Table 6.5).

6.3.4.3 Model 3

As in Model 2 no significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 38% proximal tibia (Table 6.5). Baseline height was found to be a significant positive predictor of change in cortical BMC, cortical CSA, and cortical thickness (all $p<0.05$).

Table 6.5 Summarising results of linear regression models testing for group differences in change over time at tibia with pQCT in PABS Cambridge

			Tibia 4% (n=77)				
Dependant variable	**Group β_1**	**p-value**	**Group β_2**	**p-value**	**Group β_3**	**p-value**	**Covariates in parsimonious model**
Total vBMD	**-0.65 (0.22)**	**p=0.004**	**-0.68 (0.23)**	**p=0.004**	**-0.65 (0.22)**	**p=0.004**	**-**
Trabecular vBMD	-0.40. (0.23)	p=0.08	-0.40 (0.23)	p=0.1	**-0.50 (0.23)**	**p=0.03**	weight, parity, ever smoked
Total CSA	-0.36 (0.23)	p=0.1	-0.33 (0.24)	p=0.2	-0.36 (0.23)	p=0.1	-
Cortical subcortical vBMD	**-0.60 (0.22)**	**p=0.008**	**-0.63 (0.23)**	**p=0.007**	**-0.60 (0.22)**	**p=0.007**	height, **ever smoked***
			Tibia 14% (n=75)				
Dependant variable	**Group β_1**	**p-value**	**Group β_2**	**p-value**	**Group β_3**	**p-value**	**Covariates in parsimonious model**
Cortical vBMD	-0.26 (0.23)	p=0.3	-0.13 (0.25)	P=0.2	-0.27 (0.23)	p=0.2	**weight***, contraceptives (ever)
Cortical BMC	-0.08 (0.24)	p=0.7	-0.34 (0.23)	P=0.61	-0.09 (0.24)	p=0.7	-
Cortical CSA	0.10 (0.24)	p=0.7	-0.13 (0.25)	P=0.7	0.08 (0.24)	p=0.7	weight, contraceptives (ever)
Cortical thickness	0.14 (0.24)	p=0.6	0.10 (0.24)	P=0.5	0.17 (0.24)	p=0.5	weight
Total CSA	-0.14 (0.24)	p=0.6	0.16 (0.24)	P=0.6	-0.24 (0.24)	p=0.3	parity, contraceptives (ever)
Periosteal circumference	-0.09 (0.24)	p=0.7	-0.19 (0.24)	P=0.6	-0.09 (0.24)	p=0.7	-
Endosteal circumference	-0.15 (0.24)	p=0.5	-0.13 (0.25)	P=0.4	-0.15 (0.24)	p=0.5	-
			Tibia 38% (n=77)				
Dependant variable	**Group β_1**	**p-value**	**Group β_2**	**p-value**	**Group β_3**	**p-value**	**Covariates in parsimonious model**
Cortical vBMD	-0.28 (0.23)	p=0.2	-0.24 (0.23)	p=0.3	-0.14 (0.23)	p=0.6	age, height, contraceptives (ever)
Cortical BMC	-0.02 (0.23)	p=0.9	-0.13 (0.23)	p=0.6	-0.13 (0.23)	p=0.6	weight, **height***
Cortical CSA	0.10 (0.23)	p=0.7	-0.01 (0.23)	p=0.9	-0.01 (0.23)	p=0.9	weight, **height***
Cortical thickness	0.13 (0.23)	p=0.5	0.05 (0.24)	p=0.8	0.003 (0.23)	p=0.9	**height***, contraceptives (ever)
Total CSA	-0.07 (0.23)	p=0.8	-0.11 (0.24)	p=0.7	-0.06 (0.23)	p=0.8	smoked (ever)
Periosteal circumference	-0.06 (0.23)	p=0.8	-0.12 (0.24)	p=0.6	-0.06 (0.23)	p=0.8	-
Endosteal circumference	-0.12 (0.23)	p=0.6	-0.11 (0.24)	p=0.7	-0.12 (0.23)	p=0.6	-

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. β = beta coefficient, vBMD = volumetric bone mineral density, BMC = bone mineral content, CSA = cross sectional area, *p<0.05; **p<0.01; *** p<0.001

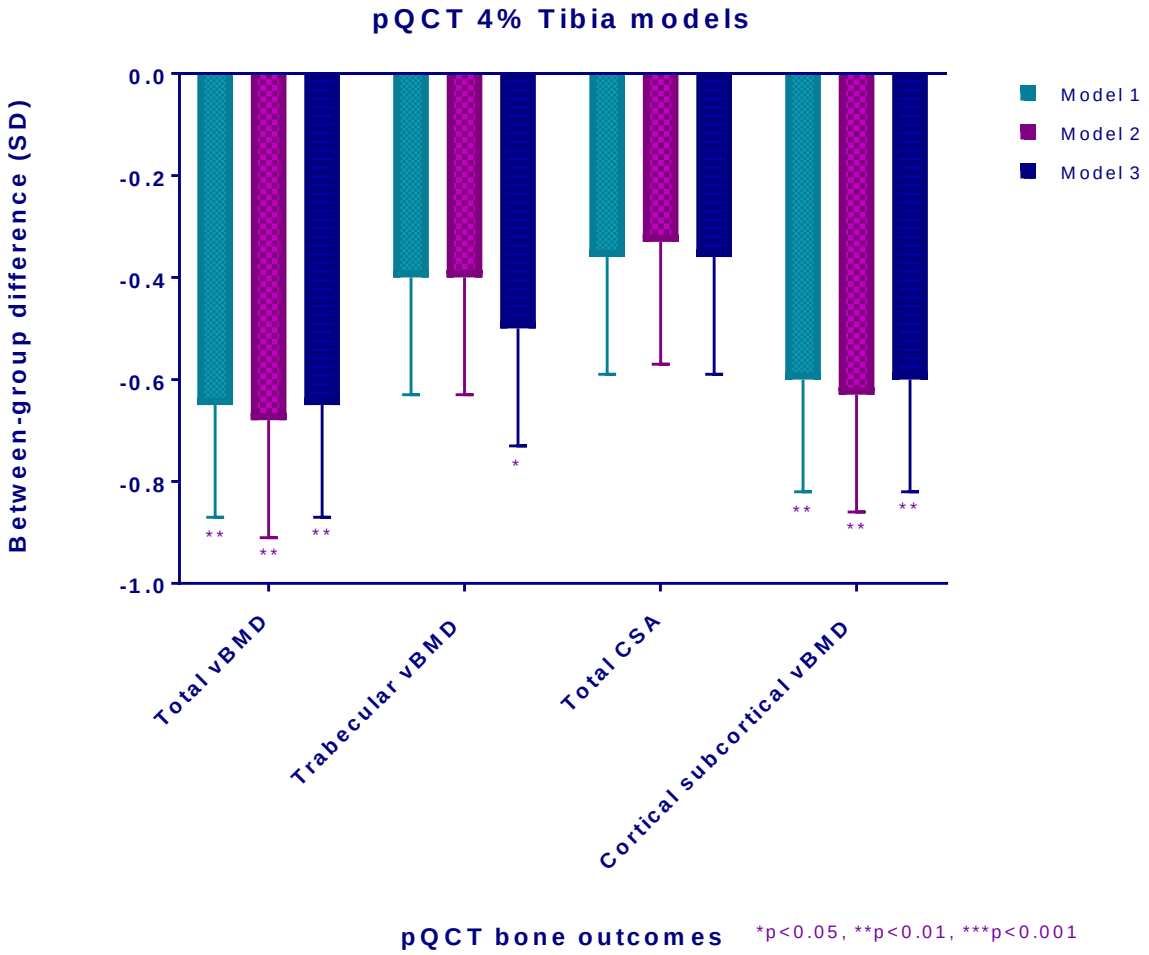

Figure 6.4 Beta-coefficients of between-group differences from models applied to pQCT bone outcomes at the distal tibia.

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. In these parsimonious models total vBMD was adjusted for group only; trabecular vBMD was adjusted for baseline weight, parity and ever smoked; total CSA was adjusted for group only; cortical subcortical vBMD was adjusted for baseline height and ever smoked. vBMD = volumetric bone mineral density, CSA = cross-sectional area.

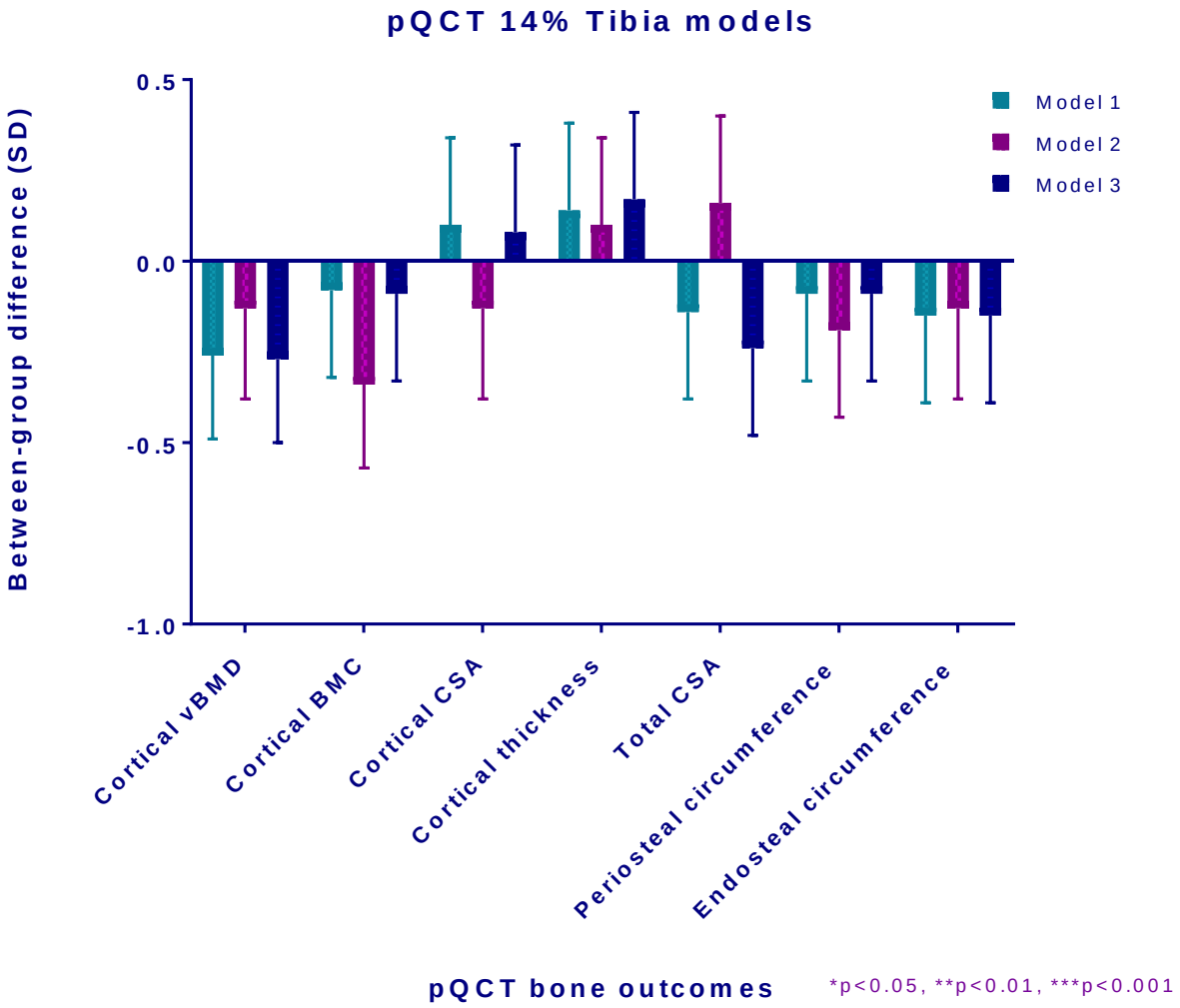

Figure 6.5 Beta-coefficients of between-group differences from models applied to pQCT bone outcomes at the 14% proximal tibia.

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. In these parsimonious models cortical vBMD was adjusted for weight and contraceptives (ever); cortical BMC was adjusted for group only; cortical CSA was adjusted for weight and contraceptives (ever); cortical thickness was adjusted for weight; total CSA was adjusted for parity and contraceptives (ever); both periosteal and endosteal circumferences were adjusted by group only. vBMD = volumetric bone mineral density, BMC = bone mineral content, CSA = cross-sectional area.

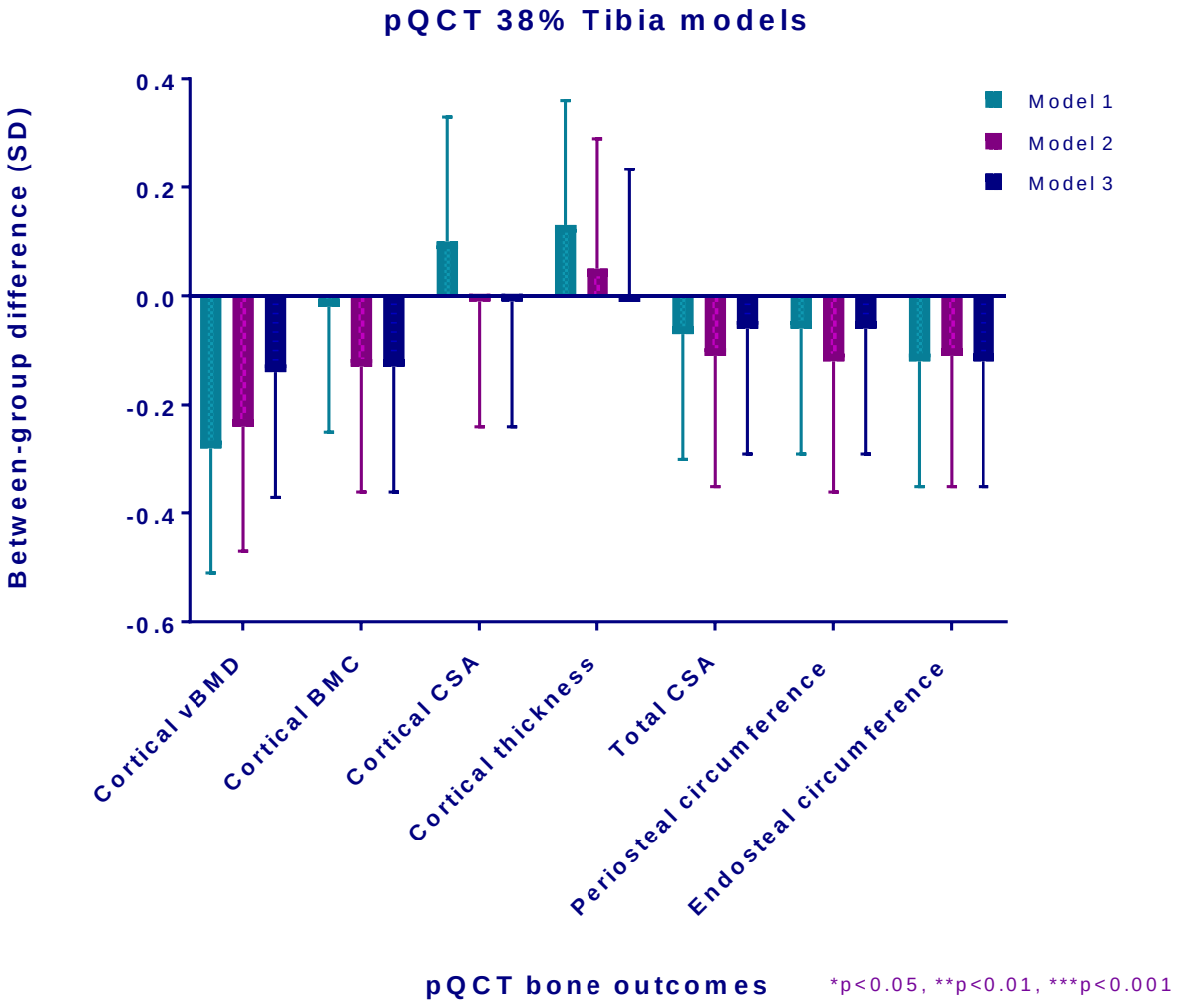

Figure 6.6 Beta-coefficients of between-group differences from models applied to pQCT bone outcomes at the 14% proximal tibia.

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. In these parsimonious models cortical vBMD was adjusted for age, height, and contraceptives (ever); cortical BMC was adjusted for weight and height; cortical CSA was adjusted for weight and height; cortical thickness was adjusted for height and contraceptives (ever); total CSA was adjusted for smoked ever; both periosteal and endosteal circumferences were adjusted by group only. vBMD = volumetric bone mineral density, BMC = bone mineral content, CSA = cross-sectional area.

6.3.5 HRpQCT at the distal radius

6.3.5.1 Model 1

No significant between-group differences were found for change in any HRpQCT bone outcome measures at the distal radius when the models were adjusted for group only (Table 6.6).

6.3.5.2 Model 2

After adjusting for size and age, no significant between-group differences were found at the distal radius (Table 6.6). Baseline weight was a positive predictor of trabecular number ($p<0.05$) but a negative predictor of trabecular separation and cortical vBMD (both $p<0.05$). Age was a positive predictor of change in cortical thickness ($p<0.05$).

6.3.5.3 Model 3

The parsimonious models did not significant between-group differences in change at the distal radius (Table 6.6). Weight was found to be a positive predictor of trabecular number but a negative predictor of both trabecular separation and cortical vBMD at the distal forearm (all $p<0.05$). Maternal age was found to be a positive predictor of total vBMD and cortical thickness (both $p<0.05$). Parity was found to be a negative predictor of trabecular number and a positive predictor of trabecular separation (both $p<0.05$).

Table 6.6 Summarising results of linear regression models testing for group differences in change over time at the distal radius with HRpQCT in PABS Cambridge

	Distal Radius (n=60)						
Dependant variable	Group β_1	p-value	Group β_2	p-value	Group β_3	p-value	Covariates in parsimonious model
Total vBMD	0.04 (0.26)	P=0.9	0.00 (0.26)	P=0.9	0.05 (0.26)	P=0.9	**age***
Trabecular vBMD	0.13 (0.26)	P=0.6	0.17 (0.27)	P=0.5	0.13 (0.26)	P=0.6	-
BV/TV	0.11 (0.26)	P=0.7	0.17 (0.27)	P=0.5	0.11 (0.26)	P=0.7	-
Trab. number	-0.18 (0.26)	P=0.5	-0.09 (0.26)	P=0.7	-0.10 (0.24)	P=0.7	**weight*, parity***
Trab. thickness	0.20 (0.26)	P=0.4	0.14 (0.27)	P=0.6	0.15 (0.26)	P=0.6	weight, parity
Trab. separation	0.11 (0.27)	P=0.7	0.03 (0.26)	P=0.9	0.04 (0.25)	P=0.9	**weight*, parity***
Cortical vBMD	-0.09 (0.26)	P=0.7	-0.15 (0.26)	P=0.6	-0.16 (0.25)	P=0.5	**weight***, parity
Cortical thickness	-0.26 (0.26)	P=0.3	-0.28 (0.26)	P=0.3	-0.24 (0.25)	P=0.3	**age***
Cortical porosity	0.49 (0.26)	P=0.06	0.48 (0.26)	P=0.07	0.48 (0.25)	P=0.06	age

Model 1 adjusted for group only; Model 2 adjusted for group, height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever), model of best fit selected by comparing the model AIC values. β = beta coefficient, vBMD = volumetric bone mineral density, BMC = bone mineral content, CSA = cross sectional area, *p<0.05; **p<0.01; *** p<0.001

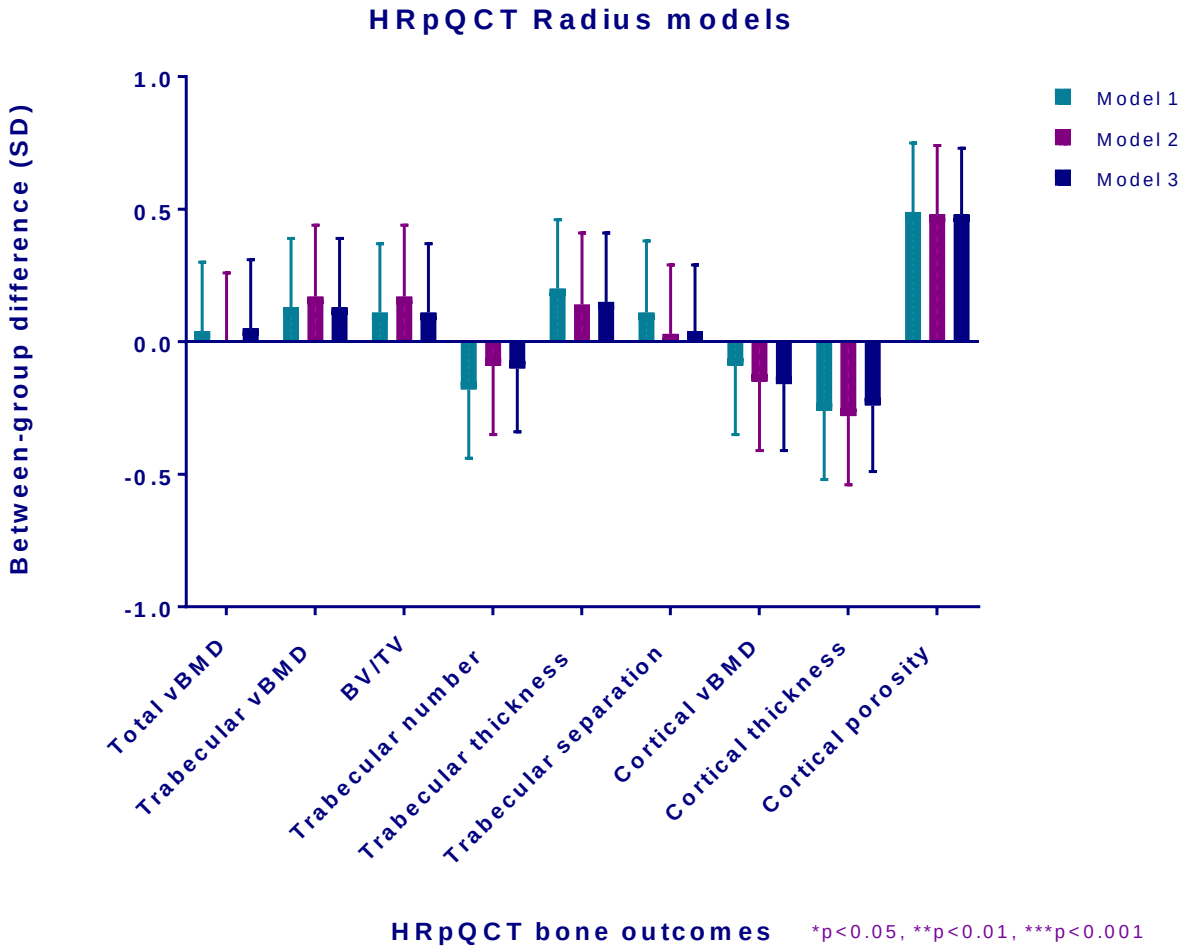

Figure 6.7 Beta-coefficients of between-group differences from models applied to HRpQCT bone outcomes at the distal radius.

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. In these parsimonious models total vBMD was adjusted for age; trabecular vBMD and BV/TV were adjusted for group only; Trabecular number, thickness and separation were adjusted for weight and parity; cortical vBMD was adjusted for weight and parity; cortical thickness and porosity were both adjusted for age .vBMD = volumetric bone mineral density, BV/TV = trabecular bone volume/total volume.

6.3.6 pQCT at the 4% distal radius

6.3.6.1 Model 1

No significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 4% distal radius when the models were adjusted for group only (Table 6.7).

6.3.6.2 Model 2

No significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 4% distal radius when the models were adjusted for group, baseline height and weight, and age (Table 6.7). The size and age covariates in Model 2 were not predictive of changes in pQCT bone outcome measures.

6.3.6.3 Model 3

No significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 4% distal radius when the models were adjusted for group, baseline height and weight, and age. None of the covariates in the models of best fit were significant predictors of change in pQCT bone outcome measures (Table 6.7).

6.3.7 pQCT at the 33% proximal radius

6.3.7.1 Model 1

No significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 33% proximal radius when the models were adjusted for group only. However a trend towards increase was found in cortical vBMD and total CSA ($p=0.06$, $p=0.08$ respectively). A trend towards a decrease in cortical thickness was also found ($p=0.07$).

6.3.7.2 Model 2

No significant between-group differences were found in the between visit change in any pQCT bone outcome measures at the 33% proximal radius when the models were adjusted for group only. However, when adjusting for baseline height, weight and age a trend towards increase was found in cortical vBMD and endosteal circumference (both $p=0.08$, Table 6.7). A trend towards a decrease in cortical thickness was also found ($p=0.06$). Age was found to be a negative predictor of cortical thickness ($p<0.05$) and a positive predictor of endosteal circumference ($p<0.05$).

6.3.7.3 Model 3

At the 33% proximal radius pregnant woman had thinner cortices -0.5 (0.23) SD and a higher endosteal circumference 0.46 (0.23) SD compared to NPNL (both $p<0.05$, Table 6.7). In addition cortical vBMD was found to increase by 0.5 (0.22) SD ($p<0.05$). Age was a significant predictor for both cortical thickness and endosteal circumference ($p<0.05$). Parity was a significant negative predictor of cortical BMC ($p<0.05$), cortical CSA ($p<0.01$), and total CSA ($p<0.05$).

