UNIVERSITY OF SOUTHAMPTON

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Mechanistic determinants of musculoskeletal health throughout childhood: findings from the Southampton Women's Survey and the MAVIDOS trial

By

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ABSTRACT

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Elizabeth M Curtis

Poor intrauterine and childhood growth have been linked with the risk of osteoporosis in later life, a relationship mediated through a variety of interacting factors. The aim of this work was to use epidemiological methods to contribute to our understanding of the mechanisms early in life which relate to offspring musculoskeletal development, focusing on epigenetic change, vitamin D and alterations in maternal bone turnover. These were investigated through a large prospective mother-offspring cohort, the Southampton Women's Survey (SWS) and a unique randomised, double-blind, placebo-controlled trial of vitamin D supplementation in pregnancy (MAVIDOS).

In the SWS, DNA methylation at a locus linked to ageing and cell cycle regulation *(CDKN2A)* has been shown to be associated with childhood bone mass. Higher levels of methylation in umbilical cord of specific CpG dinucleotides within the *CDKN2A* gene locus were associated with lower total whole body minus head bone area, BMC and areal BMD at 4 and 6 years [a 10% increase in methylation was associated with a decrease in BMC of 4-9 g at age 4 years, $p \leq 0.001$, n = 538].

Then, building on previous observations in the SWS, methylation at another locus, linked to vitamin D signalling *(RXRA),* was altered by maternal vitamin D supplementation, and associated with neonatal bone mass, in the MAVIDOS trial. For example, mean difference in % methylation between the supplemented and placebo groups at one CpG site, CpG 5 [-1.98% (95%CI: -3.65 to -0.32), p = 0.02, n = 447].

In the same trial, the relationship between maternal vitamin D supplementation and a urinary marker of maternal bone resorption was studied (C-terminal telopeptide of type 1 collagen, CTX). Median CTX increased in both placebo and cholecalciferol supplemented groups, but the increase from early to late pregnancy was greater in the placebo group [111% (IQR 47, 211%) n = 188] than

the cholecalciferol supplemented group [89% (IQR 23, 83%) n = 184; p difference = 0.02]. Higher maternal CTX was associated with lower DXA measures of bone mass at the total hip and lumbar spine in the early postpartum period.

In MAVIDOS, maternal pregnancy cholecalciferol supplementation (vs placebo) led to greater offspring neonatal bone indices in winter births only. However, on reassessment at age 4 years, it was found to be associated with greater offspring measures of whole body (less head) bone mineral density [0.18 SD, 95% CI 0.00, 0.35] and lean mass [0.17 SD, 95% CI 0.00, 0.34], regardless of season of birth.

These findings inform our understanding of the early life mechanisms related to maternal vitamin D status, epigenetic marks, maternal bone health and offspring musculoskeletal development and may guide future public health interventions aimed at preventing osteoporotic fractures and sarcopenia.

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Declaration of Authorship

I, Elizabeth Mary Curtis declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Mechanistic determinants of musculoskeletal health throughout childhood: findings from the Southampton Women's Survey and the MAVIDOS trial

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
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Project Outputs

Findings presented in this work

- **Chapter 4:** **Elizabeth M Curtis*,** Robert Murray*, Philip Titcombe, Rebecca Clarke-Harris, Paula Costello, Emma Garratt, Joanna Holbrook, Sheila Barton, Hazel Inskip, Keith M Godfrey, Christopher Bell, Cyrus Cooper, Karen A Lillycrop, Nicholas C Harvey. *Perinatal methylation at CDKN2A locus and offspring bone mass: Findings from the Southampton Women's Survey.* Journal of Bone and Mineral Research. 2017 Oct;32(10):2030-2040

- **Chapter 5:** **Elizabeth M Curtis***, Nevena Krstic*, Eloïse Cook, Stefania D'angelo, Sarah R Crozier, Rebecca J Moon, Robert Murray, Emma Garratt, Paula Costello, Jane Cleal, Brogan Ashley, Nicholas J Bishop, Stephen Kennedy, Aris T Papageorghiou, Inez Schoenmakers, Robert Fraser, Saurabh V Gandhi, Ann Prentice, M Kassim Javaid, Hazel M Inskip, Keith M Godfrey, Christopher G Bell, Karen A Lillycrop, Cyrus Cooper, Nicholas C Harvey, MAVIDOS Trial Group. *Gestational vitamin D supplementation leads to reduced perinatal RXRA DNA methylation: Results from the MAVIDOS trial.* Journal of Bone and Mineral Research. 2019 Feb; 34(2):231-240