Table 6.7 Summarising results of linear regression models testing for group differences in change over time with pQCT in PABS Cambridge

				Radius 4% (n=71)			
Dependant variable	**Group β_1**	**p-value**	**Group β_2**	**p-value**	**Group β_3**	**p-value**	**Covariates in parsimonious model**
Total vBMD	-0.31 (0.24)	P=0.2	-0.30 (0.25)	P= 0.2	-0.18 (0.24)	P=0.5	height, parity, contraceptives (ever)
Trabecular vBMD	-0.18 (0.24)	P=0.5	-0.20 (0.25)	P=0.4	-0.17 (0.25)	P=0.5	weight, parity, smoked (ever)
Total CSA	0.07 (0.24)	P=0.8	0.07 (0.25)	P=0.8	0.07 (0.24)	P=0.8	-
Cortical subcortical vBMD	-0.30 (0.24)	P=0.2	-0.30 (0.25)	P=0.2	-0.17 (0.24)	P=0.5	height, parity, contraceptives (ever)
				Radius 33% (n=76)			
Dependant variable	**Group β_1**	**p-value**	**Group β_2**	**p-value**	**Group β_3**	**p-value**	**Covariates in parsimonious model**
Cortical vBMD	0.43 (0.23)	P=0.06	0.42 (0.24)	P=0.08	**0.50 (0.23)**	**P=0.03**	parity, smoked (ever)
Cortical BMC	0.21 (0.23)	P=0.4	0.24 (0.24)	P=0.3	0.14 (0.23)	P=0.6	**parity***
Cortical CSA	-0.08 (0.23)	P=0.7	-0.05 (0.24)	P=0.8	-0.17 (0.23)	P=0.5	weight, smoked (ever), **parity****
Cortical thickness	-0.41 (0.23)	P=0.07	-0.43 (0.22)	P=0.06	**-0.50 (0.22)**	**P=0.03**	**age***
Total CSA	0.40 (0.23)	P=0.08	0.39 (0.24)	P=0.1	0.34 (0.23)	P=0.1	**parity***
Periosteal circumference	0.34 (0.23)	P=0.1	0.38 (0.24)	P=0.1	0.40 (0.23)	P=0.09	age
Endosteal circumference	0.38 (0.23)	P=0.1	0.41 (0.23)	P=0.08	**0.46 (0.23)**	**P=0.047**	**age***

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. β = beta coefficient, vBMD = volumetric bone mineral density, BMC = bone mineral content, CSA = cross sectional area, *p<0.05; **p<0.01; *** p<0.001

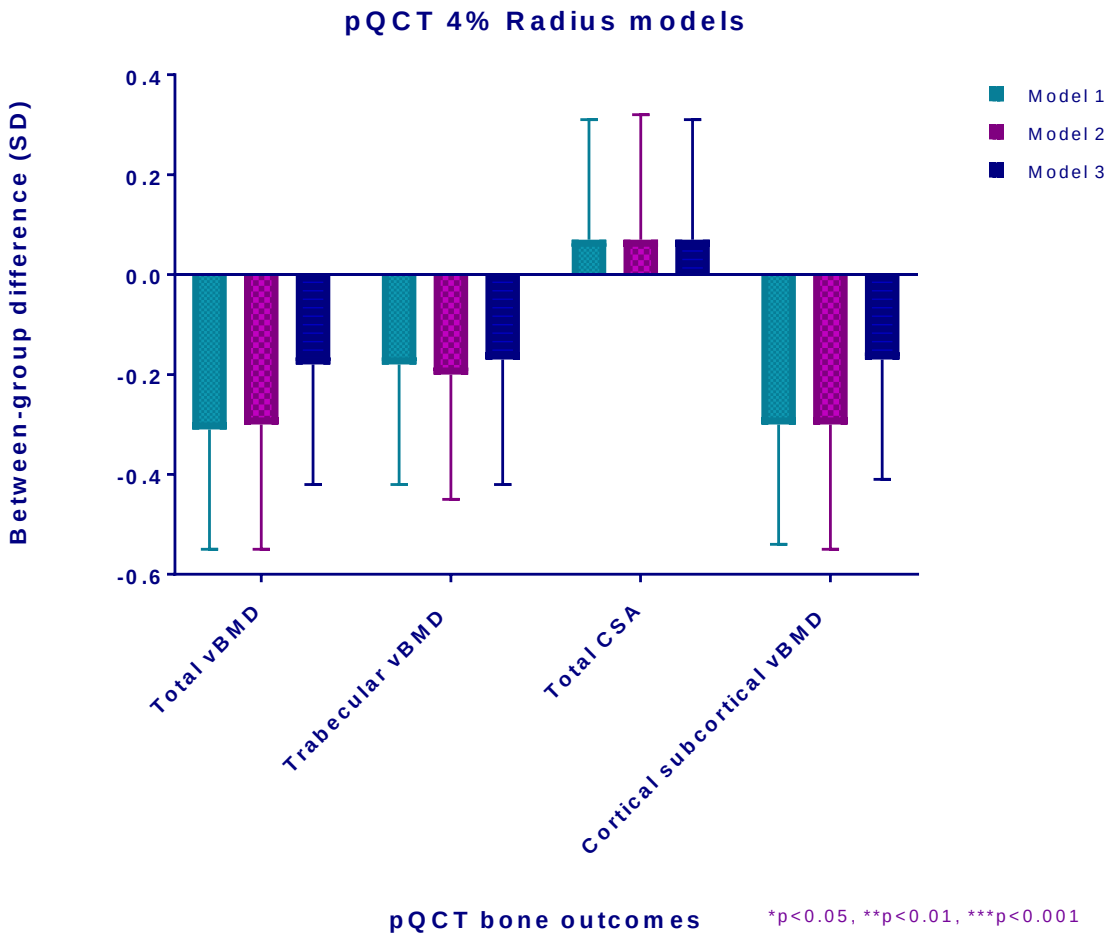

Figure 6.8 Beta-coefficients of between-group differences from models applied to pQCT bone outcomes at the distal radius.

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. In these parsimonious models total vBMD was adjusted for height, parity, and contraceptives (ever); trabecular vBMD was adjusted for weight, parity, and smoked (ever); total CSA was adjusted for group only; cortical subcortical vBMD was adjusted for height, parity, and contraceptives (ever). vBMD = volumetric bone mineral density, CSA = cross-sectional area.

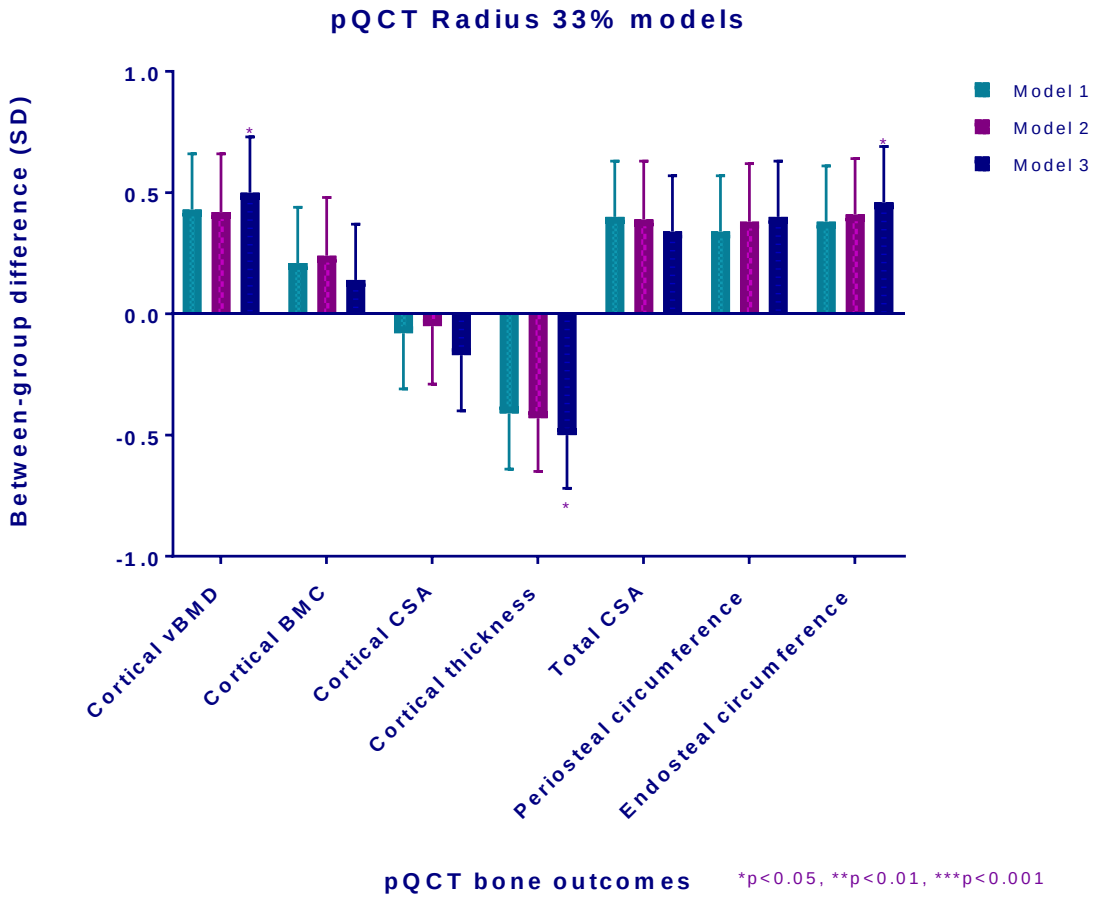

Figure 6.9 Beta-coefficients of between-group differences from models applied to pQCT bone outcomes at the 33% proximal radius.

Model 1 adjusted for group only; Model 2 adjusted for group, baseline height, weight, and age; Model 3 was ran with group, baseline height, weight, age, parity, smoking (ever), and contraceptives (ever) with the model of best fit selected by comparing the model AIC values. In these parsimonious models cortical vBMD was adjusted for parity and smoked (ever); cortical BMC was adjusted for parity; cortical CSA was adjusted for weight, smoked (ever), and parity; cortical thickness was adjusted for age; total CSA was adjusted for parity; both periosteal and endosteal circumferences were adjusted by age. vBMD = volumetric bone mineral density, BMC = bone mineral content, CSA = cross-sectional area.

6.4 Discussion of results

These data provide for the first time compelling evidence of pregnancy-induced alterations in maternal bone mineral density and microarchitecture at both the radius and tibia with two novel pQCT scanning modalities. My primary objective was to determine whether maternal bone mineral was mobilised from trabecular-rich sites, through decreases in trabecular vBMD (HRpQCT and pQCT) and the potential reorganisation of trabecular microarchitecture (HRpQCT). Changes in trabecular vBMD were found at the distal 4% tibia with pQCT, where a 0.5 SD decrease was found, but no significant changes were found in either HRpQCT measured BV/TV or trabecular vBMD. As outlined above significant changes in trabecular microarchitecture were found at the distal tibia with HRpQCT, however, two interesting findings in relation to trabecular architecture warrant discussion.

Firstly, the significant between-group baseline differences in parameters of trabecular microarchitecture may provide indirect evidence of early microarchitectural changes during the first trimester of pregnancy: trabecular number was 8% lower in the pregnant group compared to NPNL; while trabecular thickness was 12% higher in pregnant women than NPNL. Secondly, pregnancy-induced changes in trabecular microarchitecture at the distal tibia may require interpretation in the context of cortical compartment alterations, where pregnancy-induced reductions in cortical vBMD and cortical thickness alongside an increase in cortical porosity were observed. It is possible that the trabecular microarchitectural changes are artefacts of changes in the cortical compartment at the trabecular-cortical interface. Endosteal resorption may lead to the endocortical surface becoming "trabecularised" with a thinning and increasingly porous shell leading to the detection of pores as trabecular struts. Such struts would increase the trabecular number, but would also decrease the trabecular thickness and increase porosity. An alternative explanation may also relate to the limits of the techniques resolution because at baseline some trabeculae may have been too close to one another to be detected as independent branches. As mineral is resorbed from the trabecular surface thickness decreases, subsequently leading to the

detection of an "increase" in trabecular number. These newly detected trabeculae will also be closer together which accounts for the declining trabecular separation. In the light of finding changes in the trabecular microarchitecture but a lack of change in trabecular vBMD, both Dinn (trabecular vBMD of the inner 60% of the trabecular area) and DMeta (the remaining section of the compartment) were explored to investigate whether this was due to intra-trabecular compartment change rather than cortical changes. No changes were found either in these trabecular parameters or in the ratio of Dinn/Dmeta.

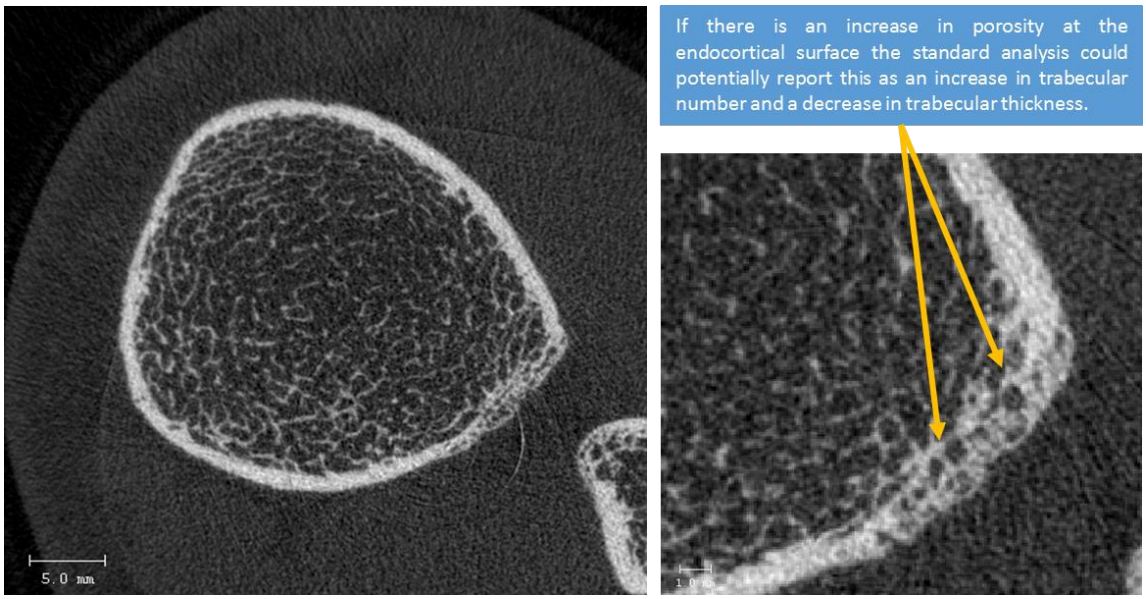

Figure 6.10 The endosteal surface can be difficult to define with standard analysis.

The images above are from the distal tibia of a healthy non-pregnant non-lactating women but highlight the difficulties in accurately defining the junction between the trabecular and cortical compartments. An increase in porosity in this region could possibly be interpreted as change in trabecular microarchitecture with standard analysis.

As noted, my data suggest marked adaptation within the maternal cortical compartment during pregnancy. Pregnancy results in the mobilisation of bone mineral from the cortical compartment indicated by decreases in vBMD and adaptations in cortical microarchitecture. This evidence of pregnancy-induced cortical adaptation was documented with two distinct imaging techniques, at both distal (trabecular-rich) and proximal (cortical-rich) skeletal sites, in both weight bearing and non-weight bearing limbs. At the distal tibia HRpQCT revealed a number of significant changes in the cortical shell where cortical vBMD and cortical thickness were found to decrease, while the cortex became increasingly porous.

No significant between-group differences were found at the distal radius during pregnancy in keeping with some of the scientific literature discussed in Chapter 2 (Christiansen et al., 1976, Kent et al., 1993, Clark et al., 1995, Kolthoff et al., 1998, Black et al., 2000, More et al., 2001, Moller et al., 2012) though the scientific literature is inconclusive as (Wisser et al., 2005) reported wide inter-individual changes in distal radius trabecular vBMD with pQCT and (Moller et al., 2012) reported a significant decrease in distal radius aBMD during pregnancy. Olausson and colleagues did not find a difference at the forearm between pre-pregnancy and postpartum scans, which is in keeping with several studies suggesting the conservation of bone mineral during lactation at the distal radius which in later life is a fracture prone site. However, at the 33% proximal radius a statistically significant decrease in cortical thickness alongside a significant increase in endosteal circumference suggested the resorption of bone mineral from the endosteal surface. If we consider that the changes in trabecular number and thickness at the distal tibia may be artefacts of cortical mineral mobilisation it is possible these are evidence of endosteal resorption from endocortical surfaces at two peripheral skeletal locations. These data at the forearm would seem to support the hypothesis of Garn and others that bone mineral accrued during growth may act as a reservoir for use during reproduction.

In the parsimonious models several of the independent variables were found to be significant predictors of change. With HRpQCT at the distal tibia, baseline weight was a positive predictor of change in trabecular number ($p<0.05$) but a negative predictor of cortical vBMD ($p<0.05$); with pQCT at the 3 tibial sites weight was only found to be a significant negative predictor of cortical vBMD at the 14% tibia ($p<0.05$). As at the distal tibia, baseline weight was found to a positive predictor of change in trabecular number and a negative predictor of trabecular separation and cortical vBMD at the distal radius (all $p<0.05$). As maternal age was one of the primary recruitment criteria of the study and a relatively narrow age band (30- 45 years) was selected, age was still found to be a significant predictor of change in several of the models at the distal and proximal radius although its effects were modest and no age effect was seen at the tibia. Age was a positive predictor

for HRpQCT parameters of total vBMD and cortical thickness at the distal radius (both $p<0.05$). However, in contrast at the proximal radius it was found to be a negative predictor of change in cortical thickness but a positive predictor of endosteal circumference (both $p<0.05$). Interestingly, parity emerged as a rather strong predictor of change in several bone outcome measures in both limbs. At both the distal tibia and radius parity was a significant negative predictor of trabecular number ($p<0.05$) and at the radius a positive predictor of trabecular separation ($p<0.05$). At the proximal radius it was found to be a strong negative predictor of change in cortical BMC ($p<0.05$), cortical CSA ($p<0.01$), and total CSA ($p<0.05$).

The primary findings of this study suggest that at the distal tibia bone mineral changes are occurring in both bone compartments, however, as we begin to move slightly more proximally to the HRpQCT scan region cortical changes predominate (decreasing cortical vBMD and thickness with increasing porosity) with the possibility of some trabecular microarchitectural reorganisation. No changes were found with pQCT at the proximal tibia at either of the 2 cortical-rich proximal sites investigated. At the forearm, measurements from both techniques showed the conservation of bone mineral at the distal radius, however, in contrast a pattern of change suggesting endosteal resorption through a decrease in cortical thickness and endosteal circumference (with an artificial increase in cortical vBMD) was found at the proximal radius.

My findings of a decrease of -0.5 SD in pQCT trabecular vBMD supports my primary hypothesis of mineral mobilisation from within the trabecular compartment, in keeping with the findings of Olausson et al. (2008) who found significant change in maternal SA-BMC at the lumbar spine of 2.4% between pre-pregnancy and 2 weeks postpartum. These results are also in line with similar studies in the literature (Drinkwater and Chesnut, 1991, More et al., 2001, Pearson et al., 2004, Moller et al., 2012). HRpQCT trabecular vBMD did not decrease, however, microarchitectural changes were found in several trabecular bone outcome measures at the distal tibia. Whether this reflects trabecular bone mineral mobilisation at the distal tibia or is an artefact of pregnancy-induced changes in the cortical compartment; cortical vBMD (-0.67 (0.23) SD), cortical thickness

(-1.01 (0.21) SD), and cortical porosity (0.83 (0.23) SD) is uncertain. Decreases in total vBMD at the distal tibia were observed with both HRpQCT and pQCT (-0.49 SD (0.24) and -0.65 (0.22) SD respectively). Pregnancy-induced changes at the forearm were all cortical in nature and confined to the proximal radius where cortical thickness decreased by -0.50 (0.22) SD and endosteal circumference increased by 0.46 (0.23) SD and suggest endosteal resorption at the cortical-rich proximal radius. The clinical relevance of these data will depend on the extent to which subsequent mobilisation of bone mineral during lactation if these women chose to breastfeed, the length of breastfeeding, and their ability to replete this mineral fully before the onset of menopause.

7 In-vivo correlation of pQCT to HRpQCT outcome measures

7.1 Rationale for analysis

As discussed in Chapter 1 HRpQCT and pQCT are versatile research tools for bone imaging of the appendicular skeleton, providing estimates of vBMD in addition to bone mass, geometry and distribution. Few research centres possess or routinely use both modalities and no in-vivo between-modality correlation or agreement data have been published, however, ex-vivo data have been published which show high correlation in vBMD and geometric parameters in cadaveric tibiae at the same anatomical location (Lala et al., 2014, Lala et al., 2012). In practical use, however, pQCT and HRpQCT scanners do not routinely image the same sites. One of my secondary objectives was to explore the in-vivo correlation and agreement of pQCT and HRpQCT techniques; focusing on measures of trabecular vBMD, cortical vBMD, and cortical thickness. The major technical differences between the techniques are:

1. Scanner resolution (voxel size) pQCT (0.2-0.5 mm) vs HRpQCT (XtremeCTI, 0.082 mm)
2. Scan sites for trabecular and cortical outcomes (Figure 7.2):
    a. pQCT and HRpQCT measure trabecular parameters at distinct but physiologically similar trabecular-rich distal scan sites.
    b. pQCT cortical measures are obtained at cortical-dominant proximal sites, whereas HRpQCT cortical parameters are acquired simultaneously with the trabecular data.

My aim was to explore correlation and agreement between pQCT and HRpQCT in typical in-vivo use. HRpQCT obtains cortical and trabecular parameters at the distal radius and tibia from a volume of interest (VOIs, a stack of 104 CT slices), in this analysis all parameters were acquired with the manufacturer's software (Version 6.6). The HRpQCT VOI is determined at a fixed distance from the reference line. Cortical parameters represent the mean cortical vBMD and cortical thickness of the software-defined cortical region of the VOI, and trabecular vBMD is the

mean vBMD of the trabecular region. Sub-regional trabecular Dinn (inner 60%), and Dmeta (outer part of the trabecular compartment) are also reported in addition to their ratio. pQCT trabecular and cortical compartment data are obtained at physiologically different scan slices (thickness 2 mm), cortical vBMD is obtained at proximal sites following the removal of non-cortical tissues by thresholding (Chapter 1.4), while cortical thickness is derived using a "circular ring model" which is discussed in Chapter 1.4. pQCT trabecular vBMD is obtained from the inner 45% of the total CSA, a voxel size of 0.5 mm is insufficient to discern cortical from trabecular compartment at their ill-defined junction. In PABS many of the observations in the trabecular and cortical compartments at both the distal and proximal tibia were similar in direction, and at the distal tibia the magnitude of change in pQCT total vBMD and HRpQCT total vBMD were similar. This encouraged me to further explore my a priori objective of investigating correlation and agreement between techniques.

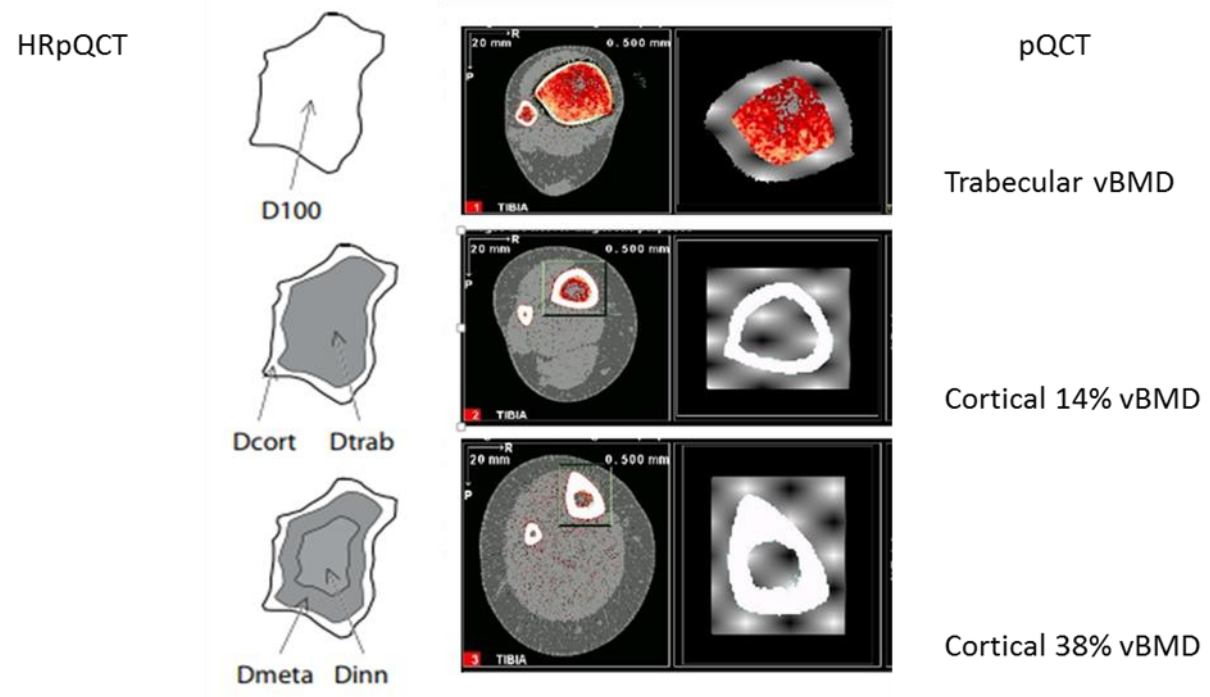

Figure 7.1 Summary of volumetric BMD parameters obtained from HRpQCT and pQCT.

HRpQCT (right) schematic illustrates the sub regions measured to provide various vBMD parameters from the distal 104 slice volume of interest. pQCT scans (centre) following segmentation and thresholding (left) show compartment specific vBMD parameters that are site-specific. vBMD = volumetric bone mineral density, D100 = total vBMD, Dcort = cortical vBMD, Dtrab = trabecular vBMD, Dmeta = , Dinn = inner trabecular vBMD.

7.2 Statistical approach

The criterion for data inclusion in this analysis was participant baseline data on pQCT and HRpQCT of analysis grade (i.e. pQCT scans ≤ grade 2, HRpQCT scan ≤ grade 4). Tibial data were selected because more usable pairs (n=85) were available. Between-scanner differences for selected outcome measures were tested by paired t-tests; statistically significant differences were present for comparisons (all $p<0.05$). Scatter plots were produced to visualise the relationship between similar parameters (Figures 1.1, 1.2 & 1.3) a line of equality was added (i.e. on which all points would lie if the two scanner gave exactly the same reading every time) as a visual aid and simple linear regression was used to determine the correlation between the pairs (adjusted R^2) with the regression line plotted. These provided a simple but visually powerful way of interpreting the data to a) explore how the real fit differed from the perfect, and b) if systematic differences were present between the techniques. Perfect correlation can be present if the points all lie along any straight-line, perfect agreement can only be present if the points lie along the line of equality (Bland and Altman, 1986). Bland and Altman caution that data which seem to be in poor agreement can produce quite high correlations and conceal considerable lack of agreement (Bland and Altman, 1986). I used mean-difference (Bland Altman) plots to visualise any possible relationship between two measurements. The mean of the two measurements is the best estimate of the true value, and the difference should be plotted against this rather than either value separately because the difference will be related to each (Gill et al., 1985). The bias between techniques can be seen from these plots estimated by the mean difference (δ) and the standard deviation of the differences (s) (Bland and Altman, 1986). These plots can also be expressed in Z-scores where the data have been standardised, this can be useful when dealing with a number of comparisons in different units and on different scales and displays the data adjusted from bias, however, although a helpful visualisation particularly when the data show high systematic difference (correlation plots) the absolute mean-difference plot provide a clearer interpretation of between-scanner bias.

7.3 Correlation between tibial pQCT and HRpQCT parameters

7.3.1 Correlation between cortical vBMD and thickness

Weak to poor correlation found between HRpQCT cortical vBMD and 14% and 38% pQCT cortical vBMD (respectively adjusted R^2=0.36 and R^2=0.15, Figure 7.2) with pQCT showing a consistent, but decreasing, bias to higher values within the range or values measured. Good correlation (R^2=0.68) was found between HRpQCT and 14% pQCT cortical thickness, also with a bias to higher values in pQCT. As the regression line initially tracks relatively parallel to identity line but diverges slightly at higher values this suggests an increasing systematic difference at very high values of cortical thickness (Figure 7.2). In contrast at the 38% pQCT tibia cortical thickness was found to show poor correlation to HRpQCT (R^2=0.07) (Figure 7.2).

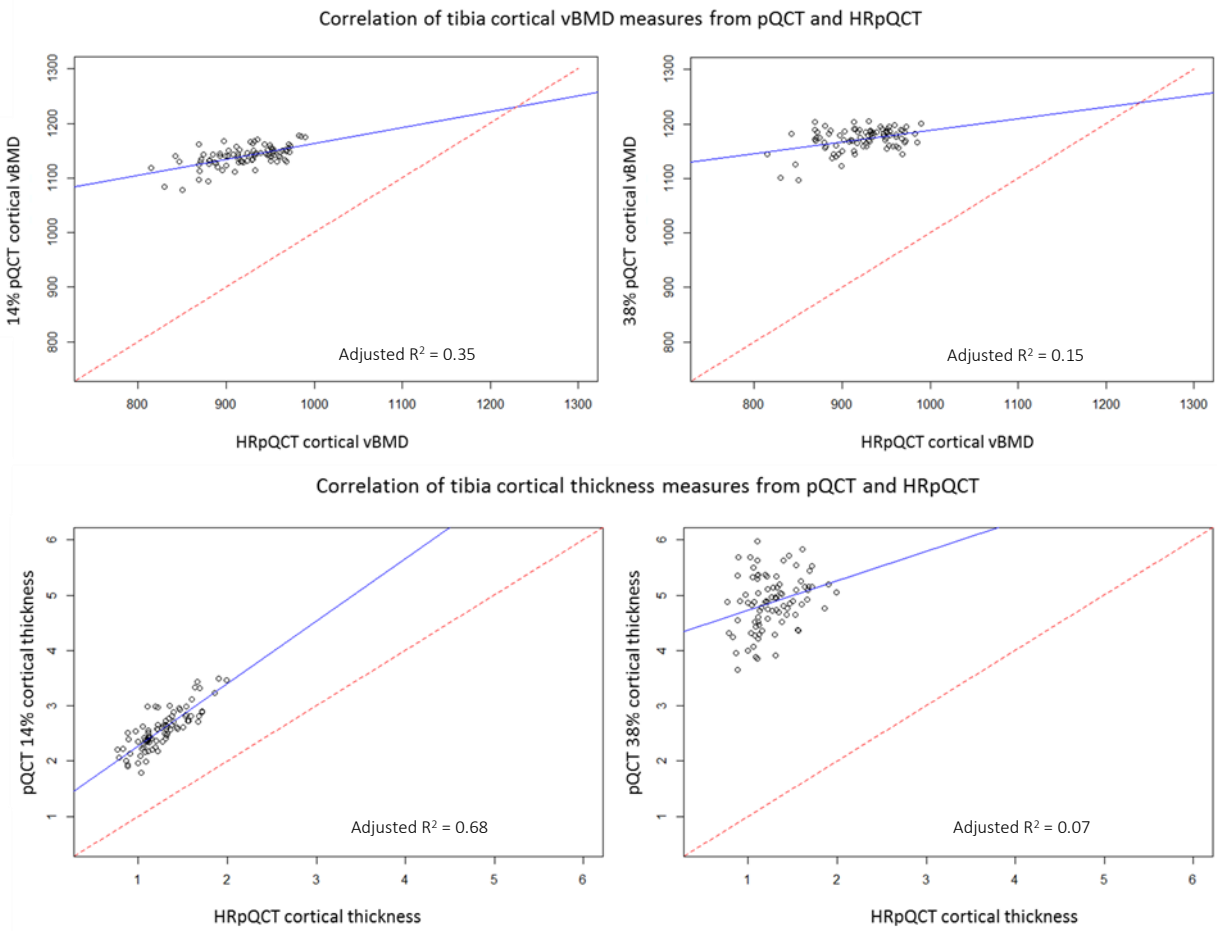

Figure 7.2 Scatter plots of the relationship between pQCT and HRpQCT cortical parameters at the tibia.

Top: cortical vBMD from 14% pQCT vs HRpQCT (left); 38% pQCT vs HRpQCT (right). Bottom: cortical thickness form 14% pQCT vs HRpQCT (left); 38% pQCT vs HRpQCT (right). Blue lines represent the regression line and dashed red lines the line of equality vBMD = volumetric bone mineral density

7.3.2 Trabecular vBMD parameters at the distal tibia

Very-strong correlation was found between pQCT and HRpQCT trabecular vBMD (R2=0.87), while a lower correlation was found when trabecular inner vBMD (Dinn) replaced trabecular vBMD the correlation with pQCT remained strong (R^2=0.83). Although there is high correlation between the data, in both cases when the plotted the regression line and equality line appear roughly parallel suggesting a constant systematic difference between the techniques with pQCT data consistently higher than either HRpQCT trabecular vBMD variable, across the range of values measured. This systematic difference was slightly greater between pQCT trabecular vBMD and Dinn.

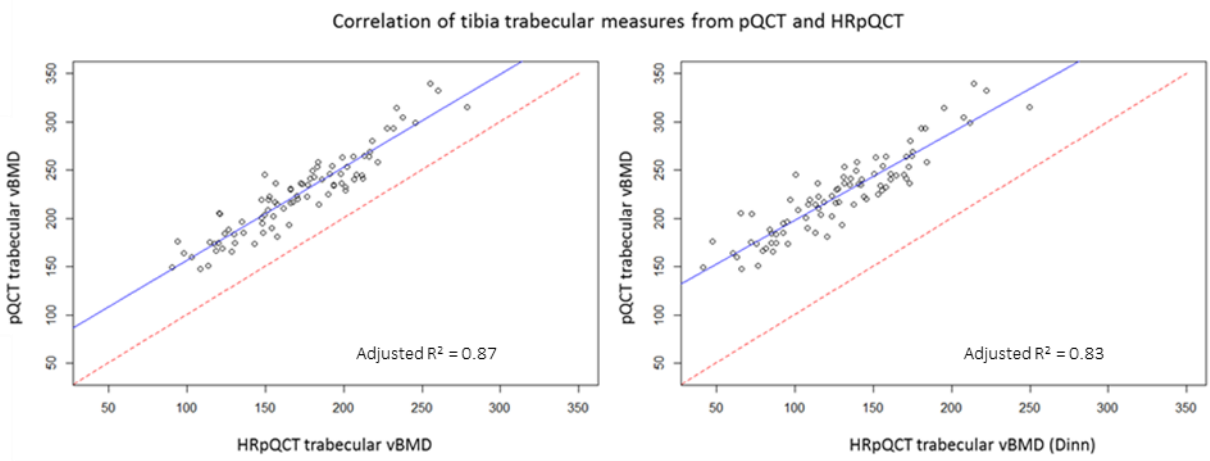

Figure 7.3 Scatter plots of the relationship between pQCT and HRpQCT trabecular vBMD parameters at the distal tibia.

Left: pQCT trabecular vBMD and HRpQCT trabecular vBMD; Right: pQCT trabecular vBMD and Dinn (i.e. inner 60% of vBMD). Blue lines represent the regression line and dashed red lines the line of equality. vBMD = volumetric bone mineral density

7.4 Agreement between pQCT and HRpQCT parameters

BA plots only define the intervals of agreements, not whether these limits are acceptable or not, this must be decided a priori. In all cases below the data were first plotted with the absolute values and subsequently with the data standardised into Z scores (SD scores).

7.4.1 Agreement between cortical vBMD and cortical thickness at the tibia

Plots for HRpQCT measured and pQCT 14% & 38% cortical vBMD show poor agreement with a mean difference of -219 mg/cm^3 (95% CI -280;-158) and -251 mg/cm^3 (95% CI -320; -181) respectively. These large mean-differences are in-line with the very poor correlation found above but highlight that between-scanner agreement differs with a bias towards worse agreement at higher mean values (x-axis) (Figure 7.4). The standardisation of these Bland-Altman plots show that even when these large biases are adjusted for the distribution of data are in both cases roughly fall within 2SD of the mean differences.

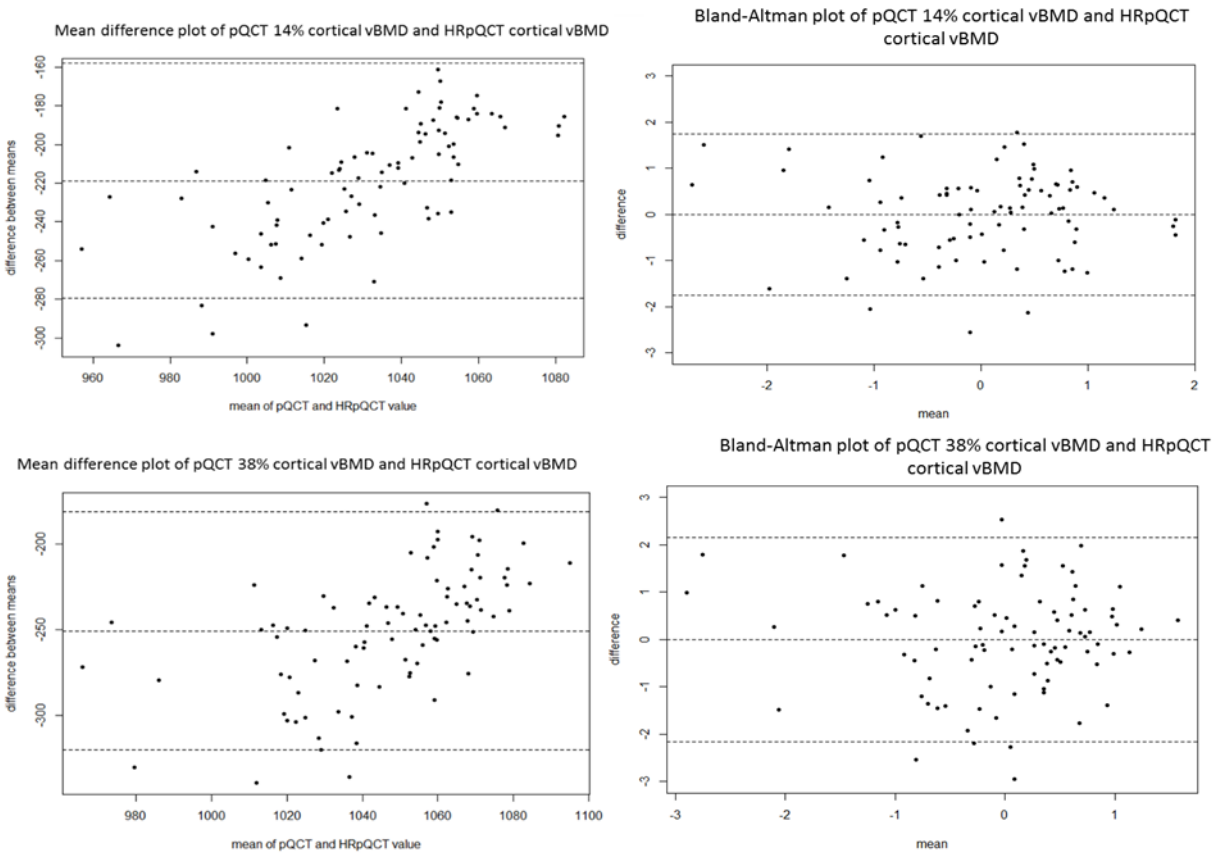

Figure 7.4 Agreement between cortical vBMD from pQCT and HRpQCT.

Mean-difference and Bland-Altman plots of the pQCT measures of cortical vBMD at 14% (left) and 38% (right) compared to HRpQCT measured cortical vBMD. Poor agreement was found between pQCT and HRpQCT cortical vBMD at both sites. vBMD = volumetric bone mineral density

Better agreement was found between 14% pQCT and HRpQCT parameters of cortical thickness, mean difference -1.31 mm (95% CI -1.72;-0.89), than for 38% pQCT and HRpQCT parameters of cortical thickness, mean difference -3.61 mm (95% CI -4.56;-2.65). The larger bias seen at the

38% tibia vs HRpQCT is understandable given the physiological differences between the scan sites. Standardisation reinforced this interpretation as we can see that the differences between means fall almost within 1SD of the mean for the 14% site but exceed 2SD at the 38% tibia.

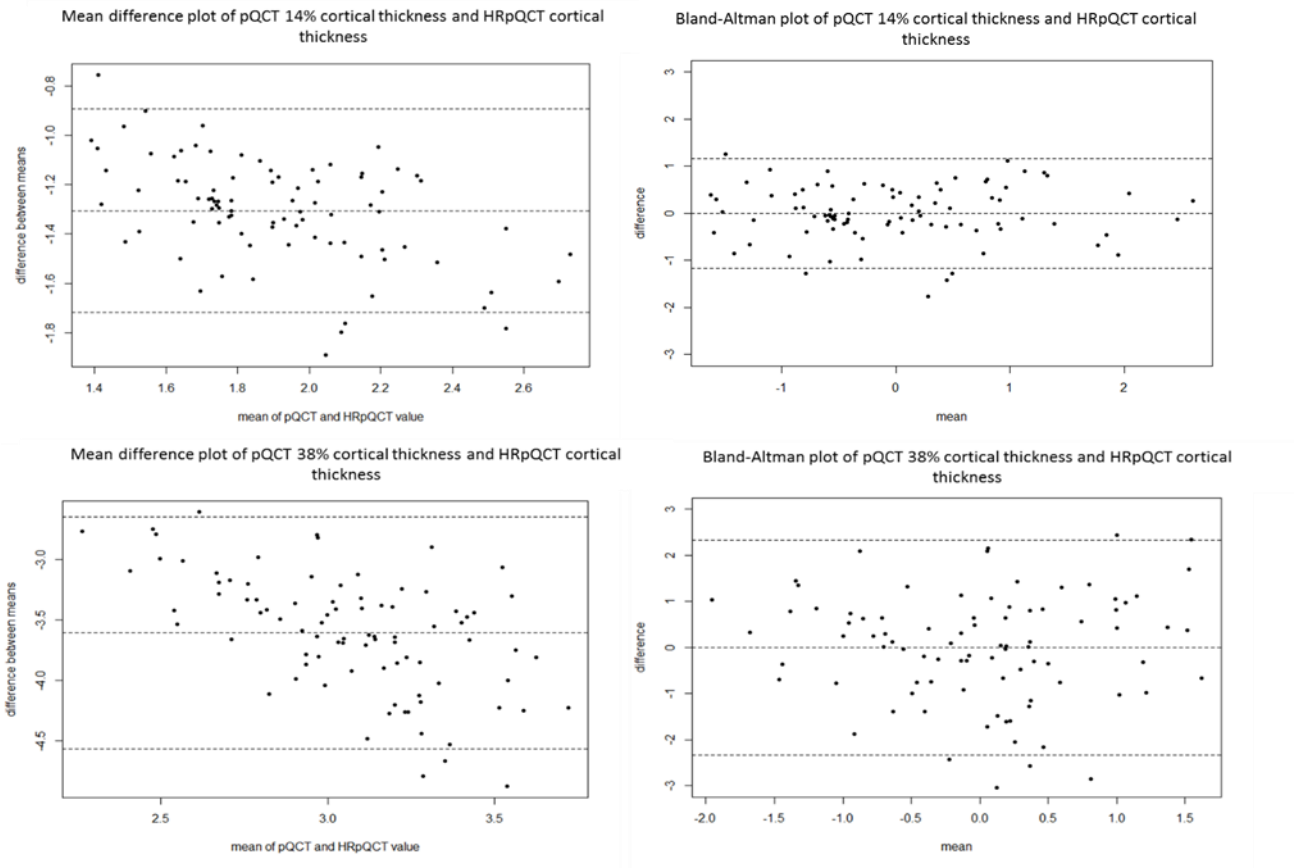

Figure 7.5 Agreement between cortical thickness from pQCT and HRpQCT.

These plots show better agreement at the 14% than the 38% tibia for cortical thickness compared to HRpQCT cortical thickness. The greater agreement seen at the 14% above is due to its greater similarities in location and structure to the distal region measured on the HRpQCT

7.4.2 Agreement between trabecular vBMD parameters at the tibia

As shown form the mean difference plots the HRpQCT trabecular vBMD and Dinn parameters were both lower consistently lower than pQCT trabecular vBMD, mean difference of -54.16 mg/cm^3 (95% CI: -24.32;-84.01) and -95.60 mg/cm^3 (-61.01;-130.18) respectively. When the data were standardised 95% of the data fell within 1SD of the mean suggesting reasonable agreement.

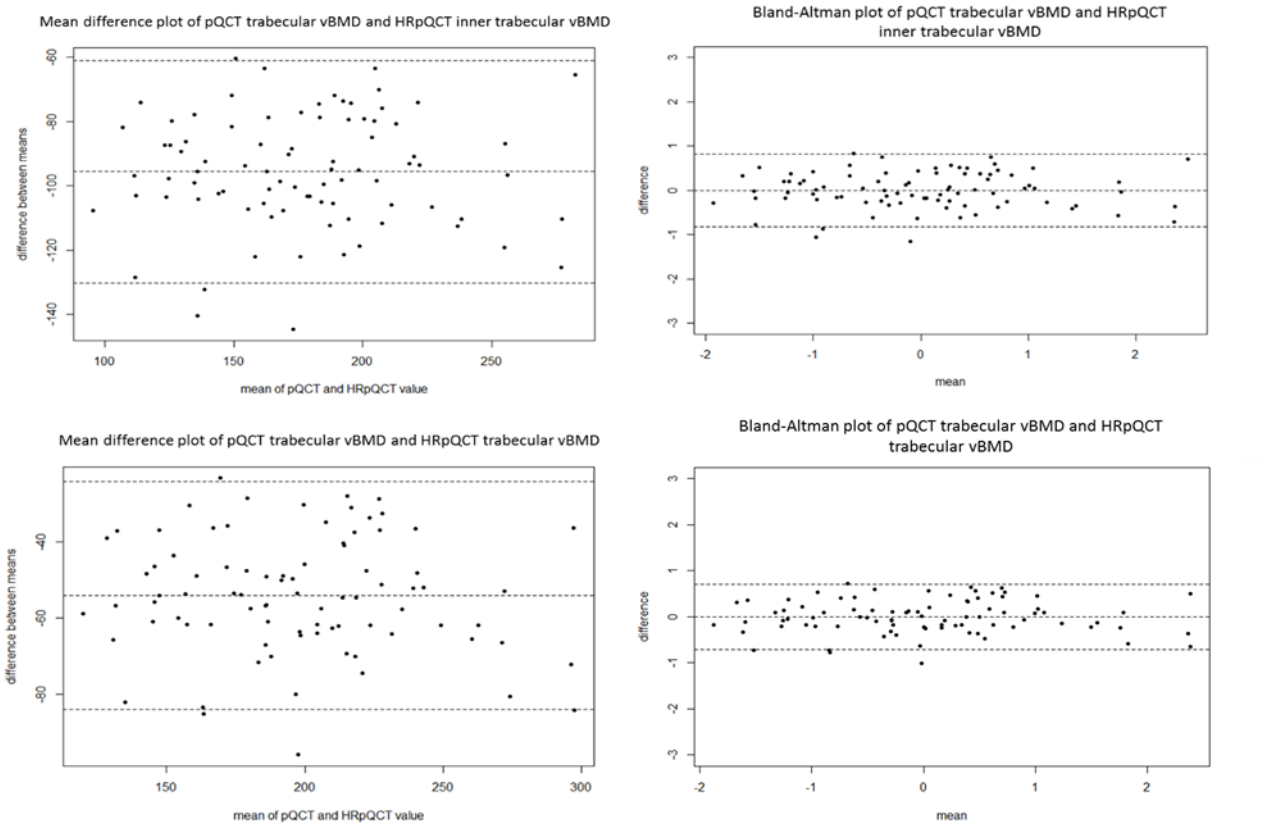

Figure 7.6 Agreement between parameters of trabecular vBMD from pQCT and HRpQCT

Bland-Altman plots show good agreement for pQCT measured trabecular vBMD and HRpQCT parameters of trabecular vBMD and Dinn. vBMD = volumetric bone mineral density, Dinn = Density of inner 60%

7.5 Discussion and conclusions

These These analyses formed a secondary objective of this thesis and explored the correlation and agreement between the two pQCT modalities in "typical use" due to the paucity of published in-vivo data. Both modalities are optimised using different approaches to provide biologically meaningful estimates of bone density, mass, shape, strength, geometry, and in the case of HRpQCT also microarchitecture. pQCT scanners have a relatively large voxel-size (0.5 mm) but this limitation can be, in part, overcome through strategic scan site selection and the application of appropriate segmentation and thresholding to produce compartment-specific data. Data are typically obtained from sites that are trabecular-rich (4%) or cortical-rich (14%, 38%, 50%, or 66% in the case of the tibia), although due to a lack of ISCD guidelines for pQCT many research groups use different sites. Although trabecular vBMD is obtained from a site containing an admixture of

cortical and trabecular bone, a trabecular only region is segmented from the total bone volume by defining an inner trabecular region (45% of total CSA). Cortical parameters are obtained at sites that predominantly contain cortical bone and appropriate thresholds can be applied to exclude non-cortical bone. A major advantage of focusing on cortical-rich sites to measure cortical parameters is that factors such as bone shape and geometry can be expanded on to provide indices of bone strength where the bone is under greatest torsional loading. In contrast HRpQCT parameters of cortical and trabecular bone are obtained simultaneously at a VOI containing a mixture of trabecular and cortical bone. First generation HRpQCT (XtremeCTI) does not image at proximal cortical-rich sites and therefore it is debatable whether this is a true reflection of how cortical bone reacts at sites where it is under greatest mechanical strain and loading forces, newer generation scanners offer an additional more proximal scan site addressing this limitation. HRpQCT scanning protocols had originally been set at a fixed distance from the end plate (unless the data were acquired in children), this meant that unlike pQCT there was much greater consistency in the region scanned. However, a limitation of this was that people have different sized limbs and using a fixed distance from the endplate of the radius and tibia may result in scans at sites of rather different composition and which can vary across genders, ages, and ethnicities (Ghasem-Zadeh et al., 2017). This has led to the development of protocols that, as with pQCT, a scan is performed at a predefined percentage of the length of the radius or tibia.

Similar between-group differences both in direction and magnitude were found in PABS for several bone parameters obtained with pQCT and HRpQCT from Model 2 and Model 3. In this chapter the observed high correlation and good agreement at baseline of several outcome measures from pQCT and HRpQCT helps contextualise such similarities. Ex-vivo work which has found very high correlation when the same site on a bone is scanned (Lala et al., 2012, Lala et al., 2014), in this analysis the highest correlation and best agreement between techniques was found at anatomically similar sites (similar proportion of cortical-to-trabecular bone). In-vivo interpretation is complicated as in "typical use" SOPs differ on how the site (% vs fixed mm) is defined from the

bone's endplate meaning there is some slight variation in the inter-individual correlation and agreement. This variation will be small and likely fall within the coefficient of variation (CV) and precision error (PE) of the inter-operator error. Despite these limitations this exploratory analysis is useful in illustrating, with the PABS longitudinal findings, the of the ability of pQCT to perform well in comparison to the much higher resolution HRpQCT when investigating change in bone outcome measures of trabecular vBMD and cortical thickness at physiologically similar sites. pQCT has the advantage of being less expensive, more mobile, and arguably more flexible than HRpQCT.

8 Summary and discussion of thesis results

The overarching aim of this thesis was to determine whether maternal bone mineral is mobilised from the appendicular skeleton during pregnancy. Prior to beginning ENID Bone data analysis or PABS study design I conducted a review (Chapter 2) of the available scientific literature to help form my research questions, which suggested that if pregnancy-induced bone mineral changes occurred they may not be detectable with bone densitometry techniques until late-gestation when Ca transfer to the fetus peaks. Both of my main analyses were constructed on this basis; of comparing data obtained from early- or mid- pregnancy when fetal Ca demands are low, to late-pregnancy when these demands peak with fetal Ca accrual (Kovacs and Kronenberg, 1997). Much of the available scientific literature on pregnancy, in particular the increases in BTMs of resorption, tend to align to a considerable extent with the much larger body of lactation data where mobilisation of maternal bone mineral has been consistently documented from trabecular-rich sites (Sowers et al., 1993, Cross et al., 1995, Laskey and Prentice, 1997, Kolthoff et al., 1998, Laskey et al., 1998, Laskey and Prentice, 1999, Akesson et al., 2004, Bjornerem et al., 2017).

Two major considerations: 1) timing, i.e. when during pregnancy and lactation bone mobilisation is occurring; and 2) compartment, i.e. whether from trabecular-rich skeletal sites alone or from axial-sites alone; shaped my primary research question and my approach to the analysis of the ENID pQCT data and the study design of PABS. Both studies presented in this thesis share a common overall aim using similar but distinct methodologies and both addressed my primary research question: is bone mineral mobilised from the maternal skeleton between early- and late pregnancy?