- **Chapter 6:** **Elizabeth M Curtis**, Camille Parsons, Kate Maslin, Stefania D'Angelo, Rebecca J Moon, Sarah R Crozier, Fatma Gossiel, Nicholas J Bishop, Stephen Kennedy, Aris T Papageorghiou, Robert Fraser, Saurabh Gandhi, Ann Prentice, Hazel M Inskip, Keith M Godfrey, Inez Schoenmakers, M Kassim Javaid, Richard Eastell, Cyrus Cooper, Nicholas C Harvey. *O29 Bone turnover in pregnancy, measured by urinary C-terminal telopeptide of type I collagen (CTX), is influenced by vitamin D supplementation and is associated with maternal bone health: findings from the MAVIDOS trial.* Rheumatology, Volume 58, Issue Supplement_3, April 2019

Related publications in the field of osteoporosis and developmental origins of disease, to which the candidate has contributed

Original Research

- Moon RJ, **Curtis EM**, Cooper C, Davies JH, Harvey NC. *Vitamin D supplementation: are multivitamins sufficient?* Archives of Disease in Childhood. 2019 Feb 25 [Epub ahead of print]

- Nicholas Fuggle, Joseph Singer, Michael Clynes, **Elizabeth Curtis**, Pallavi Wyawahare, Hayley Denison, Moira Gilmour, Geoffrey Robinson, Elaine Dennison. *Risk factors for fracture in alcohol dependent, premenopausal females.* International Journal of Alcohol and Drug Research. 2018 Nov; 7(2):2-7

- Julien Paccou, Stefania D'Angelo, Amanda Rhodes, **Elizabeth M Curtis**, Zahra Raisi-Estabragh, Mark Edwards, Karen Walker-Bone, Cyrus Cooper, Steffen E Petersen, Nicholas C Harvey. *Prior fragility fracture and risk of incident ischaemic cardiovascular events: Results from UK Biobank.* Osteoporosis International. 2018 Jun;29(6):1321-1328

- Nicholas C Harvey, Stefania D'Angelo, Julien Paccou, **Elizabeth M Curtis**, Mark Edwards, Zahra Raisi-Estabragh, Karen Walker-Bone, Steffen E Petersen, Cyrus Cooper. *Calcium and vitamin D supplementation are not associated with risk of incident ischaemic cardiac*

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    - Chapter 8 (Developmental plasticity, epigenetic mechanisms and early life influences on adult health and disease)
    - Chapter 9 (Epigenetic mechanisms in bone development)
    - Chapter 14 (Therapeutic approaches to bone protection in adulthood)

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Definitions and Abbreviations

$1,25(OH)_2D$	1, 25-dihydroxyvitamin D; calcitriol
25(OH)D	25-hydroxyvitamin D; calcidiol
aBMD	Areal bone mineral density
BMAD	Bone mineral apparent density
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
Ca^{2+}	Ionised calcium concentration
CaSR	Calcium sensing receptor
CDKN2A	Cyclin Dependent Kinase Inhibitor –2A
CI	Confidence interval
CSA	Cross-sectional area
CT	Computed tomography
CTX	C-terminal telopeptide of type 1 collagen
CV	Coefficient of variation
DBP	Vitamin D binding protein
DoH	Department of Health
DOHAD	Developmental origins of health and disease
DXA	Dual energy X-ray Absorptiometry
FGF-23	Fibroblast growth factor-23
FM	Fat mass
GDM	Gestational diabetes mellitus
IQR	Interquartile range

IU	International units
LM	Lean mass
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
LS	Lumbar spine
MAVIDOS	Maternal Vitamin D Osteoporosis Study
MSC	Mesenchymal stem cell
MUAC	Mid upper-arm circumference
NCD	Non-communicable disease
OFC	Occipto-frontal circumference
PBM	Peak bone mass
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
pQCT	Peripheral quantitative computed tomography
RCT	Randomised controlled trial
ROI	Region of interest
RXR	Retinoid X receptor
SD	Standard deviation
SFT	Skinfold thickness
SNP	Single nucleotide polymorphism
SWS	Southampton Women's Survey
T2DM	Type 2 diabetes mellitus
UK	United Kingdom
USA	United States of America