My secondary questions were chosen based on the contrasting nature of the discrete populations I studied, and would allow me to address what I had identified as major gaps in the available literature. The ENID Bone Study pQCT data offered me the opportunity to explore the maternal skeletal response to gestation in women with high parity (vs average parity in the UK) living in a

Sub-Saharan subsistence farming community with a habitually low Ca intake (Prentice et al., 1993, Prentice, 1994a, Redmond et al., 2014). With few exceptions most studies I encountered during my review were conducted in HIC in populations with what could be regarded as mid- to high-Ca intakes (Wisser et al., 2005, Olausson et al., 2008, Moller et al., 2012, Olausson et al., 2012, Moller et al., 2013). The ENID Bone Study was unparalleled in terms of sample size and although Ca intake data were not available for my analysis, all previous work in this population has shown intake to be habitually low (Prentice et al., 1993, Prentice, 1994a, Sawo et al., 2013). The inclusion criteria for PABS aimed to ensure a higher mean participant age than the majority of previously published pregnancy and bone studies in the light of rising maternal age in the UK and possible implications for recovery of bone loss. I aimed to explore the extent to which maternal bone mineral mobilisation may occur in women aged 30-45 years, this gave my study a much higher mean age than many previously published. To my knowledge this was also the first application of HRpQCT in pregnancy. This population were likely to be Ca replete, as previous studies have shown pregnant and lactating women in Cambridge to have a habitually high intake of Ca (Laskey et al., 1998, Olausson et al., 2008) in contrast to the Gambian women in ENIID Bone.

8.1 Primary thesis objective – trabecular bone

Trabecular bone is more metabolically active than cortical bone in part due to its structure, orientation and much greater surface area. During lactation, studies have consistently reported significant transient bone mineral mobilisation from trabecular-rich axial sites such as the hip and spine, while newer pQCT techniques have found trabecular microarchitectural change in the appendicular skeleton also (Kent et al., 1990, Drinkwater and Chesnut, 1991, Sowers et al., 1993, Prentice, 1994a, Kalkwarf and Specker, 1995, Kolthoff et al., 1998, Ritchie et al., 1998, Laskey and Prentice, 1999, Pearson et al., 2004, Bjornerem et al., 2017). My primary objective was to explore whether a similar pregnancy-induced bone mineral mobilisation could be observed in both studies using novel non-invasive pQCT techniques from mid- to late-pregnancy. In contrast to my

hypothesis, pregnancy-induced decreases in trabecular vBMD reflecting the potential mobilisation of Ca from the maternal skeleton were not observed in the ENID Bone pQCT analysis. To the contrary, small but statistically significant increases were found at both the radius and tibia in trabecular vBMD before adjusting for any potential supplement effects. With the addition of supplements to the model these small increases were no longer statistically significant. In contrast in PABS, significant decreases in trabecular vBMD were with pQCT in the pregnant group compared to the background aging of the NPNL group. Although decreases in HRpQCT trabecular vBMD and BV/TV were not found, this may be due to fewer data being available or more likely the relative anatomical differences between the pQCT and HRpQCT scan sites.

Interesting trabecular microarchitectural observations emerged in PABS a) cross-sectionally at baseline, and b) with data were modelled longitudinally. Statistically significant baseline differences were found between groups in trabecular microarchitectural parameters which could not be investigated further due to the study design but are somewhat supportive of the only histomorphology data published in pregnancy (Purdie et al., 1988, Shahtaheri et al., 1999) which suggested early-pregnancy changes in trabecular microarchitecture from iliac crest biopsies. My parsimonious models of change in HRpQCT found statistically significant between-group differences in trabecular number (increasing during pregnancy), trabecular separation (decreasing in pregnancy), and a trend towards decreasing trabecular thickness in pregnant women compared to NPNL. Without significant changes in trabecular vBMD, it is possible that microarchitectural differences reflect a potential redistribution of mineral within the trabecular compartment. However, it is also possible that these between-group differences could be an artefact of cortical mineral mobilisation: with the cortex becoming more porous and cortical thickness decreasing, enlarged pores close to the endocortical surface may be detected as thin struts of trabecular bone (Figure 6.10). This would artificially increase the mean trabecular number, while decreasing the mean trabecular thickness, essentially creating a "trabecularised" surface at the endosteal surface which at follow up is registered as part of the trabecular compartment (Figure 6.10).

The findings from both studies present a contrasting picture of the pregnancy-induced changes in the trabecular compartment of the appendicular skeleton between mid- to late-pregnancy. In ENID no decreases in trabecular vBMD were observed at either the distal tibia or radius with pQCT. In PABS, where pregnant and NPNL women aged 30-45 years old were scanned a significant decrease of 0.5 SD in trabecular vBMD (pQCT) was found in pregnant women exceeding possible age-effects at the distal tibia. HRpQCT microstructural trabecular change at the tibia may reflect cortical rather than trabecular adaptation, no decreases in trabecular vBMD or BV/TV were observed. At the radius no significant changes in the trabecular compartment were found with either technique at the forearm in contrast to Wisser et al. (2005).

8.2 Cortical compartment changes

The cortical compartment, in particular the endocortical surface, may act as a potential reservoir of Ca that can be drawn upon in times when physiological requirements are greatest. This hypothesis stems from the work of Stanley Garn and others who observed that during growth mineral accrued at the endosteal surface, which would not "strengthen" the bone compared to if that mineral was accrued in the periosteum (Frost, 1987, Schiessl et al., 1998, Schoenau et al., 2001, Ward et al., 2005b, Ward et al., 2005c, Wojtys et al., 2015), however, evidence of such change proved elusive in the metacarpals when assessed using radiographs (Frisancho et al., 1970, Frisancho et al., 1971, Greer and Garn, 1982). The seemingly "preferential" loss of bone mineral from the endosteal surface has been observed during menopause (Seeman, 2013). In the context of female reproduction and bone health, the findings from my research seem to support a preferential release of bone mineral from the endocortical surface of metaphyseal and diaphyseal sites during pregnancy, minimising the potential reduction in bone strength as bone total CSA does not decrease.

Statistically significant decreases in cortical vBMD and cortical thickness, with an increase in cortical porosity were observed with HRpQCT at the tibia compared to NPNL. As noted above

the changes observed in trabecular microarchitecture (increasing trabecular number and decreasing trabecular thickness) in the absence of a significant decrease in trabecular vBMD or BV/TV may indicate that these are an artefact of resorption from the endosteal surface at the tibial scan region rather than true trabecular adaptation. pQCT observations in PABS at the 33% proximal radius provide further evidence of pregnancy-induced endocortical resorption i.e. decreasing cortical and increasing endosteal circumference. The increase in cortical vBMD at the proximal radius can be explained by the loss of mineral at the endosteal surface, as voxels containing demineralised bone fall below the analysis threshold the remaining voxels contain bone tissue of higher attenuation and therefore density. In the absence of a change in either total CSA or periosteal circumference this can be viewed as the preferential mobilisation of bone mineral from a site within the cortex that would compromise bone strength the least. In contrast in ENID Bone small but statistically significant increases in cortical vBMD and BMC were found at both the radius and tibia, with minor supplement effects only found at the forearm.

8.3 Predictors of pregnancy-induced skeletal adaptation

In addition to my primary and secondary aims of determining whether maternal bone mineral was mobilised from the trabecular and cortical compartments of the skeleton, I also wanted to investigate whether any predictors of any such changes could be identified in both studies.

8.3.1 ENID Bone predictors

In the ENID Bone Study the analysis of pQCT bone data I had to control for the fact that antenatal supplementation was a major aspect of the ENID Trial. Potential mechanisms for maternal supplementation modulating any pregnancy-induced skeletal change were considered in the context of a) potential dietary Ca displacement in the two arms receiving LNS (i.e. 746 kcal addition energy and 20.6 g protein), or b) influencing maternal weight gain and modulating any potential skeletal response particularly in load-bearing bones. Additionally any potential modulation of

maternal weight gain across gestation may translate into differences in maternal body composition which may in turn influence several endocrine pathways. While I lack detailed figures of compliance in the ENID Trial, there were suggestions of variable compliance particularly in the LNS arms. As I did not have dietary intake data available to me for my pQCT analysis I cannot confirm whether supplement consumption actually displaced Ca from the maternal diet. However, what my analyses show is that no substantial supplement effects on maternal bone mineral were seen between Booking and P30. Although miniscule increases, all below 1%, in cortical vBMD and BMC were seen in all groups these are likely of negligible clinical importance at this stage of the lifecourse. However, if such increases were to occur across multiple pregnancies there could perhaps be some cumulative benefit as documented in one retrospective study in a population of American women with high parity (Specker and Binkley, 2005). When within-group weight change for the 4 supplement arms was plotted the distribution of the data were similar in all groups. I performed multiple regression also to investigate whether there was a supplement effect on maternal weight gain between Booking and P30 and found no supplement effect in this timeframe. In the ENID Bone study women aged 18-45 years old were recruited, however, only minor age effects were found at the proximal radius for cortical vBMD and cortical BMC when the when the data were modelled suggesting that in this population there is little variation in bone change as a result of maternal age or parity. In the context of very low dietary intake this may suggest that physiological adaptations are present that preserve maternal bone mineral during pregnancy.

8.3.2 PABS determinants of change in HRpQCT and pQCT outcomes

At the distal tibia where I found evidence of pregnancy-induced change in total vBMD, age was found to be a positive predictor of change ($p<0.05$). Bone mineral mobilisation was also observed at the 33% proximal radius through a thinning of the cortical shell. Maternal age was found to be a significant predictor of these changes where it was a negative predictor of cortical thickness and a positive predictor of endosteal circumference (both $p<0.05$). Although no between-group

differences were found at the distal radius with HRpQCT, age was a significant positive predictor of total vBMD and cortical thickness (both $p<0.05$).

While no significant between-group differences were found at either the 14% or 38% cortical-rich tibia, height was a significant positive predictor of several cortical parameters at the 38% distal tibia: cortical BMC, cortical CSA, and cortical thickness (all $p<0.05$). At the load-bearing tibia weight emerged as a significant predictor for a number of HRpQCT and pQCT bone outcome measures. While decreases in both total vBMD and cortical vBMD were found, baseline weight was found to be a positive predictor of total vBMD but a negative predictor of cortical vBMD (both $p<0.05$). As with HRpQCT cortical vBMD, baseline weight was a negative predictor of pQCT cortical vBMD at the 14% tibia ($p<0.01$). As seen at the tibia with HRpQCT baseline weight was found to be a significant negative predictor of cortical vBMD at the non- weight bearing radius ($p<0.05$). Baseline weight was also a positive predictor of trabecular number and a negative predictor of trabecular separation (both $p<0.05$).

At the tibia parity was only found to be a significant predictor of HRpQCT measured trabecular number where it was a negative predictor ($p<0.05$). This was also seen at the distal radius with HRpQCT ($p<0.05$) but parity was a positive predictor of trabecular separation ($p<0.05$). At the cortical rich 33% radius parity was found to be a negative predictor of cortical BMC ($p<0.05$), cortical CSA ($p<0.01$) and total CSA ($p<0.05$) suggesting that small cumulative loss may occur across multiple pregnancies.

The current or historical use of hormonal contraceptives was not found to be a significant predictor at any skeletal site although there were very few women who had never used any form of hormonal contraceptive in either of the groups (Pregnant n= 6, NPNL n= 3). I did not stratify by variety of contraceptive in the analysis in part due to the overlap of methods used. Smoking was a negative predictor of change in cortical subcortical vBMD at the distal tibia ($p<0.05$).

8.4 Correlation of pQCT and HRpQCT parameters at the tibia

My comparison of the scanning techniques in the baseline PABS data showed trabecular vBMD variables were highly correlated at the tibia, with good agreement found between the variables however they diverged at very high values. In contrast cortical vBMD showed poor to weak correlations between modalities, and poor agreement at all sites. Cortical thickness, however, had good correlation and better agreement between the HRpQCT VOI and the 14% pQCT site. The opposite was the case between the 38% pQCT site and the HRpQCT VOI. In the longitudinal PABS analysis conventional pQCT detected significant changes in total and cortical vBMD as did HRpQCT. These observations on both scanners also gives us greater confidence that if pregnancy-induced bone mineral mobilisation had occurred in the ENID Bone Study via decreases in volumetric BMD or cortical thickness they would have been detectable with pQCT which provides an effective method of acquiring 3D size-independent bone measures at physiologically optimal sites to measure both cortical and trabecular compartments.

8.5 Limitations

There are several limitations that should be addressed in the presentation of these data, I'll begin with limitations common to both projects. Both studies were constrained by practical considerations which dictated the stage of pregnancy when scan data were acquired. An ideal study design would include pre-pregnancy data collection where data would be obtained from a large number of women planning to become pregnant. Women who became pregnant, would have baseline pre-pregnancy data from which pregnancy-induced change could be calculated. This would make the investigation of first trimester changes in the maternal skeleton possible, of which there are some indications of in the literature. Those who did not conceive would be scanned longitudinally to account for any natural age-related bone changes. The greatest requirement for Ca by the fetus is in the second half of pregnancy, when skeletal growth and developments are at their greatest, by comparing maternal densitometry data collected in pregnancy when fetal

requirements are low and when they peak there would be the greatest chance of detecting maternal bone mineral mobilisation. Both of the studies presented here scanned into the third trimester of pregnancy. The ENID Bone Study was constrained by the time points of the ENID Trial, this tied the final pQCT time point during gestation to the P30 or 30 weeks pregnancy visit of the main trial. In the context of the lack of significant decreases in the maternal skeleton found in ENID Bone, it may be that by 30 weeks any changes in the maternal skeleton are not detectable with pQCT, conceivably in the absence of changes in vBMD and BMC microarchitectural redistribution could occur as seen in PABS. In PABS where the follow up visit occurred approximately 4-6 weeks later, at 34-36 weeks gestation, significant pregnancy-induced changes were observed with both pQCT and HRpQCT.

As discussed at length in Chapter 2, BTM do not present a consistent pattern of bone mineral homeorhesis during pregnancy but can with densitometry data provide further context. Both of my main analyses solely focus on densitometry outcomes without any complementary bone biochemistry or bone turnover markers, this was a pragmatic decision particularly in PABS where no bloods were obtained. In my ENID Bone analysis I did not have biochemistry and BTM data. In PABS BTM may have proved helpful at baseline where significant between-group differences in trabecular microarchitecture were detected but could not be explored further.

8.6 Conclusions

This thesis has presented novel pQCT data from two separate studies in contrasting populations of pregnant women, which suggest that there does not appear to be a uniform maternal skeletal response to pregnancy in the appendicular skeleton. These studies showed pregnancy-induced bone loss in women accustomed to high Ca intake, and in contrast a lack of bone mineral mobilisation in women with habitually low Ca intakes. Although I have not set out to compare these two very different groups of pregnant women, the contrast between the pregnancy-induced bone mineral mobilisation seen in PABS and the possible conservation of bone mineral seen in

ENID is intriguing. Although we observed some negligible supplement-modulated increases in cortical bone parameters in ENID Bone, the most striking finding in these rural Gambian women was the complete lack of bone mineral mobilisation between mid- to late- pregnancy. These data suggest that fetal Ca demand is satisfied through physiological adaptations other than maternal bone mineral mobilisation from the appendicular skeleton.

In PABS, the release of bone mineral from well-nourished women aged 30-45 years was found at both the tibia and radius, however, this varied by site. At the distal tibia, decreases in total and trabecular vBMD were found with pQCT, however HRpQCT found no evidence of trabecular vBMD falling at the slightly more proximal scan VOI. Despite this, significant changes in trabecular microarchitecture were found which support mobilisation but may likely be artefacts of the changes in taking place within the cortex as evidenced by decreasing total vBMD, cortical vBMD, and cortical thickness and the increase in cortical porosity at the tibia. Similar cortical changes were not found in cortical vBMD or thickness with pQCT at the proximal tibia. In contrast to the changes found at the distal tibia, no changes were found at the distal forearm which may suggest some potential conservation of bone mineral at a more fracture prone site. Evidence of bone mineral mobilisation from the proximal radius took the form of decreasing cortical thickness coupled with an increasing endosteal circumference, which explains the increase in cortical vBMD.

The clinical significance of these findings is as of yet unclear but will largely depend on the reversal of any mineral losses post-lactation (if they breastfeed) and prior to the beginning of menopause. The extent to which cumulative cycles of pregnancy and lactation may interact with the maternal skeleton remain unclear, however, a failure to replenish mobilised mineral may be detrimental to future bone health. While my research has focused on bone mineral changes across a single pregnancy many women will go through more than one cycle of pregnancy and lactation. Further research is required to determine potential pregnancy-induced skeletal adaptation in women at different stages of the lifecource, of difference ethnicities, and with different environmental exposures.

8.7 Thesis outputs

8.7.1 Awards

Bone Research Society Conference Winchester 2018: Best Oral Presentation

"Pregnancy induced changes in bone microarchitecture and density at the tibia as determined by single-slice and high-resolution peripheral Quantitative Computed Tomography."

8.7.2 Peer-reviewed abstracts

Bone Research Society Conference Winchester 2018: Oral presentation

"Pregnancy induced changes in bone microarchitecture and density at the tibia as determined by single-slice and high-resolution peripheral Quantitative Computed Tomography."

Bone Research Society Conference Winchester 2018: Poster

"In-vivo correlation of single-slice peripheral Quantitative Computed Tomography (pQCT) and high resolution pQCT for volumetric bone mineral density and geometry at the tibia."

Nutrition Society Summer Conference Leeds 2018: Poster presentation

"Changes in maternal volumetric bone mineral density in the peripheral skeleton during pregnancy as determined by single-slice peripheral Quantitative Computed Tomography."

Nutrition Society Summer Conference London 2017: Poster presentation

"Maternal bone mineral changes during pregnancy as measured by peripheral QCT in rural Gambian women with a habitually low calcium intake."

Pregnancy induced changes in bone microarchitecture and density at the tibia as determined by single-slice and high-resolution peripheral Quantitative Computed Tomography. Mícheál Ó Breasail[1], Ann Prentice[1], Kate Ward[1,2]

[1]MRC Elsie Widdowson Laboratory, [2]MRC Lifecourse Epidemiology Unit

Objectives

The newborn skeleton contains 25-30g of calcium, although the source of this has not been fully qualified. Evidence from DXA studies before and shortly after pregnancy suggests that some of this calcium may originate from the maternal skeleton. The aim of this study was to use single- slice and high-resolution peripheral quantitative computer tomography (pQCT, HRpQCT) to characterise whether pregnancy-induced changes occur in the appendicular skeleton.

Methods

Pregnant women were scanned twice, at 14-16 and 34–36 weeks gestation. Premenopausal controls were also scanned approximately 20 weeks apart. pQCT scans (XCT 2000L) were obtained at the distal (4%) and proximal (14,38%) sites of the non-dominant tibia. HRpQCT (XtremeCTI) scans of the distal tibia were also obtained. Outcomes were: distal total and trabecular vBMD, total cross-sectional area (CSA), plus for HRpQCT distal cortical vBMD and thickness, trabecular number and thickness; pQCT proximal outcomes were cortical vBMD, cortical CSA, cortical thickness, and total CSA. Linear regression models were fitted, and the model of best fit selected; covariates were group, height, change in weight, baseline age, time between visits, parity, smoking history, oral contraceptive use, group*time difference. Groups differences are presented as mean (SE).

Results

Ninety (53 pregnant) women, mean(SD) age 35.4(3.8)years, were recruited. No significant differences were found between groups at baseline except trabecular thickness which was lower in the pregnant group ($p<0.05$). At follow up pregnant women had significantly lower distal total vBMD (-1.9(0.6)%); proximal cortical vBMD (14%-0.9(0.2)%), 38%-0.5(0.2)%). HRpQCT distal total (-1.8(0.5)%) and cortical vBMD (-1.8 (0.5)%) were lower in pregnant women, as were distal cortical and trabecular thickness (-2.3(0.5)%, -3.9(1.9)% respectively). Trabecular number was greater (3.6(1.8)%) in pregnant women.

Conclusion

During pregnancy bone mineral is mobilised predominantly from the cortical compartment of the distal tibia. These data also suggest there may be early pregnancy changes in trabecular bone which have previously been reported in one study of bone histomorphology.

In-vivo correlation of single-slice peripheral Quantitative Computed Tomography (pQCT) and high resolution pQCT measures at the tibia. Mícheál Ó Breasail[1], Ann Prentice[1], Kate Ward[1,2]

[1]MRC Elsie Widdowson Laboratory, [2]MRC Lifecourse Epidemiology Unit

Objectives

Single-slice pQCT (pQCT) and high-resolution pQCT (HRpQCT) are available for in-vivo measurements of cortical (Ct) and trabecular (Tb) compartments. These techniques measure different sites, single slice pQCT at any site along the bone and first generation HRpQCT at distal sites. The aim was to explore the agreement between the methods for measures of cortical vBMD, cortical thickness, and trabecular vBMD.

Methods

The non-dominant tibiae of 76 healthy premenopausal women (mean(SD) age 35.4(3.6) years) were measured. Outcomes were: pQCT (XCT 2000L, voxel size 0.5mm) 4% Tb-vBMD, 14% and 38% Ct-vBMD and thickness; HRpQCT (XtremeCTI, voxel size 0.82μm) a 9.02mm volume of interest (110 contiguous slices) at the distal tibia (Tb-vBMD, Dinner (Dinn), Ct-vBMD and thickness) Correlations between comparable parameters were obtained, and Bland-Altman (BA) plots used to assess the agreement between the two methods and to detect systematic bias.

Results

Ct-vBMD measures on pQCT and HRpQCT were poor-fairly correlated (R2=0.17, 0.37 for 38% and 14% tibia respectively). There was good correlation between cortical thickness at 14% pQCT site and HRpQCT (R2=0.68) but not at the 38% tibia (R2=0.09). BA plots showed increasing systematic difference at both sites with increasing cortical thickness. There were high correlations between Tb-vBMD by pQCT and both HRpQCT Tb-vBMD and Dinn (R2 = 0.87, 0.83 respectively). There was good agreement between the Tb outcomes; pQCT Tb-vBMD was lower than both HRpQCT Tb measures mean(95%CI) difference of Tb-vBMD 54 mg/cm^3 (-26;-82 mg/cm^3) and Dinn 95 mg/cm^3 (-62;-128 mg/cm^3).

Conclusion

Correlations between cortical vBMD and thickness measured at the proximal (38% pQCT site) and distal (HRpQCT) tibia are weak. However cortical measures at the 14% and distal site agreed well. Trabecular measures agreed well but cannot be interchanged. To understand cortical bone adaptations to environment, a proximal site should be used.

Changes in maternal volumetric bone mineral density in the peripheral skeleton during pregnancy as determined by single-slice peripheral Quantitative Computed Tomography.

By M. Ó Breasail[1], A. Prentice[1] and K. Ward[1,2], [1]Medical Research Council Elsie Widdowson Laboratory (MRC EWL), CB1 9NL and [2]Medical Research Council Lifecourse Epidemiology Unit (MRC LEU), University of Southampton, SO16 6YD

At birth the fetus contains 25-30 g of calcium (Ca), although whether the maternal skeletal stores of calcium are a source (in addition to increased intestinal absorption and reduced excretion) is not fully known. There is limited evidence from DXA studies before and shortly after pregnancy suggesting some mobilisation of maternal Ca stores (1, 2); such mobilisation has been consistently observed during lactation (1, 3-5). Peripheral QCT scanners measure volumetric bone mineral density (vBMD) and geometry in the appendicular skeleton. To determine if pregnancy induced bone mineral changes occur in the appendicular skeleton we scanned pregnant women at the distal tibia and radius at two time points during pregnancy (14-16 weeks and 34 – 36 weeks). Healthy premenopausal non-pregnant non-lactating controls were also scanned with a similar length between visits.

Data were obtained from 90 (53 pregnant) healthy premenopausal women (mean age 35.4 (SD 3.8) years). Scans with pQCT (XCT 2000L, Stratec) were obtained at 4% of the length of the lower leg and forearm. Independent t-tests tested for unadjusted differences between change in vBMD. Linear regression models were then fitted for total and trabecular vBMD and total cross-sectional area (CSA) at the radius and tibia with the terms: (1) height, baseline weight, group and (2) model 1 plus age, weight change, parity, ever smoked and previous contraception use. In each case the model of best fit was selected using backwards stepwise regression for each outcome measure of interest. Data are presented from model 2.

At baseline there were no statistically significant difference between the two groups ($p < 0.05$). Between visits the pregnant participants gained 9 (SD 3.4) kg. There were significant decreases in the pregnant group compared to non-pregnant controls of: total vBMD 1.8% (SE 0.9) ($P < 0.001$) and in trabecular vBMD 1.4% (SE 0.5) ($P < 0.001$). These decreases exceed the precision error of the scanning technique. No significant changes in tibia total CSA. Testing at the distal radius found no statistically significant changes during pregnancy in any of the measured parameters of interest. During pregnancy bone mineral is mobilised from the distal tibia, a trabecular rich site. In contrast there appeared to be conservation of mineral at the distal radius during pregnancy. The basis for the site differences requires further investigation but is consistent with previous DXA studies.

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Maternal bone mineral changes during pregnancy as measured by peripheral QCT in rural Gambian women with a habitually low calcium intake. By M. Ó Breasail[1], S. Schoenbuchner[1], L. Jarjou[2], S.E. Moore[3], A. Prentice[1,2], and K.A. Ward[1,4], [1]MRC EWL, 120 Fulbourn Road, Cambridge, CB1 9NL, UK; [2]MRC Keneba, MRC The Gambia Unit, The Gambia; [3]Division of Women's Health, Kings College London SE1 7EH, [4]MRC Lifecourse Epidemiology, University of Southampton, Tremona Rd, Southampton SO16 6YD

Evidence from studies of pre- and post-pregnancy bone densitometry or bone turnover markers suggest maternal bone mineral is mobilised to satisfy the calcium demands of the fetus(1), which at birth contains 25 - 30 g Ca. There are now techniques available that can be used during pregnancy to measure changes in the maternal skeleton. We aimed to quantify these changes in a supplementation trial (The Early Nutrition and Immune Development (ENID) trial) that randomized pregnant women before 20 weeks gestation to receive one of 4 supplements daily until term(2). The supplement groups were: control receiving 60 mg iron and 400 μg folate as per standard Gambian guidelines, multi-micronutrient (MMN: 15 micronutrients at twice RDA), protein energy (PE: 746 kcal and 20.8g protein) or combined PEMMN(2). It was hypothesised that there would be a significant decrease in maternal BMD and structure between early and late pregnancy. We used peripheral QCT to describe these changes and to explore whether supplement type predicted change in bone outcomes.

814 pregnant rural Gambians aged 18-45 years (mean [SD] 29.6 [6.7] years, median parity 4 (IQR 2 - 6)) were included in this study. Anthropometric and pQCT data at the distal and proximal radius and tibia were obtained at a pre-intervention "booking" visit (mean [SD] 13 [3] wks), week 20, and week 30; (only change between booking and P30 will be described). These pQCT measures were for distal sites: total and trabecular vBMD, total CSA; for proximal sites: total CSA, cortical vBMD, cortical content (BMC), cortical CSA, cortical thickness, and stress-strain index (SSI), an estimate of bone torsional strength. A paired samples t-test was used to determine if change between booking and P30 was statistically significant. Linear regression models tested whether supplement was a significant predictor of change between booking and P30.

No significant differences were observed between booking and P30 in the whole cohort for total vBMD, trabecular vBMD, and total CSA at the either the distal radius or tibia or in total CSA, cortical vBMD, cortical thickness, or SSI at the proximal radius. At the proximal radius there was a small but significant increase in BMC of 0.4% (SE 0.2%) between baseline and P30 in the whole cohort ($p<0.05$). Cortical vBMD also increased by 0.2% (SE 0.1%) ($p=0.07$). A similar pattern was observed at the proximal tibia where increases occurred in parameters of cortical bone, BMC, cortical CSA, and SSI, ranging from 0.1-0.6% (all $p<0.05$). Cortical vBMD also increased (0.2% SE 0.1%), but was not statistically significant ($p=0.09$). Supplement type predicted change at the proximal radius where PE supplementation was a significant negative predictor of cortical BMC ($p<0.05$) and of cortical vBMD ($p<0.001$). In contrast, PEMMN supplementation was a significant positive predictor of cortical vBMD ($p<0.001$) at the proximal radius. Supplement group was not a predictor of change in any of the outcomes at the distal tibia.

These findings suggest that during pregnancy there is no evidence of bone loss from the trabecular-rich distal radius or tibia and that small increases in cortical bone mineral may occur at the proximal radius and tibia that might be modified by supplementation. The lack of substantial change is somewhat surprising as in lactation release of bone mineral from trabecular sites has been observed consistently and was hypothesised to be the source of bone mineral during pregnancy. Mobilisation of bone mineral may occur in pregnancy through other mechanisms such as changes in maternal trabecular microarchitecture or cortical porosity, however, these are not detectable with pQCT due to limitations of resolution. These may be overcome with the use of more advanced modalities such as high resolution-pQCT.

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Appendix A: Training

Training undertaken during PhD

Courses and workshops attended	
European Calcified Tissue Society PhD training course 2016	Sienna, Italy
The Sub-Saharan African MuSculOskeletal Network Training Workshop 2018	Harare, Zimbabwe
Good Clinical Practice	Cambridge, UK
The Ionising Radiation (Medical Exposure) Regulations (IR(ME)R) 2000	Online
Human Tissue Act	Cambridge, UK
Good Clinical Practice (GCP)	Cambridge, UK
General Data Protection Regulation (GDPR) training 2018	Online
An Introduction to R: Software For Statistical Analysis	Cambridge, UK
An Introduction to Solving Biological Problems with R	Cambridge, UK
R: Introduction for Beginners	Cambridge, UK

Training of staff and students

During my PhD I was actively involved in supporting several other studies, which in addition to performing scans including the training of staff and students at MRC EWL, and colleagues in The Gambia and Zimbabwe. I continue to provide technical pQCT support to scanning facilities in The Gambia, Zimbabwe, and South Africa.

Training of staff and students at MRC EWL, Cambridge, UK	
Carla Greenwood (Placement Student)	pQCT scan grading
Lauren Oliver (Research Assistant)	pQCT scan grading and image analysis
Dominique Taylor (Placement Student)	pQCT scan acquisition, pQCT scan grading, HRpQCT scan analysis
Rebecca McGrath (Research Assistant)	pQCT scan acquisition, pQCT scan grading, pQCT scan analysis, HRpQCT scan acquisition
Matthew Harvey (Physician's Assistant)	pQCT scan acquisition, HRpQCT scan acquisition
Sophie Yelland (Placement Student)	HRpQCT scan grading and analysis
Elise Orford	pQCT scan acquisition, pQCT scan grading, pQCT scan analysis, HRpQCT scan acquisition, DXA scan acquisition
Training of staff at MRC The Gambia, Keneba, The Gambia	
Michael Mendy	Musculoskeletal functional tests training
Mustapha Ceesay	Musculoskeletal functional tests training
Set up of XCT 2000 system and training of staff at Parirenyatwa Hospital, Harare, Zimbabwe	
Radiographers employed by the University of Zimbabwe/Parirenyatwa Hospital	pQCT scan acquisition, pQCT scan grading, pQCT data analysis and extraction, Standard operating procedure design

Appendix B: ENID Bone Study documents

ENID Bone Participant Information Sheet

Medical Research Council Laboratories
MRC Keneba Field Station

ENID Bone add-on

SCC/EC Number L2009.66 Version 1 (September 2009)

Information and consent form

To be translated by the fieldworker into a language understood by the subject

Purpose of the study

The Calcium, Vitamin D and Bone Health (CDBH) team at MRC Keneba would like to study the women and their babies who are taking part in the 'ENID' study. We would like to add-on to this study because we would like to understand more about what happens to the bones of Gambian women before during and after pregnancy, and the growth of infants and children, and how this affected by different kinds of nutrition.

Before you decide to participate in this add-on study it is important for you to understand what will be involved. Please take time to read or listen to this carefully and discuss it with others if you wish. If you have any questions, please ask. It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

The Gambia Government / MRC Ethics Committee have approved this study. All information which is collected during the course of this study will be kept strictly confidential.

What does participation in the study mean for you and your infant ?

The CDBH team running this study will be working closely with the investigators running the main ENID study. We will share some of the information and samples they collect from you during pregnancy, after labour, and later from you and your baby. In addition to this information and samples we would like to add the following:

Before you become pregnant

We would like to measure you before you become pregnant and whilst you are not breastfeeding. The member of the project team who asks you monthly questions about your periods will tell us if you are not pregnant. We will then invite you to come to MRC Keneba to have your bones scanned by DXA (whole-body, hip, spine and forearm) and pQCT (forearm and lower leg) and collect a small blood sample (about 2 teaspoonsful). We will also measure your weight and height, waist and hip circumference and body composition in the same way as in the main ENID study. After you return home a fieldworker will arrange to visit you to collect a 24 hour urine sample and do 2-day dietary and complete a calcium intake questionnaire.

When you are pregnant

In addition to the measurements made for the main ENID study we will ask you to collect a 24 hour urine sample at home at 12 and 30 weeks of pregnancy and we will do dietary measurements. We would also like to collect some additional blood (approximately an extra teaspoonful); this will be done at the same time as the main ENID measurements and so will not need an extra blood sample (blood draw). We would also like to do pQCT measurements of your forearm and lower leg.

After your baby is born

When your baby is 1 week old - when you visit MRC Keneba, in addition to the measurements made for the main ENID study, measure your weight and height, waist and hip circumference and body composition. We would also like to collect a small blood sample (about 2 teaspoonsful), and do DXA and pQCT scans of your bones (the same as before pregnancy), and a whole-body DXA scan of your baby. We will ask you to collect a 24 hour urine sample when you return home and a fieldworker will do dietary.

When your baby is 12 weeks old -when you visit MRC Keneba, in addition to the measurements made for the main ENID study we would like to collect a small blood sample (about 2 teaspoonsful), and ask you to collect a small additional amount of breast milk. We will use some of the blood collected from your infant but not take an additional amount. We would also like to do DXA and pQCT scans of your bones (the same as before pregnancy and), and a whole-body DXA scan of your baby. We will ask you to collect a 24 hour urine sample when you return home and a fieldworker will do dietary.

Your participation in this add-on study and/or that of your child is entirely voluntary and if you wish not to take part in the study after signing the consent form you can discontinue at

any stage. This will not affect your participation in the main ENID study and will not affect the health care the MRC Keneba provides you or to your child.

Potential risks and benefits

There are minimal risks associated with participation in this add-on study. The DXA and pQCT tests involve exposure to some additional radiation. We cannot avoid being exposed to natural radiation from the air and soils that surround us. This is called background radiation. The additional radiation that you will receive from the DXA and pQCT tests on any one occasion is less than the amount you receive from background radiation on one day. The additional radiation that your baby will receive from a DXA scan on any one occasion is approximately the same as they receive from background on one day. The pQCT measurements during pregnancy involve arm and leg measurements of the mother only and so the baby will not be in the x ray beam when the scans are made.

ENID Bone Study Consent Form

Medical Research Council Laboratories
MRC Keneba Field Station

ENID Bone add-on

SCC/EC Number L2009.66 Version 1 (September 2009)

Consent

This explanation of the study was given by:.....

Date: |____|_____|____|____|_____|_____| Village:.....

The information sheet has been read to me and I understand it /I have read and understood the information sheet.

I understand what participation in this study means for me and my infant.

I understand that the information regarding me and when I have a baby that is collected in the course of this study will remain confidential.

I understand that the Calcium, Vitamin D and Bone Health (CDBH) investigators running this study will use some of the results and samples collected in the main ENID study.

I understand the CDBH investigators running this study will be doing additional laboratory tests on the blood, breast milk and placental samples from me and my infant, and that some of these samples will be sent to the UK and stored for laboratory testing.

I understand that I am free to take part in the study or refuse, and that I can withdraw either myself or my infant from the study at any time, and without giving any reason. Deciding not to take part or to withdraw from the study will not affect my participation in the main ENID study or affect the care that I or any of my family are normally entitled to .

I have had a chance to ask questions and have them answered.

This form has been read by /
I have read the above to _____ (Name of volunteer)

in a language that she understands. I believe that she has understood what I explained and that she has freely agreed to take part in the study.

I agree to participate in this study. _____

[Signature or thumb print of volunteer]

Name of field worker: ----------

Signature of field worker: _____

Appendix C: ENID Bone pQCT Study supplementary material

Baseline descriptive statistics for all participants

Baseline descriptive statistics at Booking for the 4 supplement groups in the ENID Bone Study, mean (SD). Between- group baseline differences were tested by ANOVA with posthoc Tukey Test

	FeFol	MMN	PE	PEMMN
4% Distal Radius	n = 171	n = 170	n = 171	n = 171
Season ratio (wet/dry)	89/82	86/85	86/85	86/84
Total vBMD (mg/cm^3)	317.39 (42.44)	322.61 (45.91)	320.71 (44.48)	318.33 (44.08)
Trabecular vBMD (mg/cm^3)	159.30 (33.84)	162.37 (36.46)	160.81 (36.01)	158.60 (36.13)
Total CSA (mm)	337.88 (40.85)	333.74 (39.91)	340.60 (43.82)	334.36 (44.86)
33% Proximal Radius	n = 171	n = 173	n = 171	n = 170
Season ratio (wet/dry)	90/81	88/85	87/84	83/87
Total CSA	120.31 (16.95)	120.12 (16.38)	122.19 (17.14)	118.98 (16.75)
Cortical vBMD (mg/cm^3)	1239.57 (39.15)	1243.61 (39.97)	1241.13 (36.86)	1240.02 (37.08)
Cortical BMC (mg/mm)	93.51 (11.54)	94.34 (10.61)	95.26 (11.11)	92.75 (10.47)
Cortical CSA (mm^2)	75.42 (8.80)	75.83 (7.91)	76.72 (8.37)	74.80 (8.14)
Cortical Thickness (mm)	2.42 (0.23)	2.45 (0.22)	2.45 (0.25)	2.42 (0.23)
SSI (mm^3)	228.01 (45.82)	232.80 (44.86)	231.82 (47.51)	225.55 (42.81)
4% Distal Tibia	n = 156	n = 159	n = 147	n = 159
Season ratio (wet/dry)	83/73	80/79	79/68	81/78
Total vBMD (mg/cm^3)	282.19 (37.62)	283.78 (34.93)	286.50 (35.20)	280.97 (34.76)
Trabecular vBMD (mg/cm^3)	190.55 (33.15)	191.85 (27.89)	193.75 (30.81)	189.97 (29.24)
Total CSA (mm^2)	949.95 (109.66)	941.18 (106.73)	948.02 (110.54)	938.06 (114.70)
38% Proximal Tibia	n = 155	n = 162	n = 147	n = 158
Season ratio (wet/dry)	83/72	82/80	79/68	81/77
Total CSA (mm^2)	382.98 (52.55)	379.03 (50.45)	380.06 (44.15)	374.85 (48.76)
Cortical vBMD (mg/cm^3)	1233.50 (37.73)	1233.48 (38.54)	1231.07 (38.64)	1231.44 (35.35)
Cortical BMC (mg/mm)	280.86 (37.63)	281.31 (37.97)	283.43 (35.12)	275.69 (32.72)
Cortical CSA (mm^2)	227.63 (29.17)	227.93 (28.76)	230.04 (26.03)	223.72 (24.28)
Cortical Thickness (mm)	4.03 (0.44)	4.07 (0.45)	4.12 (0.47)	4.01 (0.39)

*p<0.05 FeFol = iron folate, MMN = multiple micronutrients, PE = protein energy, PEMMN = protein energy multiple micronutrients, vBMD = volumetric bone mineral density, CSA = cross sectional area, SSI = stress strain index. Season coded as Wet: July-Oct, Dry: Nov-June

Proportion of participants scanned/supplemented by season of Booking visit

The following three tables are provided to illustrate the roughly even proportion of pregnant Gambians:

1. At Booking in Wet and Dry season;
2. Supplement by Booking season;
3. Scanned on the XCT2000/2000L by Booking season.

Proportion of scans at each site by season at Booking

	Distal radius	Proximal Radius	Distal Tibia	Proximal Tibia
Wet Season	347	348	323	325
Dry Season	336	337	298	297

Season coded as Wet: July-Oct, Dry: Nov-June

Proportion of participants in each supplement arm by Booking season.

	Distal radius				Proximal Radius				Distal Tibia				Proximal Tibia			
SEASON	PE	PEMMN	FEFOL	MMN	PE	PEMMN	FEFOL	MMN	PE	PEMMN	FEFOL	MMN	PE	PEMMN	FEFOL	MMN
Wet	86	86	89	86	87	83	90	88	79	81	83	80	79	81	83	82
Dry	85	84	82	85	84	87	81	85	68	78	73	79	68	77	72	80

Season coded as Wet: July-Oct, Dry: Nov-June

Proportion of participants scanned on the XCT2000/2000L by Booking season.

	Radius distal scan/proximal scan		Tibia distal scan/proximal scan	
	XCT2000	XCT2000L	XCT2000	XCT2000L
Wet	137/143	206/203	127/128	196/197
Dry	154/158	175/172	128/129	167/165

Season coded as Wet: July-Oct, Dry: Nov-June

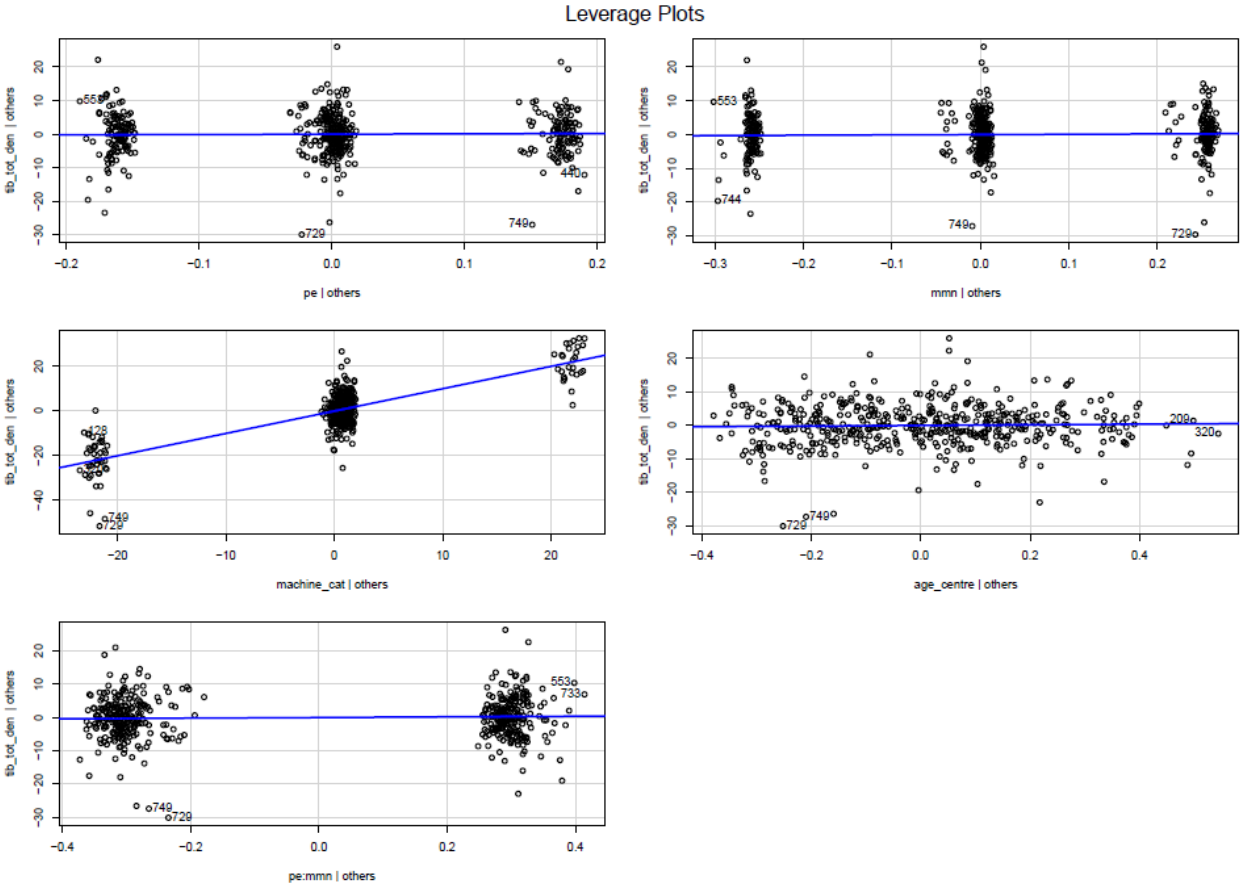
Absolute change between Booking and P30 for women who did not change scanner

Baseline pQCT bone outcome measures (Booking) for the all women in the ENID Bone Study who did not change scanners between Booking and P30, mean (SD).

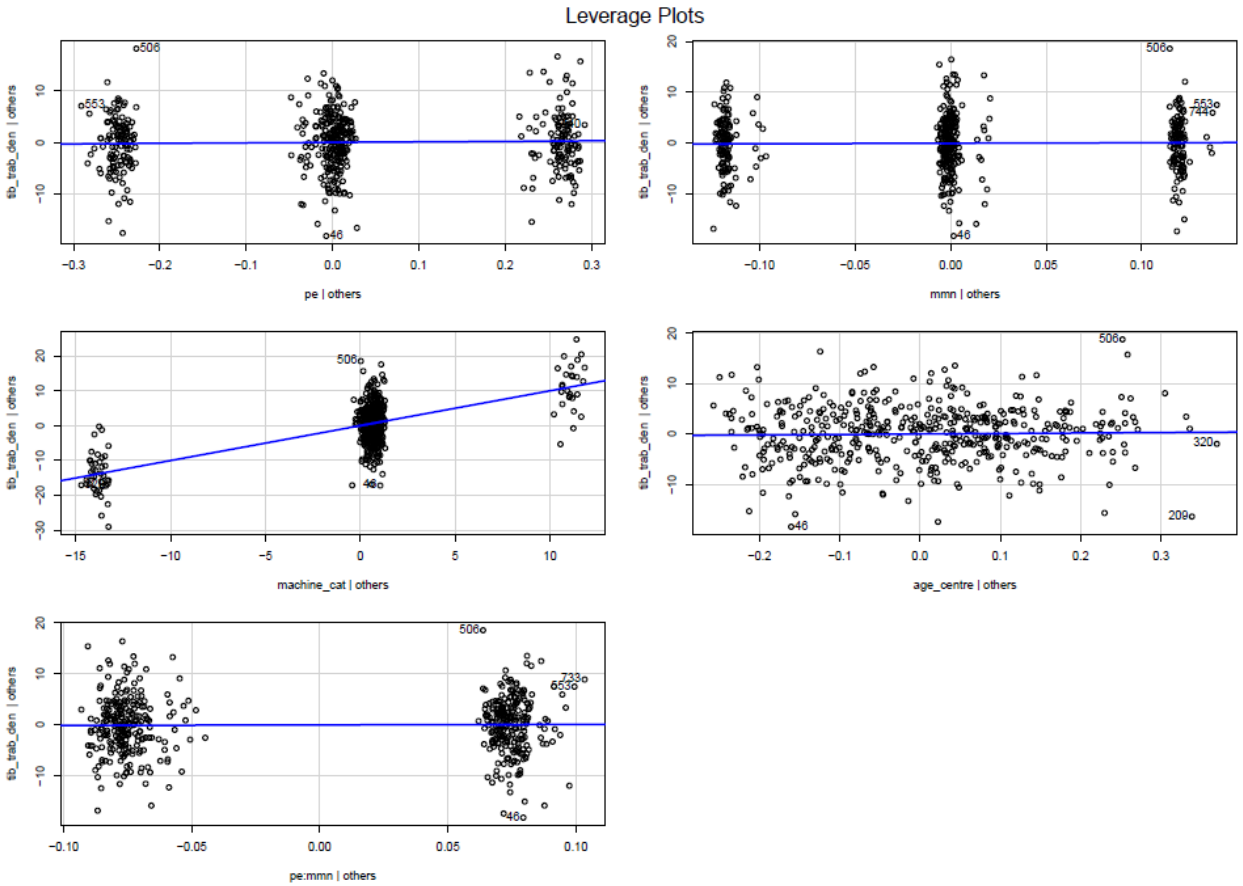
	Booking	Change by P30
4% Radius	n=435	
Total vBMD (mg/cm^3)	319.23 (44.98)	0.89 (16.81)
Trabecular vBMD (mg/cm^3)	160.51 (35.63)	1.52 (9.03)*
Total CSA (mm)	335.94 (42.09)	1.91 (21.72)
33% Radius	n=440	
Total CSA (mm^2)	119.82 (16.12)	-0.33 (11.00)
Cortical vBMD (mg/cm^3)	1239.66 (35.17)	5.09 (22.44)*
Cortical BMC (mg/mm)	94.05 (10.77)	0.46 (2.13)*
Cortical CSA (mm^2)	75.84 (8.17)	0.06 (1.67)
Cortical Thickness (mm)	2.45 (0.23)	0.01 (0.17)
4% Tibia	n=407	
Total vBMD (mg/cm^3)	281.86 (36.08)	0.91 (5.74)
Trabecular vBMD (mg/cm^3)	190.49 (31.19)	0.77 (5.43)
Total CSA (mm^2)	945.65 (112.59)	-1.19 (25.34)
38% Tibia	n=409	
Total CSA (mm^2)	377.60 (47.95)	0.69 (11.67)
Cortical vBMD (mg/cm^3)	1230.23 (35.20)	4.38 (11.25)
Cortical BMC (mg/mm)	280.01 (34.20)	1.52 (4.02)*
Cortical CSA (mm^2)	227.52 (26.26)	0.42 (2.51)*
Cortical Thickness (mm)	4.07 (0.41)	0.00 (0.11)

*p<0.05 Independent T-test change vs zero, vBMD = volumetric bone mineral density, CSA = cross sectional area

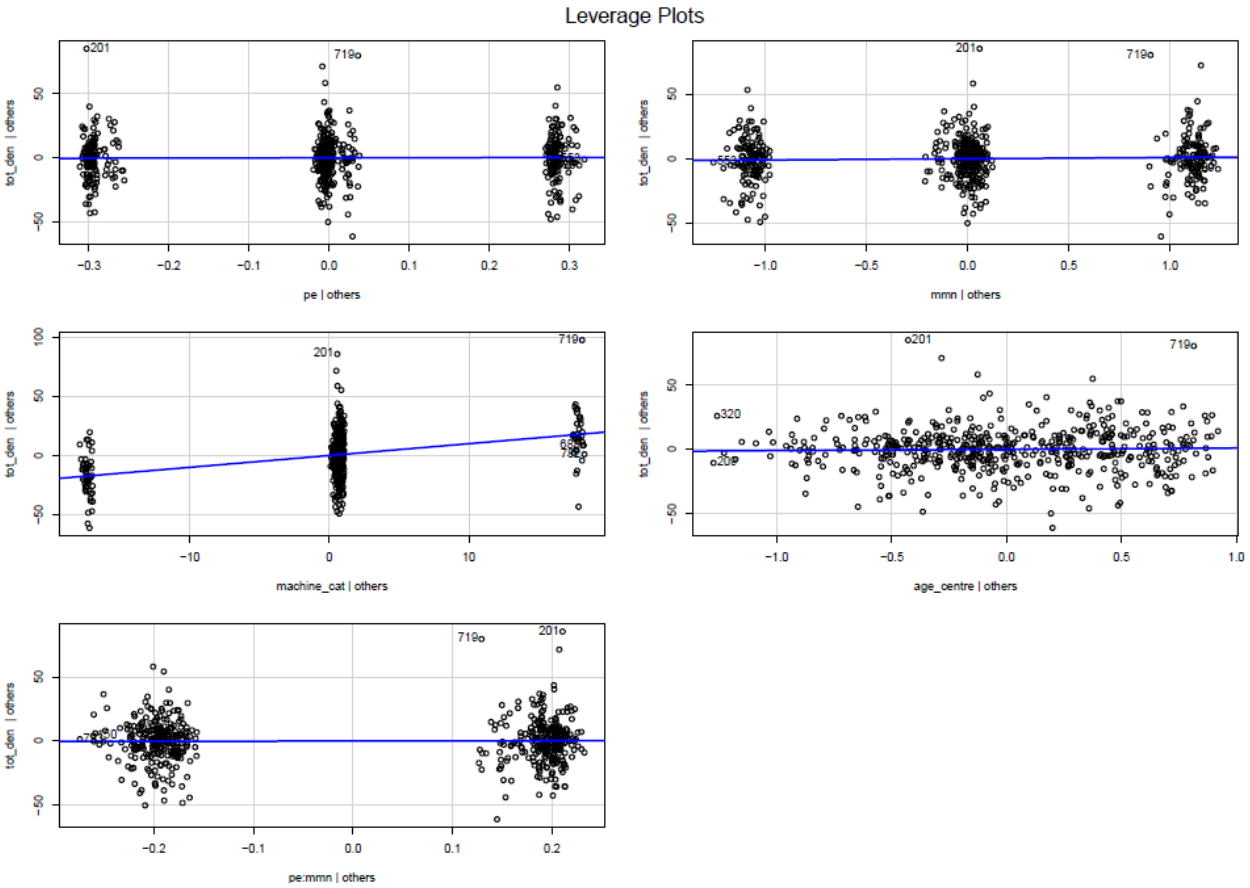
Additional diagnostic plots of ENID pQCT models