UVB	Ultraviolet B
VDD	Vitamin D deficiency
VDDR	Vitamin D dependent rickets
VDR	Vitamin D receptor
WBLH	Whole body less head
WHO	World Health Organisation

Chapter 1: Literature Review

1.1 Context: The Developmental Origins of Disease Hypothesis and Osteoporosis

Increasing life expectancy due to improvements in healthcare, diet and sanitation worldwide have led to a growing burden of chronic, non-communicable diseases (NCD). The most widely recognised include cardiovascular disease, type 2 diabetes mellitus (T2DM), chronic respiratory conditions, malignancy, and, of course, a deterioration in musculoskeletal health (1). Whilst traditional approaches to management of such conditions have involved targeting those at highest risk in later life, emerging evidence suggests that there are opportunities for prevention of non-communicable diseases early in the lifecourse, even prior to conception, and during pregnancy. The developmental origins of health and disease (DOHAD) hypothesis proposes that exposure to environmental and nutritional factors during sensitive periods in development, including in utero or during early childhood, influences the risk for specific diseases in adult life. Therefore, a population-based approach to reducing the non-communicable disease burden could be undertaken, through the identification of critical factors which might influence, or program, developmental trajectories. There is evidence that changes to the epigenome, influenced by the early environment, may be the basis for developmental programming. This work aims to elucidate associations between epigenetic changes, through DNA methylation, and bone health throughout childhood. It also aims to study whether maternal vitamin D supplementation during pregnancy is associated with differences in DNA methylation in the offspring, and to study associations between vitamin D supplementation, DNA methylation and offspring musculoskeletal outcomes. Studies of bone turnover markers may also provide an improved understanding of the relationship between 25(OH)D status in pregnancy, maternal and offspring bone health.

Such approaches might represent a novel strategy to assess an individual's risk of later osteoporosis through an understanding of epigenetic biomarkers for bone health, and enable us to understand potential mechanisms behind the developmental origins of osteoporosis.

1.1.1 Introduction to the Developmental Origins of Disease Hypothesis

The fetal programming hypothesis is often attributed to Professor David JP Barker (1938-2013), widely recognised as one of the most influential epidemiologists of our time. His hypothesis has transformed our thinking about a variety of chronic diseases, including diabetes, cardiovascular disease, cancer, osteoporosis and various psychiatric illnesses. The idea that these diseases

cannot be explained solely by genetic predisposition and unhealthy adult lifestyles, but rather that their roots lay in the early environment, was initially thought to be controversial. The new developmental model for the origins of disease proposed that maternal, infant and childhood nutrition, health, exposure to infections, and lifestyle permanently "programmes" the body's metabolism and growth, thereby determining the pathologies of old age.

Though these ideas were met with widespread criticism when first proposed, there is now a wealth of evidence into adverse developmental conditions and later disease to support this hypothesis, the "developmental origins of health and disease", or DOHAD. The idea that the poorer health of people in lower socioeconomic groups or living in poverty is linked to poorer welfare of mothers and babies has led to changes in the approach to maternal, fetal and childhood health worldwide (2). The premise of developmental plasticity – that a single genotype, influenced by specific intrauterine events, has the capability to produce different phenotypes – is widely accepted (3). Specific developmental periods are known to exist whereby an organism is sensitive, or plastic, to its environment. The organism, in general, develops a phenotype which is best suited to its environment – for example, a malnourished fetus will alter the structure and function of various organs – including the cardiovascular, endocrine and musculoskeletal system to preserve neurodevelopment and enable its survival in the womb. Such adaptations prepare the baby for life in a nutrient poor environment where additional stressors may be encountered. These changes may persist through an individual's lifespan and may be advantageous or disadvantageous to the health and survival of the individual, dependent on the environmental and nutritional challenges they face. If mismatch occurs between the environment experienced in utero and the environment experienced in postnatal life, disease may result. The most effective time to intervene and reduce the risk of chronic non-communicable diseases may be early in life when plasticity is at its greatest; this concept is illustrated in Figure 1.1.