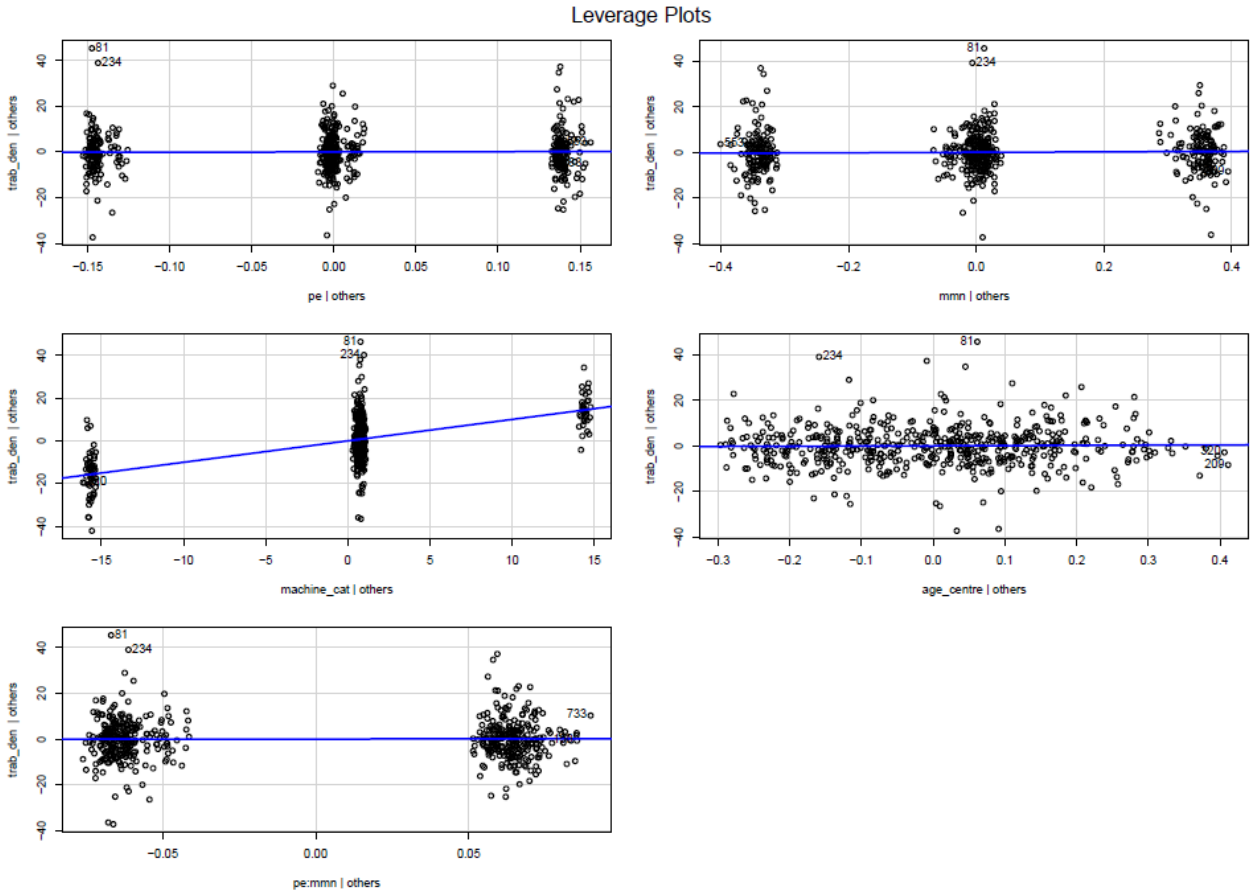
*Leverage plots that show the main effects for Model 2 i.e. linear regression of between-visit difference in pQCT total vBMD (tibia). Model 2: $Y_i=\beta_0+\beta_1$machine $+\beta_2$age$+\beta_3$PE*MMN$+\epsilon$. Yi is the absolute change in total vBMD for participant I. The error ε represents the difference between what is explained by the systematic part of the model and what is observed. Model 2 included supplement effects (as binary dummy variables, Figure 3.15) to allow MMN, PE, and a PE x MMN interaction term to be explored. Machine_cat was also included in this model. PE = protein energy, MMN = multiple micronutrients.*



Leverage plots that show the main effects for Model 2 i.e. linear regression of between-visit difference in pQCT trabecular vBMD (tibia). Model 2: $Y_i=\beta_0+\beta_1 machine+\beta_2 age+\beta_3 PE*MMN+\epsilon$. Yi is the absolute change in total vBMD for participant I. The error ε represents the difference between what is explained by the systematic part of the model and what is observed. Model 2 included supplement effects (as binary dummy variables, Figure 3.15) to allow MMN, PE, and a PE x MMN interaction term to be explored. Machine_cat was also included in this model. PE = protein energy, MMN = multiple micronutrients.



*Leverage plots that show the main effects for Model 2 i.e. linear regression of between-visit difference in pQCT total vBMD (radius). Model 2: $Y_i=\beta_0+\beta_1$machine $+\beta_2$age$+\beta_3$PE*MMN$+\epsilon$. Yi is the absolute change in total vBMD for participant I. The error ε represents the difference between what is explained by the systematic part of the model and what is observed. Model 2 included supplement effects (as binary dummy variables, Figure 3.15) to allow MMN, PE, and a PE x MMN interaction term to be explored. Machine_cat was also included in this model. PE = protein energy, MMN = multiple micronutrients.*



*Leverage plots that show the main effects for Model 2 i.e. linear regression of between-visit difference in pQCT trabecular vBMD (radius). Model 2: $Y_i=\beta_0+\beta_1$machine $+\beta_2$age$+\beta_3$PE*MMN$+\epsilon$. Yi is the absolute change in total vBMD for participant I. The error ε represents the difference between what is explained by the systematic part of the model and what is observed. Model 2 included supplement effects (as binary dummy variables, Figure 3.15) to allow MMN, PE, and a PE x MMN interaction term to be explored. Machine_cat was also included in this model. PE = protein energy, MMN = multiple micronutrients.*

Summary of Model 1 for change in bone outcome measures at the 4% distal and 33% proximal radius between Booking and P30 for women scanned on the same machine at both visits.

	Total vBMD (mg/cm^3)	Trabecular vBMD (mg/cm^3)	Total CSA 4% (mm^2)	Total CSA 33% (mm^2)	Cortical vBMD (mg/cm^3)	Cortical BMC (mg/mm)	Cortical CSA (mm^2)	Cortical Thickness (mm)
Age	-0.18	0.04	0.14	0.07	-0.30.	-0.03a	-0.01	-0.002
	(0.13)	(0.07)	(0.16)	(0.08)	(0.17)	(0.02)	(0.01)	(0.001)
Constant	0.92	1.51[c]	1.89.	-0.34	5.13[c]	0.47[c]	0.07	0.01
	(0.81)	(0.43)	(1.04)	(0.52)	(1.07)	(0.10)	(0.08)	(0.01)
Observations	435	435	435	440	440	440	440	440
R2	0.004	0.001	0.002	0.002	0.01	0.01	0.001	0.004

Model 1 reference group: women of mean age at booking. vBMD = volumetric bone mineral density, CSA = cross-sectional area, BMC = bone mineral content, a = $p<0.05$; b = $p<0.01$; c = $p<0.001$

Summary of Model 2 for change in bone outcome measures at the 4% distal and 33% proximal radius between Booking and P30 for women scanned on the same machine at both visits

	Total vBMD (mg/cm^3)	Trabecular vBMD (mg/cm^3)	Total CSA 4% (mm^2)	Total CSA 33% (mm^2)	Cortical vBMD (mg/cm^3)	Cortical BMC (mg/mm)	Cortical CSA (mm^2)	Cortical Thickness (mm)
PE	-0.99	-0.02	3.16	2.00	-8.96[b]	-0.48.	0.14	-0.02
	(2.24)	(1.21)	(2.90)	(1.47)	(2.96)	(0.28)	(0.22)	(0.02)
MMN	3.39	0.36	-2.52	2.43.	-6.72[a]	0.12	0.50[a]	-0.01
	(2.25)	(1.21)	(2.91)	(1.46)	(2.93)	(0.28)	(0.22)	(0.02)
Age	-0.17	0.04	0.13	0.06	-0.29.	-0.04[a]	-0.01	-0.002
	(0.13)	(0.07)	(0.16)	(0.08)	(0.17)	(0.02)	(0.01)	(0.001)
PE*MMN	-2.82	0.52	0.90	-2.82	14.50[c]	0.41	-0.54.	0.02
	(3.22)	(1.74)	(4.17)	(2.10)	(4.23)	(0.40)	(0.32)	(0.03)
Constant	0.44	1.23	1.35	-1.83.	9.35[c]	0.54[b]	-0.12	0.02
	(1.55)	(0.84)	(2.01)	(1.02)	(2.06)	(0.20)	(0.15)	(0.02)
Observations	435	435	435	440	440	440	440	440
R2	0.01	0.002	0.01	0.01	0.03	0.02	0.01	0.01

Model 2 reference group: women of mean age at booking, who received FeFol. vBMD = volumetric bone mineral density, CSA = cross-sectional area, BMC = bone mineral content, a = $p<0.05$; b = $p<0.01$; c = $p<0.001$

Summary of Model 1 for change in bone outcome measures at the 4% distal and 38% proximal tibia between Booking and P30 for women scanned on the same machine at both visits.

	Total vBMD (mg/cm^3)	Trabecular vBMD (mg/cm^3)	Total CSA 4% (mm^2)	Total CSA 38% (mm^2)	Cortical vBMD (mg/cm^3)	Cortical BMC (mg/mm)	Cortical CSA (mm^2)	Cortical Thickness (mm)
Age	0.02	0.02	0.17	0.07	-0.09	-0.03	-0.01	-0.001
	(0.04)	(0.04)	(0.19)	(0.09)	(0.09)	(0.03)	(0.02)	(0.001)
Constant	0.91[b]	0.77[b]	-1.21	0.68	4.39[c]	1.52[c]	0.43[c]	0.005
	(0.28)	(0.27)	(1.26)	(0.58)	(0.56)	(0.20)	(0.12)	(0.01)
Observations	407	407	407	409	409	409	409	409
R^2	0.001	0.0004	0.002	0.002	0.003	0.003	0.0005	0.002

Model 1 reference group: women of mean age at booking. vBMD = volumetric bone mineral density, CSA = cross-sectional area, BMC = bone mineral content, [a] = $p<0.05$; [b] = $p<0.01$; [c] = $p<0.001$

Summary of Model 2 for change in bone outcome measures at the 4% distal and 38% proximal tibia between Booking and P30 for women scanned on the same machine at both visits.

	Total vBMD (mg/cm^3)	Trabecular vBMD (mg/cm^3)	Total CSA 4% (mm^2)	Total CSA 38% (mm^2)	Cortical vBMD (mg/cm^3)	Cortical BMC (mg/mm)	Cortical CSA (mm^2)	Cortical Thickness (mm)
PE	0.48	0.53	2.20	2.40	-0.84	-0.27	-0.07	-0.02
	(0.81)	(0.77)	(3.60)	(1.66)	(1.60)	(0.57)	(0.36)	(0.02)
MMN	0.49	-0.02	-2.89	2.94[.]	0.98	0.42	0.19	-0.02
	(0.79)	(0.75)	(3.50)	(1.60)	(1.54)	(0.55)	(0.35)	(0.01)
Age	0.02	0.02	0.18	0.07	-0.10	-0.03	-0.01	-0.001
	(0.04)	(0.04)	(0.19)	(0.09)	(0.09)	(0.03)	(0.02)	(0.001)
PE*MMN	1.14	0.22	-3.11	-3.59	2.13	0.12	-0.29	0.02
	(1.13)	(1.08)	(5.03)	(2.31)	(2.22)	(0.80)	(0.50)	(0.02)
Constant	0.14	0.47	-0.01	-1.09	3.74[c]	1.40[c]	0.43[.]	0.02[.]
	(0.55)	(0.53)	(2.46)	(1.14)	(1.10)	(0.39)	(0.25)	(0.01)
Observations	407	407	407	409	409	409	409	409
R^2	0.02	0.004	0.01	0.01	0.01	0.01	0.003	0.01

Model 2 reference group: women of mean age at booking, who received FeFol. vBMD = volumetric bone mineral density, CSA = cross-sectional area, BMC = bone mineral content, [a] = $p<0.05$; [b] = $p<0.01$; [c] = $p<0.001$

Appendix D: PABS Ethical Approval Letters

Research Ethics Committee favourable opinion letter

NHS
Health Research Authority

East of England - Essex Research Ethics Committee
The Old Chape
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0207 104 8069

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

08 September 2016

Mr Micheal O Breasail
Elsie Widdowson Laboratory
120 Fulbourn Road
Cambridge
CB1 9NL

Dear Mr O Breasail

Study title:	**Determining changes in bone mineral content, shape and microstructure in the trabecular and cortical compartments during pregnancy**
REC reference:	**16/EE/0273**
IRAS project ID:	**199857**

Thank you for your submission of 18 August, responding to the Committee's request for further information on the above research and submitting revised documentation. The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Helen Poole at NRESCommittee.EastofEngland-Essex@nhs.net

Confirmation of ethical opinion

The use of retained images and data for future research, with consent, is approved as part of the study. It was noted that as and when there was a desire to use such material this use must be in accordance with ethical requirements as stand at the time. Project-specific ethical approval may or may not be required, according to the details and then-current requirements. The point of the committee stipulations is to permit retention and remove the need to re-consent in such circumstances, in keeping with the spirit of GAfREC 2.3.10. The committee is satisfied with the response.

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Pregnancy and Bone Study - Poster]	1	18 April 2016
Copies of advertisement materials for research participants [Pregnancy and Bone Study - Recruitment Email]	1	14 June 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [MRC Statement of Indemnity]		
GP/consultant information sheets or letters [Pregnancy and Bone Study - GP letter]	1	29 February 2016
IRAS Application Form [IRAS_Form_18082016]		18 August 2016
Letters of invitation to participant [Pregnancy and Bone Study - Formal Invitation Letter]	1	18 April 2016
Letters of invitation to participant [Pregnancy and Bone Study - Appointment Letter]	1	18 April 2016
Letters of invitation to participant [Pregnancy and Bone Study - Reminder Letter]	1	14 June 2016
Letters of invitation to participant [Pregnancy and Bone Study - SMS Reminder]	1	14 June 2016
Non-validated questionnaire [Pregnancy and Bone Study - Telephone Screening Questionnaire]	1.1	16 August 2016
Non-validated questionnaire [Pregnancy and Bone Study - Musculoskeletal Questionnaire]	1.1	16 August 2016
Other [Response to Validation Query re Insurance]		14 June 2016
Other [Response Letter]	1	18 August 2016

Participant consent form [Pregnancy and Bone Study - Consent Form]	2	25 July 2016
Participant information sheet (PIS) [Pregnancy and Bone Study - Participant Information Sheet]	2	25 July 2016
Referee's report or other scientific critique report [PAB Study SERB review letter]	1	17 June 2016
Referee's report or other scientific critique report [Pregnancy and Bone Study - RRB Stage i Internal Review Form]	1	18 December 2015
Research protocol or project proposal [Pregnancy and Bone Study Protocol]	1.2	25 July 2016
Summary CV for Chief Investigator (CI) [Micheal Ó Breasail CV 2016]	1	02 May 2016
Summary CV for student [Micheal Ó Breasail CV 2016]	1	02 May 2016
Summary CV for supervisor (student research) [Kate Ward CV 2016]	1	
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Pregnancy and Bone Study - Study Flow Diagram]	1.1	25 July 2016
Validated questionnaire [Food Frequency Questionnaire]	1	16 June 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

16/EE/0273	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely

pp

Dr Alan Lamont
Chair

Email:NRESCommittee.EastofEngland-Essex@nhs.net

Enclosures: "After ethical review – guidance for researchers"
Copy to: *Ms Polly Page*
Mr Stephen Kelleher, Cambridge University Hospitals

NHS
Health Research Authority

Mr MÃ-cheÃ¡l Ã“ Breasail
Elsie Widdowson Laboratory
120 Fulbourn Road
Cambridge
CB1 9NL

Email: hra.approval@nhs.net

25 October 2016

Dear Mícheál Breasail

Letter of HRA Approval

Study title:	**Determining changes in bone mineral content, shape and microstructure in the trabecular and cortical compartments during pregnancy**
IRAS project ID:	**199857**
REC reference:	**16/EE/0273**
Sponsor	**MRC Human Nutrition Research**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application

procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **199857**. Please quote this on all correspondence.

Yours sincerely

Rekha Keshvara
Assessor

Email: hra.approval@nhs.net

Copy to: *Ms Polly Page, MRC Human Nutrition Research (Sponsor contact)*
Mr Stephen Kelleher, Cambridge University Hospitals (R&D contact)

Appendix A - List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Pregnancy and Bone Study - Poster]	1	18 April 2016
Copies of advertisement materials for research participants [Pregnancy and Bone Study - Recruitment Email]	1	14 June 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [MRC Statement of Indemnity]		
GP/consultant information sheets or letters [Pregnancy and Bone Study - GP letter]	1	29 February 2016
IRAS Application Form [IRAS_Form_18082016]		18 August 2016
Letters of invitation to participant [Pregnancy and Bone Study - Formal Invitation Letter]	1	18 April 2016
Letters of invitation to participant [Pregnancy and Bone Study - Appointment Letter]	1	18 April 2016
Letters of invitation to participant [Pregnancy and Bone Study - Reminder Letter]	1	14 June 2016
Letters of invitation to participant [Pregnancy and Bone Study - SMS Reminder]	1	14 June 2016
Non-validated questionnaire [Pregnancy and Bone Study - Telephone Screening Questionnaire]	1.1	16 August 2016
Non-validated questionnaire [Pregnancy and Bone Study - Musculoskeletal Questionnaire]	1.1	16 August 2016
Other [Response to Validation Query re Insurance]		14 June 2016
Other [Response Letter]	1	18 August 2016
Participant consent form [Consent form - Minor Amendment]	3	13 October 2016
Participant consent form [Pregnancy and Bone Study - Consent Form]	2	25 July 2016
Participant information sheet (PIS) [Pregnancy and Bone Study - Participant Information Sheet]	2	25 July 2016
Participant information sheet (PIS) [PIS - Minor Amendment]	3	13 October 2016
Referee's report or other scientific critique report [PAB Study SERB review letter]	1	17 June 2016
Referee's report or other scientific critique report [Pregnancy and Bone Study - RRB Stage i Internal Review Form]	1	18 December 2015
Research protocol or project proposal [Pregnancy and Bone Study Protocol]	1.2	25 July 2016
Summary CV for Chief Investigator (CI) [Micheal Ó Breasail CV 2016]	1	02 May 2016
Summary CV for student [Micheal Ó Breasail CV 2016]	1	02 May 2016
Summary CV for supervisor (student research) [Kate Ward CV 2016]	1	
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Pregnancy and Bone Study - Study Flow Diagram]	1.1	25 July 2016
Validated questionnaire [Food Frequency Questionnaire]	1	16 June 2016

IRAS project ID	199857

Appendix B - Summary of HRA Assessment

This appendix provides assurance to you, the sponsor and the NHS in England that the study, as reviewed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing and arranging capacity and capability.

For information on how the sponsor should be working with participating NHS organisations in England, please refer to the, *participating NHS organisations, capacity and capability* and *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* sections in this appendix.

The following person is the sponsor contact for the purpose of addressing participating organisation questions relating to the study:

Polly Page (Email: 01223426356, Email: polly.page@mrc-hnr.cam.ac.uk)

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	Statement of activities will act as agreement of an NHS organisation to participate.

Section	HRA Assessment Criteria	Compliant with Standards	Comments
4.2	Insurance/indemnity arrangements assessed		The study insurance cover is provided by the MRC. The MRC, as a government body, is not insured but has indemnity arrangements in place to ensure funding is provided to meet such claims where the sponsor is liable. *Ex gratia* payments may be considered in circumstances of non-negligent harm. Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional indemnity provided by their medical defence organisation covers the activities expected of them for this research study
4.3	Financial arrangements assessed	Yes	The funding details for the study are included in the Statement of Activities.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	No comments
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
This is a single NHS site study. The research activities to be carried out by the research team. The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website. If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Confirmation of Capacity and Capability

This describes whether formal confirmation of capacity and capability is expected from participating NHS organisations in England.
Participating NHS organisations in England **will be expected to formally confirm their capacity and capability to host this research**. • Following issue of this letter, participating NHS organisations in England may now confirm to the sponsor their capacity and capability to host this research, when ready to do so. How capacity and capacity will be confirmed is detailed in the *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* section of this appendix. • The Assessing, Arranging, and Confirming document on the HRA website provides further information for the sponsor and NHS organisations on assessing, arranging and confirming capacity and capability.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).
Principal Investigators to be allocated at participating research site in order to complete the research procedures as detailed in the protocol. GCP training is not a generic training expectation, in line with the HRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
Use of identifiable patient records held by an NHS organisation to identify potential participants should be undertaken by a member of the direct care team for the patient, so it would not normally be acceptable for this to be done by staff not employed by that organisation. An Honorary Research Contract (or equivalent) would be expected for any external NHS/HEI staff undertaking all of the other activities for the study once consent from the participant is in place. The pre-engagement checks should include an enhanced DBS check (including a check against the DBS 'barred list' for adults), and Occupational Health Clearance.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.
• The applicant has indicated that they do not intend to apply for inclusion on the NIHR CRN Portfolio.

Addenbrooke's R&D Authorisation Form

R&D Number: A094123

REC number: 16/EE/0273

Authorisation required prior to Trust Confirmation of Capacity and Capability

STUDY TITLE: Determining changes in bone mineral content, shape and microstructure in the trabecular and cortical compartments during pregnancy

I have reviewed the documentation supplied to me, and have discussed this study with the research team (if applicable), and can confirm that:

1. The Principal Investigator is suitably qualified to carry out the research described and has relevant experience taking into account his/her:
    - professional qualifications,
    - knowledge of the research field,
    - expertise in the procedures involved,
    - previous research experience,
    - training in research methods (including informed consent),
    - ability to take clinical responsibility for the local research team
2. The facilities required for this study, relating to the directorate that I am Divisional/Clinical Director for, are adequate and appropriate for this research
3. I agree that this study can proceed

Signature: .....

Date: 7/11/2016

Name (Block Capitals): HEVSCHLER, ROBERT

Directorate: Division E

V1 March 2009

R&D approval letter

Cambridge University Hospitals NHS
NHS Foundation Trust

Research and Development Department

R&D ref: A094123

Box 277
Addenbrooke's Hospital
Hills Road
Cambridge
CB2 0QQ

Direct Dial: 01223 348455
Switchboard: 01223 245151

E-mail:tracy.assari@addenbrookes.nhs.uk
r&denquiries@addenbrookes.nhs.uk
www.addenbrookes.org.uk

Mr Jeremy Brockelsby
Consultant in Obstetrics & Fetomaternal Medicine
Box 224 Department of Obstetrics and Gynaecology
The Rosie Hospital

22 November 2016

Dear Mr Jeremy Brockelsby

IRAS ID: 199857
Determining changes in bone mineral content, shape and microstructure in the trabecular and cortical compartments during pregnancy
REC Ref: 16/EE/0273

Thank you for sending details of the above named study.

The R&D department has received the HRA Approval letter and reviewed the study documents. The project has been allocated the internal R&D reference number of **A094123**. Please quote this in all future correspondence regarding this study.

Capacity and capability to conduct this study at Cambridge University Hospitals NHS Foundation Trust is confirmed. Recruitment can commence at this site from the date of this letter.

We would like to take this opportunity to remind you of your responsibilities under the terms of the Research Governance Framework for Researchers, Chief Investigators, Principal Investigators and Research Sponsors and to also of the requirement to notify R&D of any amendments or changes made to this study.

You will be aware that the Trust is subject to national reporting requirements for first patient recruitment within 70 days. Further details on this can be found on the NIHR website:
http://www.nihr.ac.uk/policy-and-standards/faster-easier-clinical-research.htm
If you have any questions or concerns about this, please contact me.

I wish you every success with this study.

Yours sincerely

Louise Stockley
Research Governance Manager

Letter of access for research

Cambridge University Hospitals NHS
NHS Foundation Trust

Research and Development Department
Box 277
Addenbrooke's Hospital
Hills Road
Cambridge
CB2 0QQ

R&D Manager: Stephen Kelleher
stephen.kelleher@addenbrookes.nhs.uk
HR Manager: Debbie Richards
01223 274660
deborah.richards@addenbrookes.nhs.uk
HR Advisor: Gayle Lindsay
01223 348496
gayle.lindsay@addenbrookes.nhs.uk

Mrs Jenny Woolston
Senior Research Coordinator
Medical Research Council
Elsie Widdowson Laboratory
Fulbourn Rd

1st December 2016

Dear Jenny

Letter of access for research – A094123 - Quantifying maternal bone loss during pregnancy

This letter confirms your right of access to conduct research through Cambridge University Hospitals NHS Foundation Trust for the purpose and on the terms and conditions set out below. This right of access commences on **23rd November 2016** and ends on **22nd November 2019** unless terminated earlier in accordance with the clauses below.