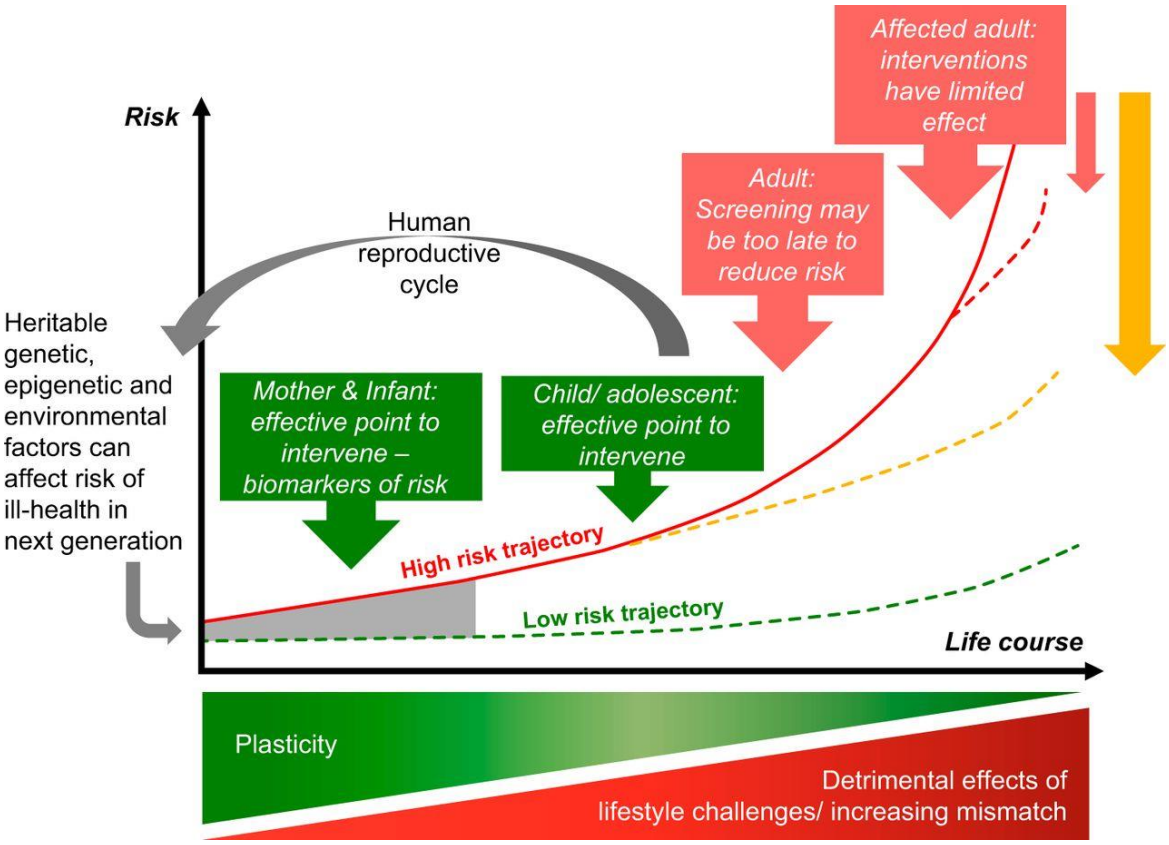


Figure 1.1 Illustration of the lifecourse strategy for noncommunicable disease risk.

Reproduced with permission from Hanson and Gluckman, 2014 (4)

To summarise Figure 1.1, the prenatal period establishes the trajectory for disease risk through interactions between genetic, epigenetic and environmental factors. NCD risk increases throughout the lifecourse in a nonlinear way due to decreasing plasticity and accumulative damage from lifestyle-imposed risk or other challenges. The effects of mismatch between the developmentally induced and evolutionarily influenced phenotype and the environment also increases throughout the lifecourse. Interventions affecting adults can be of benefit, but only to a degree, and indeed screening of adults in middle age may be too late to decrease the risk of many NCDs substantially. Interventions in childhood/adolescence are likely to be more effective and can also reduce the NCD risk in the next generation. The impact of intervention at different timepoints differs. Plasticity decreases throughout the lifecourse, so interventions in early life have the greatest impact in improving the response to environmental challenges.