You have a right of access to conduct such research as confirmed in writing in the letter of permission for research from this NHS organisation. Please note that you cannot start the research until the Principal Investigator for the research project has received a letter from us giving permission to conduct the project and you have provided the Trust's R&D department with written evidence that you have completed GCP training from an EU institution before you start your research.

The information supplied about your role in research at Cambridge University Hospitals NHS Foundation Trust has been reviewed and you do not require an honorary research contract with this NHS organisation. We are satisfied that such pre-engagement checks as we consider necessary have been carried out.

You are considered to be a legal visitor to Cambridge University Hospitals NHS Foundation Trust premises. You are not entitled to any form of payment or access to other benefits provided by this NHS organisation to employees and this letter does not give rise to any other relationship between you and this NHS organisation, in particular that of an employee.

While undertaking research through Cambridge University Hospitals NHS Foundation Trust, you will remain accountable to your place of work **University of Cambridge** but you are required to follow the reasonable instructions of **Jeremy Brocklesby** in this NHS organisation or those given on his behalf in relation to the terms of this right of access.

Where any third party claim is made, whether or not legal proceedings are issued, arising out of or in connection with your right of access, you are required to co-

operate fully with any investigation by this NHS organisation in connection with any such claim and to give all such assistance as may reasonably be required regarding the conduct of any legal proceedings.

You must act in accordance with Cambridge University Hospitals NHS Foundation Trust policies and procedures, which are available to you upon request, and the Research Governance Framework.

You are required to co-operate with Cambridge University Hospitals NHS Foundation Trust in discharging its duties under the Health and Safety at Work etc Act 1974 and other health and safety legislation and to take reasonable care for the health and safety of yourself and others while on Cambridge University Hospitals NHS Foundation Trust premises. You must observe the same standards of care and propriety in dealing with patients, staff, visitors, equipment and premises as is expected of any other contract holder and you must act appropriately, responsibly and professionally at all times.

If you have a health condition or disability which may affect your research role and which might require reasonable special adjustments to your role, if you have not already done so, you must notify your employer and the Trust's R&D HR Office prior to commencing your research role at the Trust.

You are required to ensure that all information regarding patients or staff remains secure and *strictly confidential* at all times. Personal identifiable data must be carried securely at all times and mobile devices must be encrypted. You must ensure that you understand and comply with the requirements of the NHS Confidentiality Code of Practice (https://www.gov.uk/government/publications/confidentiality-nhs-code-of-practice) and the Data Protection Act 1998. Furthermore you should be aware that under the Act, unauthorised disclosure of information is an offence and such disclosures may lead to prosecution. Data controllers could also be fined for a breach of the Data Protection Act 1998. You must familiarise yourself with the Trust's Information Governance Code of Conduct.

You must keep confidential any information regarding the design, conduct or management or results of any research unless authorised in writing by the Trust to disclose it. You must acknowledge the Trust's contribution in any publication arising out of this Agreement.

Subject to any agreement with your employer to the contrary (e.g. as part of a multi-centre study), any Intellectual Property (IP) resulting from research carried out under this Agreement will be the property of the Trust and you will do all things necessary or desirable to give effect to the assignment of this IP.

You should ensure that, where you are issued with an identity or security card, a bleep number, email or library account, keys or protective clothing, these are returned upon termination of this arrangement. Please also ensure that while on the premises you wear your ID badge at all times, or are able to prove your identity if challenged. Please note that this NHS organisation accepts no responsibility for damage to or loss of personal property.

We may terminate your right to attend at any time either by giving seven days' written notice to you or immediately without any notice if you are in breach of any of the terms or conditions described in this letter or if you commit any act that we reasonably consider to amount to serious misconduct or to be disruptive and/or prejudicial to the interests and/or business of this NHS organisation or if you are

convicted of any criminal offence. You must not undertake regulated activity if you are barred from such work. If you are barred from working with adults or children this letter of access is immediately terminated. Your employer will immediately withdraw you from undertaking this or any other regulated activity and you MUST stop undertaking any regulated activity immediately.

Your substantive employer is responsible for your conduct during this research project and may in the circumstances described above instigate disciplinary action against you.

Cambridge University Hospitals NHS Foundation Trust will not indemnify you against any liability incurred as a result of any breach of confidentiality or breach of the Data Protection Act 1998. Any breach of the Data Protection Act 1998 may result in legal action against you and/or your substantive employer.

INDUCTION AND MANDATORY TRAINING

You are responsible for familiarising yourself with the Trust's policies and mandatory training courses such as Moving and Handling, Health and Safety, Fire Training etc and be aware of the responsibility to maintain a safe environment for patients, staff and visitors

Your host Manager will ensure that you receive a comprehensive Departmental Induction. She/he will also provide you with details of Corporate Induction, research specific induction and annual Mandatory Refresher Training.

If your letter of access is for more than 3 months, you must attend Corporate Induction. Where your letter of access is for more that 12 months, you must attend annual Mandatory Refresher Training.

If your current role or involvement in research changes, or any of the information provided in your Research Passport changes, you must inform your employer through their normal procedures. You must also inform your nominated manager in this NHS organisation.

Yours sincerely

Stephen Kelleher
R&D Manager, Cambridge University Hospitals NHS Foundation Trust

cc: Jeremy Brocklesby, Clinical Director, Obs and Gynae, Division E
John Crisp, Finance & HR Manager, MRC Elsie Widdowson Laboratory, via e-mail john.crisp@mrc.ewl.cam.ac.uk

Enc: P6 form to confirm Letter of Access issued
ID Badge Form

Appendix E: Pregnancy and Bone Study forms

PABS Participant Information Sheet (PIS)

MRC | Elsie Widdowson Laboratory

Pregnancy and Bone Study (PABS)

Participant Information Sheet

Chief Investigator: Mícheál Ó Breasail PhD Student

Telephone: 01223 426356
Study email: PABS@mrc-ewl.cam.ac.uk

You are being invited to take part in a research study. Your decision whether to take part in this study will not affect your medical care. Before you decide if you wish to take part, it is important that you understand why the research is being done and what it will involve. Please read this information carefully and discuss it with others if you wish. If you have any questions about the research you can contact the Research Study Manager, Jenny Woolston, without any obligation to participate. Please take time to decide whether or not you wish to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason.

What is the purpose of the study?

Building and maintaining healthy bones is important for the prevention of osteoporosis (risk of easily breaking a bone as we age). Bone loss in women begins in their 30's and there is a possibility that the normal postpartum recovery of bone is impaired in older mothers. If bone recovery is impaired, this may be a risk factor for suffering low trauma fractures in later life. The aim of this study is to use newly developed measurement methods (bone scans using pQCT and HRpQCT – see below) to assess changes in bone between the early-second and late-third trimesters in pregnant women, aged 30-45. Healthy non-pregnant non-lactating (NPNL) women will be measured as a control group so we can look at differences in bone health over time.

What will happen to me if I take part?

Recruitment

If you are pregnant and aged 30-45 years you will be approached at the Rosie Hospital, Cambridge University Hospital (CUH) and asked if you would be interested in taking part in this study. If you wish to take part or hear more about the study you can call a member of the research team. We also wish to recruit healthy non pregnant non lactating women aged 30-45 years to take part in our study to allow us to see whether any measured changes in the pregnant group are due to pregnancy or are the normal changes we see over time.

Screening telephone call

Before we invite you to Medical Research Council Elsie Widdowson Laboratory (MRC EWL), Cambridge for a study visit we would like to ask you a few questions to ensure that you are eligible for our study. This telephone screening will take about 20 minutes. If you are eligible we will send you an appointment letter inviting you to attend MRC EWL for your first study visit. To help you manage your time you will also be asked if you wish to receive a reminder telephone call or text message ahead of your scheduled visits.

Study visits at MRC EWL

If you agree to take part in this study, we would like to invite you to visit EWL for two visits, each lasting no longer than 2.5 hours. For pregnant participants these visits will be between 14-16 weeks and 34-36 weeks of your pregnancy. For our non-pregnant participants these visits will occur over a similar time period approximately 20 weeks apart. Your partner is welcome to accompany you, and we can provide childcare in the Volunteer lounge (by arrangement) if you need to bring children with you. We hope these arrangements make it easier for you to attend. We will offer flexible appointment times and can offer rescheduling of study visits if required.

Study visits will take place in our purpose-built volunteer suite. At visit 1 we will explain the study fully and you will be asked to provide written and informed consent. Once we have obtained your consent to participate we will ask you to complete a Musculoskeletal Questionnaire (both visits) to collect information about your bone health, medication, supplement use, and lifestyle. We will also measure your height and weight at both visits. We will also ask pregnant participants for their permission to access medical records to gather data on the baby's gestational age, weight and length at birth.

At both visits we will perform bone scans on all participants at the forearm and lower leg using machines that use very low levels of x-rays (see below: ionising radiation). These scans will take place in dedicated scanning rooms with arrangements to comfortably seat and position you while you are being scanned. We will use two machines to image your bones (peripheral Quantitative Computed Tomography (pQCT) and High Resolution (HR) -pQCT). Each scan will take approximately 5 minutes but you will need to remain seated for 20 minutes. You can eat and drink normally before, during, and after visits, and can take regular breaks between procedures. It is preferable to wear loose clothing so that we can roll sleeves/trouser legs up for scanning. We will ask you to take off rings (if possible) and bracelets when your arm is being scanned.

pQCT and HR-pQCT are considered appropriate for use in healthy volunteers, and for use in pregnancy as they take measurements of the your leg or arm, meaning your baby isn't directly in the X-ray beam. X-ray scatter from these technologies is very low as these are peripheral devices and will image only the forearm and lower leg and is not considered a risk to your baby, however, we can provide extra shielding in the form of lead aprons should you wish to wear one.

HRpQCT forearm scan

HRpQCT lower leg scan

pQCT forearm scan

pQCT lower leg scan

What do I need to do if I want to take part?

If you would like to participate in the study please call or email Jenny Woolston, on 01223 437588 or at PABS@mrc-ewl.cam.ac.uk If you already have an appointment for your first visit it should have been confirmed in the letter enclosed in this pack. With your agreement we will send you a reminder by post, email or text of the date and time shortly before each of your visits to MRC EWL

What are the possible benefits of taking part?

There will be no direct health benefit to participants who take part in this study, however, you may feel proud that you are taking part in medical research and contributing to knowledge advancement. Lifestyle advice relating to bone health, healthy eating and exercise will be given to participants after the study has concluded.

What are the possible risks and disadvantages of taking part?

There are minimal risks associated with participation in this study. Every day we are exposed to natural background radiation from the air and soils that surround us. As reference Cambridgeshire is considered to be a low background radiation area in the UK (~7 μSv/day). The small additional radiation that you will receive from pQCT (<3.2 μSv/visit) and HR-pQCT (<6 μSv/visit) scans for the total study is 18.4 μSv (<9.2 μSv/visit). Participation in our study exposes a woman to the equivalent of less than three days of background radiation in Cambridgeshire (<7 μSv/day), or less than a quarter of the exposure from a return transatlantic flight (~80 μSv).

The scanners we use are designed to only scan the arms and legs so the scatter is known to be extremely low. This means that for pregnant participants, the baby will not be in the x-ray beam when the scans are made. However, any use of x-ray includes x-ray scatter beyond the beam and because X-rays pass through the body your baby will be exposed to a very small amount of radiation. In the current study this has been calculated to be 10μSv, just over a day's background radiation, which is extremely low but we do recognise that this may cause you concern. You may wish to wear a lead apron to further reduce this very small risk and we will provide one should you wish. For your information, in accordance with the Health Protection Agency's advice on the "Protection of Pregnant Patients during Diagnostic Medical Exposures to Ionising Radiation" a <10 μSv dose means the increased risk of cancer occurring in your child up to the age of 15 due to the exposure will be ~ 1 in 1,300,000. This risk is much lower than the natural risk of childhood cancer in the UK, which is 1 in 500.

All scans are reviewed by the research team (including Dr Kate Ward the Imaging Expert at MRC EWL) and it is possible that a scan may reveal unusual or abnormal findings. If any abnormal findings are observed on your scans they may be sent for review by musculoskeletal health experts at Cambridge University Hospitals who will decide if clinical follow up is required via your GP.

Participants will need to travel to MRC EWL on two occasions where free on-site parking is available. We will cover your travel expenses and taxis can be provided if this helps you to attend the visits. Each visit will be at most 2.5 hours. We will give appointments to suit you. Young children cannot be in the scan room but we can ensure childcare if needed.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any time. However if you do decide to withdraw from the study, data already obtained from you may still be used to contribute to study results.

What will happen if something goes wrong?

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence you may have grounds for legal action for compensation against MRC EWL but you may have to pay for your legal costs. For research carried out at MRC EWL, participants would be at the same position as if public liability had been taken out.

Complaints

Any complaints you might have for this study will be fully investigated. If you have any concerns about study procedures you can speak to the Research Study Manager, Jenny Woolston (Tel: 01223 437588, email: PABS@mrc-ewl.cam.ac.uk), who will answer your questions. If you remain unhappy and wish to complain formally you can contact the MRC EWL Head of Operations, Ms Polly Page (Tel: 01223 426356, email: Polly.Page@mrc-ewl.cam.ac.uk) who will address any queries you may have.

Will my taking part in the study be kept confidential?

Any information that is collected about you during the course of the study will be kept strictly confidential and MRC EWL will be the custodian of the data. Any information about you that leaves MRC EWL will have your name and address removed so that you cannot be identified from it.

MRC EWL has a standard confidentiality procedure for participants involved in research. This stipulates how personal information is collected, used, stored and disposed of during and following completion of research projects. Any information that is collected about you during the course of the study will be kept strictly confidential and secure in locked filing cabinets and/or electronic files on computers that have restricted access. Each participant is assigned a unique code that is used on all data collected during the research. This code is then used to identify data in place of personal information.

Only specified research team members will have access to personal identifying data information. However, with your agreement, your GP will be notified of your study results and copies of these letters will also be provided to you.

MRC EWL maintains a central record of all research projects but this does not include personal information on participants. With your agreement we will store data for 20 years. With your consent, retained data including the images and data obtained from them may be used for future studies of bone health through the life course. You may opt-out of the data being stored for purposes other than the current study. This will not stop you taking part in the current study.

What will happen to the results?

The overall results of the study will be presented at scientific meetings and/ or published in a scientific journal. You will not be identified in any of these presentations or publications.

Involvement of GP

With your permission, we will notify you GP of your participation in the study. In the event of any abnormal or unusual scans, we may liaise with musculoskeletal health expert colleagues at Cambridge University Hospitals who will decide if there is a need for clinical follow up via your GP.

Will I be reimbursed for my time?

Participants will be compensated for their time and contribution to the study. You will be given an honorarium of £20 after the first visit and £30 after the second visit, a total payment of £50. We will also cover reasonable travel expenses.

Further information and independent advice

If you have any questions about the study please contact the Research Study Manager, Jenny Woolston, by telephone (01223 437588) or email (PABS@mrc-ewl.cam.ac.uk). If you would like independent advice about this study please contact your local Patient Advise Liaison Service (PALS) (http://www.pals.nhs.uk/officemapsearch.aspx).

Participant ID Number: _ _ _ _ _ _ _

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Telephone Screening Questionnaire

Pregnancy and Bone Study (PABS)

Name of researcher conducting the questionnaire: _____

Screening date: ☐☐/☐☐/☐☐

Screening questionnaire to be conducted by the chief investigator or by a trained member of the research team.

Introduction:

Firstly, welcome and thank you for taking the time to go through the pre-screening questionnaire with me.

I am going to give you some more details about what is involved within the study, and provide you with the opportunity to ask any questions that you may have. Are you still interested in taking part?

Yes **No**

If **yes** continue to **section 1** of the questionnaire, if **no** thank the participant for their interest and time.

***If participant answers no, ask if they would like their details to be added to the volunteer database for future studies?**

Section 1

I need to ask some questions to find out some more about you and to check that you are eligible for the study. Could I ask you to answer the following questions please?:

1. STUDY CRITERIA			
Can you confirm that you are/have:		**YES**	**NO**
1	Aged between 30 and 45 years of age		
2	Were you referred to the study by a midwife at the Rosie or did you see an advertisement for the study?		
3	Are you pregnant?		
4	**(ONLY FOR PREGNANT WOMEN NOT REFERRED FROM THE ROSIE)** Can I ask whether the care for your pregnancy will fall under the Rosie Hospital? If no, participant isn't eligible to take part.		
5	Do you know your due date? **(ONLY PREGNANT WOMEN)** **Or if not, how many weeks pregnant** ☐☐/☐☐/☐☐		

	you are? _____		
6	Natural conception **(ONLY PREGNANT WOMEN)**		
7	Live within Cambridge (CB post code)/within reasonable travelling distance to HNR		
8	Not been exposed to x-ray recently for research purposes within the last year		
9	Not taken part in any clinical drug trials for research purposes within the last year		
10	Not lactating in the past 3 months (**NPNL ONLY**)		
11	Able to give informed & written consent e.g. write their own name		
12	Are you currently taking part in any other research studies or have done in the last year? Provide details if yes:		

2. ANTHROPOMETRIC MEASUREMENTS			
Standing height (m)			
Weight (kg)		BMI ($Kg/m^{2)}$	

Section 2

I now need to ask a few questions about your current health status…

3. GENERAL HEALTH			
Do you have or have had any of the following conditions?		**YES**	**NO**
1	Diabetes		
2	Stomach or bowel problems		
3	Asthma/Eczema/Hay fever		
4	Prolonged periods of amenorrhea*		
5	High Cholesterol		
6	Anaemia		
7	Food/Drug allergies		
8	Eating Disorder		
9	Chronic medical conditions known to affect bone mass		
	(i) Rickets		
	(ii) Osteomalacia		
	(iii) Osteoporosis		
	(iv) Osteogenesis Imperfecta		

	(v) Other Please detail:		
10	Prolonged periods of immobilisation in the last year e.g. surgery		
11	Condition making scanning difficult e.g. scoliosis		
12	Take drugs known to affect bone mass e.g. steroids, bisphosphonates		
13	Received or waiting an organ transplant		
14	Growth problems e.g. due to premature birth, hyperthyroidism, delayed puberty etc.		
If you have answered yes to any of the above, please give details here:			
*absence of period for ≥6 months in women with previously normal menses, or for 12 months in women with previous oligomenorrhoea. Or absence of menses for 3 months in women with previously normal menstruation, or for 9 months in women with previous oligomenorrhoea (NICE 2014).			
Please list any medication or supplements that you are currently taking or have been taking in the last 5 years, either prescribed by your doctor or purchased over the counter e.g. contraception pill/ injection, inhalers etc.			

4. AVAILABILITY AND TRANSPORT		
Would you have any difficulty:	**YES**	**NO**
(i) In attending MRC-HNR on two occasions (20 weeks apart)?		
(ii) With your transport arrangements?		
Do you have any preferred times/days to attend the unit?	**YES**	**NO**
If yes, please give times/days here:		

Section 3

Participant ID Number: _ _ _ _ _ _ _

Confirm participant's eligibility for the study-

Is this participant eligible for this study?		**YES**	**NO**	**UNSURE**
If YES	'You appear to be suitable for the study." 'I am now going to take your contact information and some personal details. These details will be kept strictly confidential. After that we can book your screening visit.' 'Do you have any questions at this stage?'	Formal invitation and Information sheets have been given/ sent		
If NO	'Would you like information about other studies at the MRC HNR or would you consider placing your name on the volunteer database? This database will allow us to contact you regarding future studies at HNR for which you are likely to be eligible'		**YES**	**NO**
UNSURE	'I'm sorry but from the information you provided, I am uncertain of your eligibility. I will need to check with our clinician and get back to you'. Date: Time: Name: Contact number: Email address:			

Participant ID Number: _ _ _ _ _ _ _

Section 4 (ONLY FOR ELIGIBLE PARTICIPANTS)

I now need to ask you for some contact details....

5. PERSONAL DETAILS					
Title:		**First name:**		**Last name:**	
Address:				**Date of Birth:**	
				Age:	
Phone Number:				**Prefered Contact Time:** *(Please circle)*	Day Evening
Mobile Number:				**Prefered Method of Contact:** *(Please circle)*	Post Email Telephone Text
Email Address:					

Finally, can I ask where you heard about the study?

_____

_____

_____

To be completed by research team:

Name of Investigator:
Signature:
Date:

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Participant ID Number: _ _ _ _ _ _ _

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Consent Form

Pregnancy and Bone Study (PABS)

GENERAL STATEMENTS

Please initial boxes

1. I confirm that I have read and understand the information sheet entitled "*Pregnancy and Bone Study - Participant Information Sheet v.3*" for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw my consent at any time, without giving any reason, and without my medical care or legal rights being affected. ☐
3. I consent to my doctor being notified of my participation in this research and to being informed of relevant results. ☐
4. **Non-pregnant non-lactating participants:** I understand that I cannot take part in this study if I am pregnant. I am not pregnant and will inform the research team if I become pregnant. ☐
5. **Pregnant participants:** I understand that sections of any of my medical notes may be looked at by a research midwife at the Rosie Hospital, where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
6. I am willing to be contacted again in the future about the present study and any potential follow-up from it. I understand that I am under no obligation to undergo any future additional tests and can withdraw this consent at any time by notifying the study team. ☐
7. I am willing for the scans and data generated from this study to be stored, and used for the purposes of further research, for up to 20 years which is in accordance with the Data Protection Act. ☐
8. I wish to opt-out of this data being stored for purposes other than the current study. ☐
9. I agree to take part in the above study. ☐

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Participant ID Number: _ _ _ _ _ _ _ _

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Participant's written consent:

I (name of participant) _____ hereby give permission fully and freely to participate in the Pregnancy and Bone Study.

I have read the study information leaflets and understand that I can withdraw this consent at any stage.

_____	_____	_____
Name of Participant *(Please print)*	Date	Signature

Section to be completed by member of research team:

_____	_____	_____
Name of Research Team Member *(Please print)*	Date	Signature

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Participant ID Number: _ _ _ _ _ _ _

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Musculoskeletal Questionnaire

Visit 1

Pregnancy and Bone Study (PABS)

Name of researcher conducting the questionnaire: _____

All personal details on this form will be kept confidential. Please answer the questions below. Please ask the researcher for help or for any questions you may have. Once completed, place the form into the envelope provided.

1. DETAILS OF GENERAL PRACTITIONER	
Name of Doctor:	
Doctor's Surgery Address:	
Telephone No:	

2. FAMILY HISTORY		
Is there a history of osteoporosis or broken bones in your family?	**YES**	**NO**
If yes, what is their relation to you?		
At what age were they diagnosed?		

3. FRACTURES		
Have you ever suffered a broken bone?	**YES**	**NO**
If yes, please tick which bone(s)?	✓	**What age were you?**
Wrist		
Arm		
Shoulder		

Leg		
Foot		
Other (Please state which)		

4. MEDICATION

Please answer the following questions....

- **Are you currently taking or have previously taken steroids?**

☐ **Yes**

☐ **No**

If yes, please give details below including brand name, dose, length of time taken and when they were last taken/if you are still taking them.

- **Are you currently using or have previously used contraception?**

☐ **Yes**

☐ **No**

If yes, please indicate in the table below which of the following you have used stating brand name, length of time used and when you last used them/if you are still using them (more than one option may be selected)

- ☐ Depo-Provera _____
- ☐ Combined pill _____
- ☐ Progestogen- only pill _____
- ☐ Patch (Evra) _____
- ☐ Implant _____

• Are you currently taking or have previously taken any of the supplements below?

- ☐ Yes
- ☐ No

If yes, please give details below including brand name, dose, length of time taken and when they were last taken/if you are still taking them (more than one option may be selected)

- ☐ Multi-vitamin_____
- ☐ Calcium _____
- ☐ Vitamin D _____
- ☐ Calcium + Vitamin D _____

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• Please list all medications/supplements not stated above which you are taking at present or have previously taken in the last 5 years. This should include all prescribed drugs, 'over the counter drugs' and vitamin supplements.

Name of drug or supplement	Dose	How long have you been taking this drug/ supplement? · 0-6 months · 6-12 months · 5 years or less · >5 years	Brand	Tick if still taking

5. PHYSICAL ACIVITY		
How many hours per week do you spend on the following activities?		
Activity	Hours per week	Comments
Weight bearing activity (e.g. Walking, running, tennis, weight training, field sports, etc.)		
Non-weight bearing activity (e.g. Swimming, cycling, etc.)		
Occupation		
How many hours per day do you spend sitting?	On a week day _____hrs/day	On a weekend day _____hrs/day

6. SMOKING		
Have you ever smoked cigarettes or tobacco?	**YES**	**NO**
If yes, please state date started and if applicable date stopped	**Details:**	

7. ALCOHOL INTAKE		
Do you currently drink alcohol?	YES	NO
If yes, how many units of alcohol would you consume in an average week? (1 unit= 1 small glass of wine/ 1 half pint of beer)		

8. PREGNANCY (TO BE COMPLETED BY PREGNANT PARTICIPANTS ONLY)		
Is this your first pregnancy?	YES	NO
How many weeks pregnant are you?		
When is your due date?		

9. PREGNANCY AND LACTATION		
How many times have you been pregnant? (For pregnant participants please include this pregnancy)		
How many children do you have?		
What age were you when you had your first child?		
Have you previously breastfed?	YES	NO

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Pregnancy and Bone Study
Data Collection Form
Visit 1

Participant ID:	P B S
Date:	/ /
Date of birth:	/ /

Anthropometry measurements			
Height (cm):	.	**Weight (kg):**	.
BMI (kg/m²):	.		
Arm length (mm):	.	**Leg length (mm):**	.

Scanning questions: (please circle the correct answer)

Radius		
Which is the participant's non dominant arm?	Right	Left
Has the participant ever fractured this arm?	Yes	No
***If yes, scan the dominant arm**		
CONFIRM- which side will be scanned?	Right	Left
Tibia		
Which is the participant's non dominant leg?	Right	Left
Has the participant ever fractured this leg?	Yes	No
***If yes, scan the dominant leg**		
CONFIRM- which side will be scanned?	Right	Left

0: Completed
1: Machine broken
2: Refused/not attempted
3: Physically unable but attempted
4: Other

pQCT:

Operator initials: ☐☐

Distal radius: ☐

Distal tibia:

Comments:

HRpQCT

Operator initials: ☐☐

Distal radius: ☐

Distal tibia:

PAT number: _____

Comments:

Consent form completed:	Yes / No
Justification checklist completed:	Yes / No
Scanning record books completed:	Yes / No
Musculoskeletal questionnaire returned:	Yes / No
Honorarium and travel expenses:	£_____

_____ _____ _____
Investigator Date Signature

Data entered into the database:

_____ _____ _____
Investigator Date Signature

Appendix F: Pregnancy and Bone Study

supplementary material

Tibia bone outcome measures complete baseline descriptive statistics

Baseline mean (SD) for anthropometry and bone outcome measures of interest as measured with pQCT and HR-pQCT in non-pregnant non-lactating and pregnant groups.

	NPNL	Pregnant
HRpQCT Distal Tibia	n=37	n=50
Total vBMD (mg/cm^3)	315.18 (50.91)	326.78 (70.65)
Trabecular vBMD (mg/cm^3)	164.31 (37.58)	174.27 (42.84)
Cortical vBMD (mg/cm^3)	918.71 (36.95)	923.69 (39.32)
BV/TV	0.14 (0.03)	0.15 (0.04)
Trabecular number (mm^{-1})	**2.17 (0.34)**	**2.01 (0.33)***
Trabecular thickness (mm)	**0.06 (0.01)**	**0.07 (0.01)****
Trabecular separation (mm)	0.41 (0.07)	0.44 (0.10)
Cortical thickness (mm)	1.25 (0.21)	1.27 (0.30)
Cortical porosity (%)	99.30 (7.16)	98.90 (8.24)
pQCT Distal Tibia	n = 36	n = 52
Total vBMD 4% (mg/cm^3)	301.95 (40.77)	307.15 (49.01)
Trabecular vBMD 4% (mg/cm^3)	220.18 (39.01)	227.23 (43.83)
Total CSA 4% (mm^2)	980.28 (114.93)	970.97 (114.68)
Cortical subcortical vBMD (mg/cm^3)	368.82 (45.61)	372.49 (57.60)
pQCT Proximal 14% Tibia	n=36	n=53
Total CSA (mm2)	416.70 (55.03)	411.89 (58.42)
Cortical vBMD (mg/cm^3)	1135.89 (20.06)	1142.47 (17.65)
Cortical BMC (mg/mm)	188.09 (22.68)	187.55 (23.65)
Cortical CSA (mm^2)	165.52 (19.30)	164.08 (19.97)
Cortical thickness (mm)	2.55 (0.32)	2.56 (0.40)
Periosteal circumference (mm)	73.16 (4.72)	72.69 (5.14)
Endosteal circumference (mm)	57.14 (5.51)	56.60 (6.68)
pQCT Proximal 38% Tibia	n=37	n=53
Total CSA (mm^2)	392.17 (44.35)	377.67 (42.50)
Cortical vBMD (mg/cm^3)	1169.84 (23.78)	1174.16 (18.22)
Cortical BMC (mg/mm)	324.58 (39.94)	316.50 (40.72)
Cortical CSA (mm^2)	277.48 (33.92)	269.61 (34.86)
Cortical thickness (mm)	4.90 (0.53)	4.84 (0.46)
Periosteal circumference (mm)	72.11 (4.47)	70.83 (4.24)
Endosteal circumference (mm)	41.33 (5.07)	40.43 (3.55)

vBMD = volumetric bone mineral density, CSA = cross sectional area, BV/TV = trabecular bone volume/bone volume. *p-value < 0.05, **p-value < 0.01

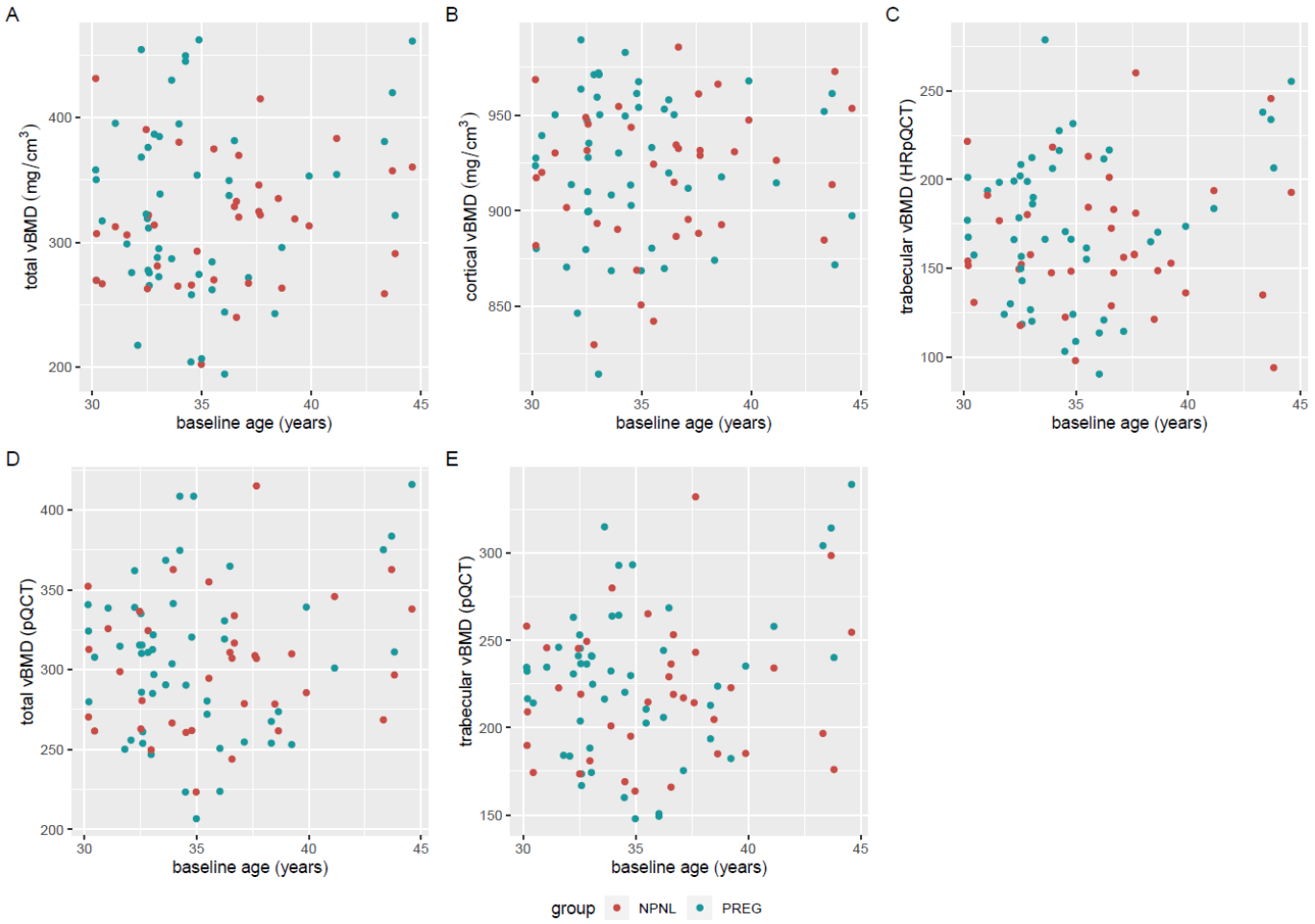
Radius bone outcome measures complete baseline descriptive statistics

Baseline mean (SD) for anthropometry and bone outcome measures of interest as measured with pQCT and HR-pQCT in non-pregnant non-lactating and pregnant groups.

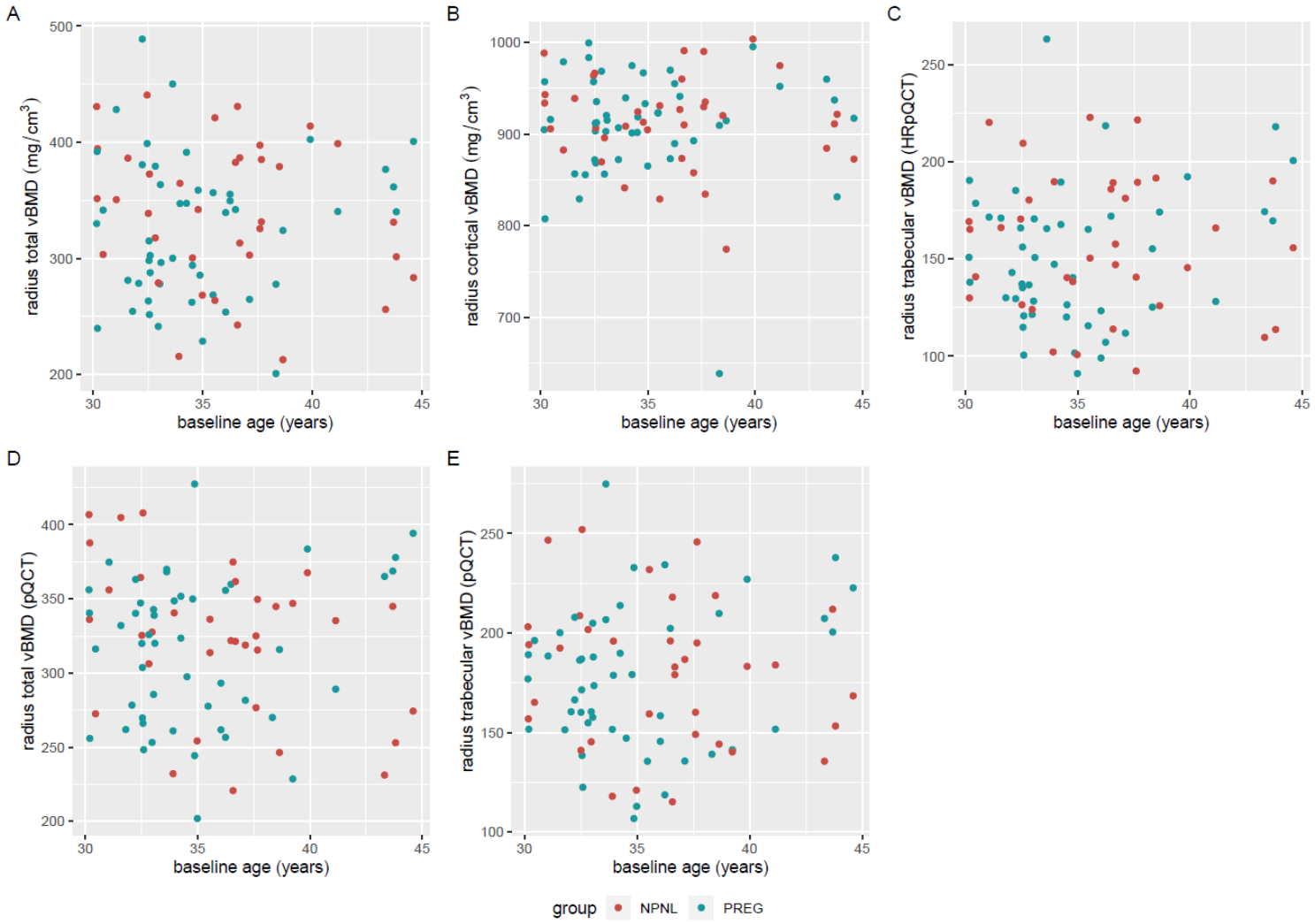
	NPNL	Pregnant
HRpQCT Radius	n=31	n=47
Total vBMD (mg/cm^3)	335.51 (59.82)	324.29 (59.50)
Trabecular vBMD (mg/cm^3)	157.89 (33.28)	150.61 (35.89)
Cortical vBMD (mg/cm^3)	912.43 (49.39)	911.23 (57.87)
BV/TV	0.13 (0.03)	0.13 (0.03)
Trabecular number (mm^{-1})	2.04 (0.25)	1.98 (0.31)
Trabecular thickness (mm)	0.06 (0.01)	0.06 (0.01)
Trabecular separation (mm)	0.43 (0.06)	0.45 (0.09)
Cortical thickness (mm)	0.80 (0.15)	0.79 (0.16)
Cortical porosity (%)	66.40 (5.15)	67.46 (6.33)
Radius 4%	n=35	n=49
Total vBMD 4% (mg/cm^3)	322.92 (50.68)	315.57 (49.93)
Trabecular vBMD 4% (mg/cm^3)	179.99 (36.84)	176.62 (36.20)
Total CSA 4% (mm^2)	325.51 (44.13)	335.59 (43.25)
Cortical subcortical vBMD (mg/cm^3)	439.66 (73.77)	429.12 (68.86)
Radius 33%	n=36	n=52
Total CSA (mm^2)	100.06 (12.72)	98.59 (11.95)
Cortical vBMD (mg/cm^3)	1217.73 (17.37)	1218.11 (15.23)
Cortical BMC (mg/mm)	89.40 (9.73)	88.26 (10.20)
Cortical CSA (mm^2)	73.42 (7.99)	72.47 (8.40)
Cortical thickness (mm)	2.55 (0.20)	2.55 (0.22)
Periosteal circumference (mm)	36.81 (2.19)	36.47 (2.34)
Endosteal circumference (mm)	20.81 (2.16)	20.47 (2.39)

vBMD = volumetric bone mineral density, CSA = cross sectional area, BV/TV = trabecular bone volume/bone volume. *p-value < 0.05, **p-value < 0.01

Distribution of pQCT and HRpQCT vBMD bone outcome measures at baseline by age



Baseline tibia HR-pQCT and pQCT vBMD parameters plotted against age at baseline for pregnant (PREG) and non-pregnant non-lactating (NPNL) women in PABS.



Baseline radius HR-pQCT and pQCT vBMD parameters plotted against age at baseline for pregnant (PREG) and non-pregnant non-lactating (NPNL) women in PABS.

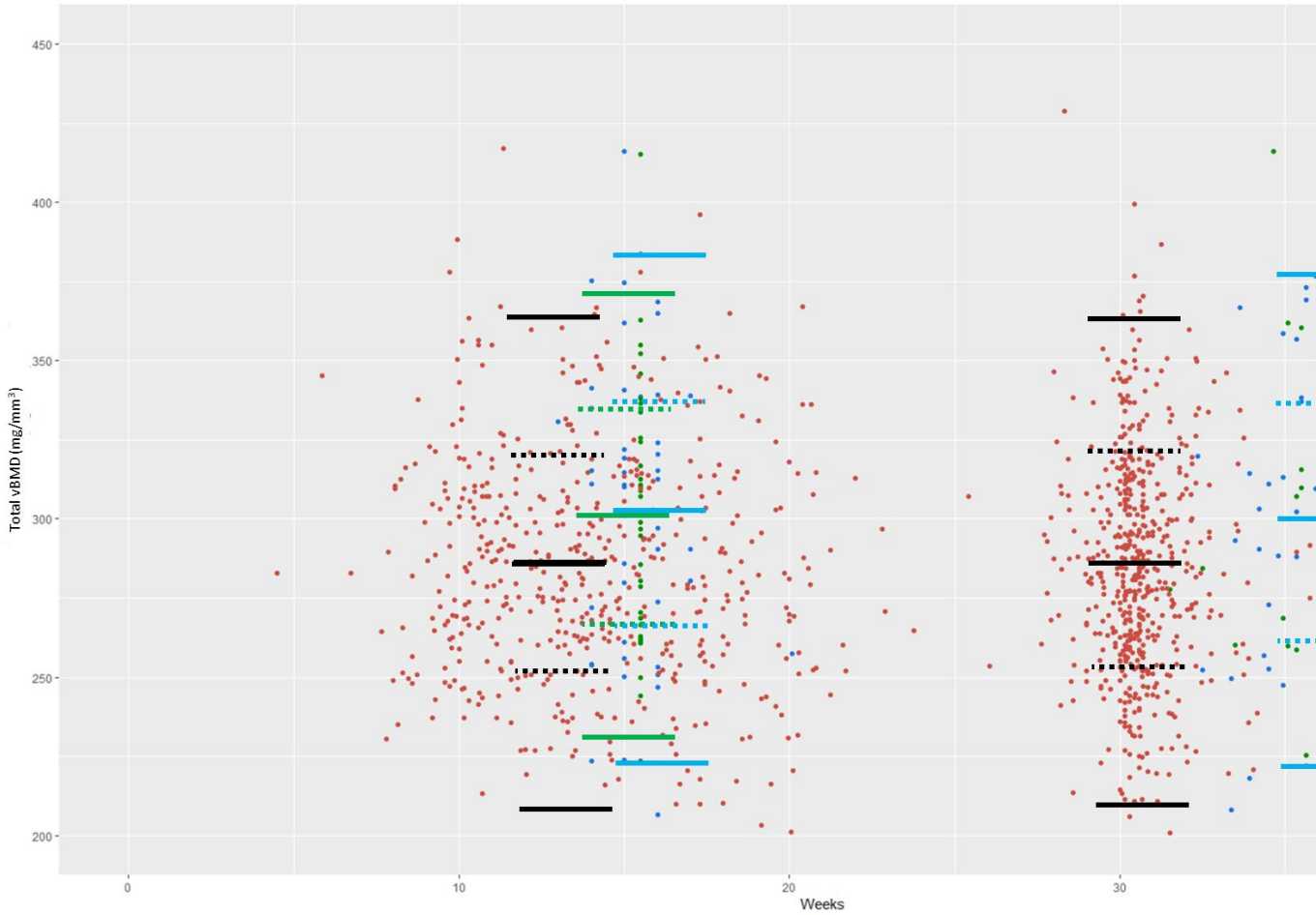
HRpQCT bone outcome measures between-visit change expressed as a percentage in PABS Cambridge

HRpQCT bone outcomes measures at baseline and change by follow-up expressed in absolute units and percentages at the tibia and radius in PABS, Cambridge

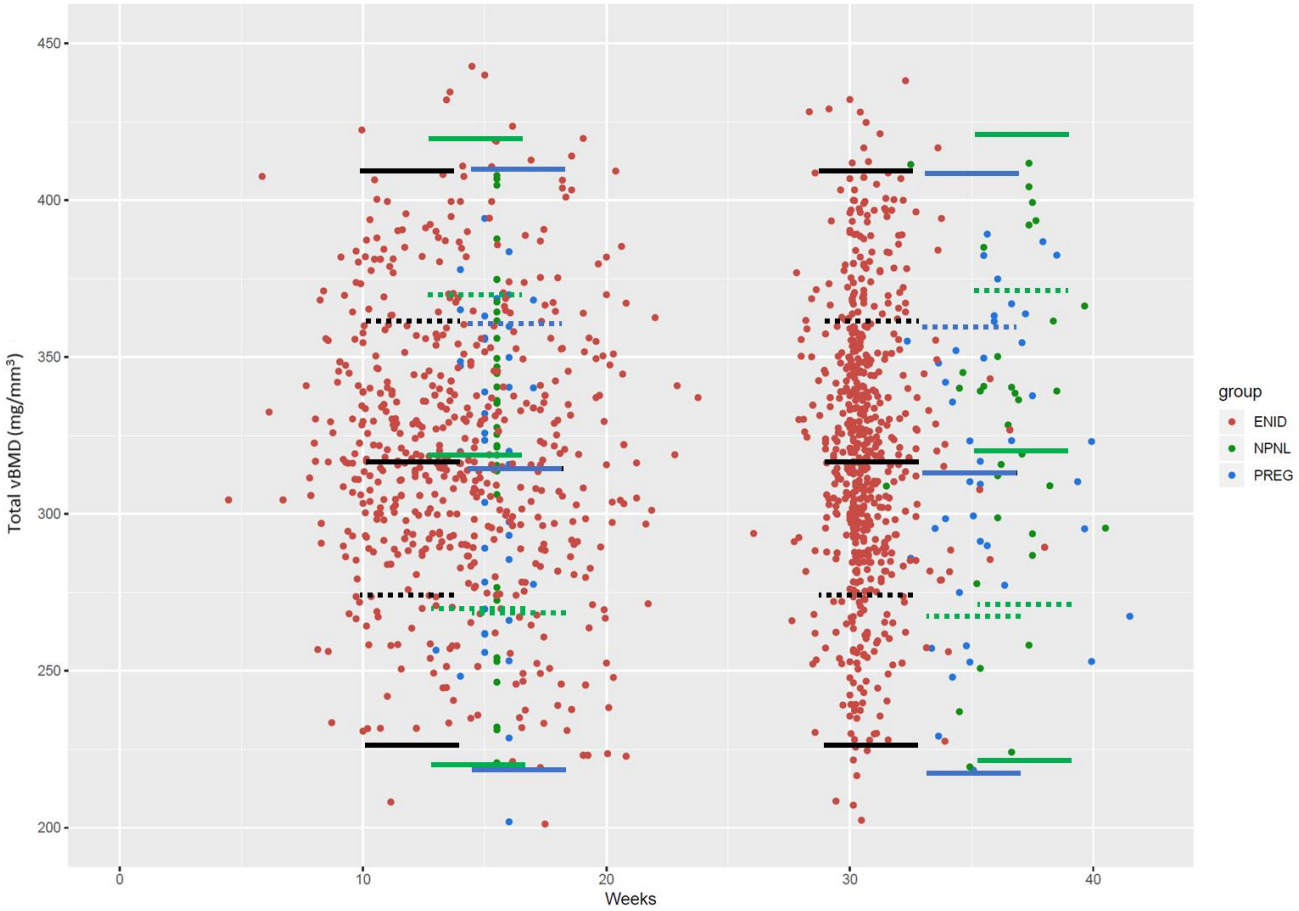
	NPNL			Pregnant		
	Visit 1	Between-visit change	Δ%	Visit 1	Between-visit change	Δ%
Tibia		n=32			n=33	
Total vBMD (mg/cm^3)	315.26 (53.64)	**-1.50 (3.02) ‡‡**	**-0.48 (1.00)**	317.43 (68.09)	**-4.69 (6.32) ‡‡‡**	**-1.39 (1.73)**
Trabecular vBMD (mg/cm^3)	165.53 (38.02)	**-2.55 (2.36) ‡‡‡**	**-1.56 (1.48)**	174.68 (41.33)	**-2.79 (3.59) ‡‡‡**	**-1.48 (1.97)**
Cortical vBMD (mg/cm^3)	921.45 (33.66)	-0.85 (6.47)	-0.08 (0.70)	916.26 (38.81)	**-7.40 (12.91)‡‡**	**-0.81 (1.37)**
BV/TV	0.14 (0.03)	**-0.002 (0.002)‡‡‡**	**-1.56 (1.48)**	0.15 (0.03)	**-0.002 (0.003) ‡‡‡**	**-1.48 (1.97)**
Trabecular number (mm^{-1})	2.13 (0.27)	-0.01 (0.15)	-0.29 (7.18)	**1.98 (0.27)***	**0.09 (0.15) ‡‡**	**4.58 (7.61)**
Trabecular thickness (mm)	0.065 (0.014)	-0.0007 (0.0043)	-0.78 (6.92)	**0.073 (0.013) ***	**-0.004 (0.005) ‡‡‡**	**-5.35 (6.68)**
Trabecular separation (mm)	0.41 (0.06)	0.00 (0.03)	1.10 (7.43)	0.44 (0.08)	**-0.02 (0.03) ‡‡‡**	**-3.66 (6.75)**
Cortical thickness (mm)	1.25 (0.22)	**0.01 (0.01) ‡‡‡**	**0.78 (1.06)**	1.21 (0.29)	**-0.01 (0.02)‡‡**	**-1.21 (1.75)**
Cortical porosity (%)	99.91 (7.16)	0.03 (0.23)	0.03 (0.24)	99.6 (8.3)	**0.25 (0.25) ‡‡‡**	**0.25 (0.25)**
Radius		n=27			n=33	
Total vBMD (mg/cm3)	334.75 (63.13)	**-3.15 (7.70) ‡**	**-0.90 (2.31)**	334.20 (58.74)	**-2.89 (6.90) ‡**	**-0.94 (2.04)**
Trabecular vBMD (mg/cm3)	155.10 (32.80)	**-2.53 (2.23) ‡‡‡**	**-1.74 (1.49)**	153.28 (39.86)	**-2.18 (3.34) ‡‡‡**	**-1.54 (2.07)**
Cortical vBMD (mg/cm3)	916.35 (49.22)	-3.45 (10.17)	-0.37 (1.12)	920.46 (42.47)	**-4.32 (9.42) ‡**	**-0.47 (1.05)**
BV/TV	0.13 (0.03)	**-0.002 (0.002) ‡‡‡**	**-1.74 (1.49)**	0.13 (0.03)	**-0.002 (0.003) ‡‡‡**	**-1.54 (2.07)**
Trabecular number (mm-1)	2.02 (0.23)	0.04 (0.20)	2.06 (10.11)	1.95 (0.33)	0.01 (0.19)	1.27 (10.99)
Trabecular thickness (mm)	0.06 (0.01)	**-0.002 (0.005) ‡**	**-2.93 (8.58)**	0.07 (0.01)	-0.001 (0.007)	-1.79 (9.76)
Trabecular separation (mm)	0.44 (0.06)	-0.005 (0.0419)	-0.86 (9.61)	0.46 (0.10)	-0.002 (0.051)	-0.01 (9.93)
Cortical thickness (mm)	0.81 (0.16)	0.003 (0.0307)	0.50 (3.98)	0.82 (0.14)	-0.005 (0.026)	-0.75 (3.40)
Cortical porosity (%)	66.74 (5.35)	-0.10 (0.36)	-0.16 (0.56)	66.68 (5.80)	0.08 (0.35)	0.07 (0.52)
Total vBMD (mg/cm3)	334.75 (63.13)	**-3.15 (7.70) ‡**	**-0.90 (2.31)**	334.20 (58.74)	**-2.89 (6.90) ‡**	**-0.94 (2.04)**

*p<0.05, **p<0.01, ***p<0.001 independent t-test between groups at baseline; ‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001 single-sided t-test vs 0. vBMD = volumetric bone mineral density, CSA = cross sectional area, BV/TV = trabecular bone volume/bone volume

Plots of ENID and PABS pQCT total vBMD at baseline and follow visits



Tibia total vBMD (pQCT) at baseline and follow up visits (by week of pregnancy) for ENID (red), PABS pregnant (PREG, blue), and PABS (NPNL, green). Dashed lines represent 1SD, solid lines represent 2SD



Radius total vBMD (pQCT) at baseline and follow up visits (by week of pregnancy) for ENID (red), PABS pregnant (PREG, blue), and PABS (NPNL, green), Dashed lines represent 1SD, solid lines represent 2SD.