1.1.3 Initial evidence for the DOHAD hypothesis

The first clues that the early life environment may be important in determining risk of adult disease came from studies of coronary heart disease. It was noted in the early 20th century that the incidence of coronary heart disease was rising rapidly, becoming the most common cause of death in Western countries. Several decades later in the 1980s and 1990s, the incidence of coronary disease was noted to also be rising in developing parts of the world, such as India and China. Genetic factors could not explain the sudden increase in coronary artery disease incidence, so studies of the lifestyles of men and women were carried out. The rising rates of heart disease were initially attributed to increasing prosperity (mirroring the rising incidence of heart disease in industrialising nations). However within Britain, paradoxically, rates were twice as high in poorer areas of the country, and in lower income groups (5, 6).

The discovery, by Richard Doll, of the powerful effects of cigarette smoking on lung cancer directed the focus towards smoking, dietary fat, obesity and blood pressure on heart disease, though these were found to only explain a limited portion of the risk (7). However, for a man falling into the lowest socioeconomic category, but also in the lowest risk groups for serum cholesterol, cigarette smoking, blood pressure and pre-existing symptoms of coronary heart disease, the most common cause of death is still coronary heart disease.

Geographical studies provided the first clues that solutions to this dichotomy lay in early life. Studies of the rates of death from coronary heart disease throughout England and Wales were shown to parallel death rates in babies. Analyses of birth registries in the county of Hertfordshire, UK, were the first to show that birthweight at the lower end of the normal range was associated with higher rates of coronary heart disease and type 2 diabetes in later life (8, 9). These findings have since been extensively replicated, in both men and women in Europe, the USA, China and India (10-12).

Low birthweight is now known to be associated with a host of other chronic diseases including cancer, osteoporosis and anxiety disorders (13-15). The effect of low birthweight or relative thinness appears to be independent of environmental risk factors as adults, such as smoking, obesity, cholesterol or socioeconomic status in the case of cardiovascular disease (11, 16).

Osteoporosis as a disease model for which there is compelling evidence for the role of the early environment in its development, and such evidence provides a foundation for this work.

1.1.4 Osteoporosis: definition and burden of disease

Osteoporosis is a skeletal disorder characterised by low bone mass and loss of the normal bone microarchitecture, leading to increased bone fragility and therefore susceptibility to fracture (WHO 1993). In adults, a diagnosis of osteoporosis is based on a measurement of bone mineral density (BMD) at the femoral neck by Dual Energy X-ray Absorptiometry (DXA). For all adults, independent of age and sex, an individual's measured BMD is related to data from a reference population comprised of healthy young adult females to generate a standard deviation "T-score". A BMD that is 2.5 standard deviations (SD) or more below the young adult female mean defines osteoporosis; a T-score between -1.0 and -2.5 SD as osteopenia (17). It is important to note that the definition of osteoporosis in childhood differs to that in adulthood due to the increase in BMD during growth. Thus, the definition includes a clinical component; one or more non-traumatic vertebral fractures is considered to represent osteoporosis independent of BMD, or sustaining multiple long-bone fractures (two or more below the age of 10 years, or 3 or more above the age of 10 years) in conjunction with low BMD on DXA (18).

The Global Burden of Disease study demonstrated a massive impact of musculoskeletal conditions on populations worldwide: the number of disability adjusted life years (DALYs) attributable to musculoskeletal disorders has increased by 17.7% between 2005 and 2013 (19). "Low back pain" ranked top, "neck pain" fourth, "other musculoskeletal" tenth, and "osteoarthritis" thirteenth in the WHO rankings of causes for years lived with disability worldwide in 2013 (1), with osteoporotic fractures playing a major part in the "back pain" and "other musculoskeletal" categories. The 2004 US Surgeon General's report estimated that 10 million Americans over the age of 50 have osteoporosis, leading to 1.5 million fragility fractures (of the hip, spine, wrist, humerus, pelvis, scapula or ribs) each year (20), with another 34 million Americans at risk of the disease. In the EU, a report estimated that in 2010, 6.6% of men and 22.1% of women aged over 50 years had osteoporosis, and that there were 3.5 million fragility fractures (21). The annual direct costs attributable to fracture treatment in the EU equate to approximately €24 billion, though when indirect costs such as long term care and fracture prevention therapies are taken into account, this figure rises to €37 billion per year (21) Table 1.1. A British study indicated similar population risks (22), with 1 in 2 women and 1 in 5 men aged 50 years expected to have an osteoporosis-related fracture in their remaining lifetime. In addition to the associated morbidity and economic cost, fractures are associated with an increased mortality, with hip fractures associated with an excess mortality of 10-20% in the first year after fracture (23), with a similar proportion requiring long term residential or nursing care (24).

Table 1.1 Impact of osteoporosis-related fractures across Europe.

Data derived from (Hernlund et al., Archives of Osteoporosis, 2013)

	Hip	***Spine***	***Wrist***
Lifetime risk in Women (%)	23	29	21
Lifetime risk in Men (%)	11	14	5
Cases / year	620,000	810,000	574,000
Hospitalization (%)	100	2-10	5
Relative survival	0.83	0.82	1.00
Costs: All sites combined ~ €37 billion			

1.1.5 Osteoporosis and the developmental origins of disease hypothesis

As osteoporosis is becoming a pressing public health issue, research has been directed towards ways of preventing osteoporotic fracture, by studying factors which affect the peak bone mass attained by an individual during growth and development and also by studying factors affecting the rate of subsequent bone loss. Peak bone mass, a key component of the mechanical strength of bone, is defined as the maximum total skeletal mass accrued at the completion of skeletal development. It increases throughout fetal, infant, childhood and early adult life reaching a peak in the third to fourth decade, the exact timing varying by site and sex, as shown in Figure 1.2 (25, 26). Peak bone mass has been shown in mathematical modelling studies to be a more powerful predictor of the age of osteoporosis development than age at menopause or rate of subsequent age-related bone loss (27). Acquiring optimal peak bone mass is therefore essential to bone health as an adult, so targeting the developmental processes which influence bone mineral accrual during early life may be the key to future prevention of the disease, and is central to this line of research.

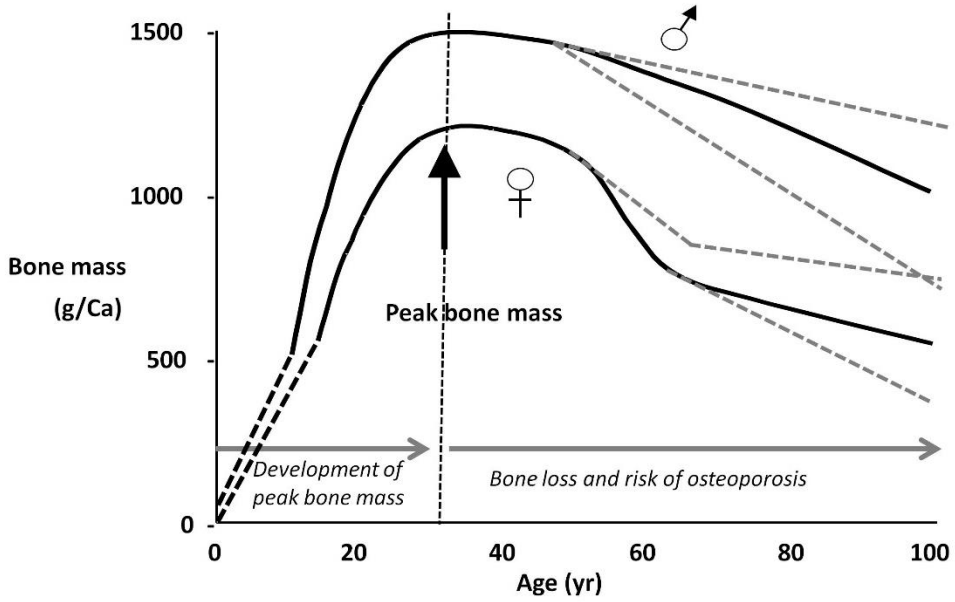


Figure 1.2 Changes in bone mass throughout the lifecourse. Bone mass increases from development in utero to a peak in early adulthood, at around age 30 years, with a plateau then decline thereafter. A rapid decline occurs in women around the time of the menopause. Dotted lines show opportunities for modulation of bone mass. Based on image from Cooper and Melton (1992) (28)

1.2 Methods for assessment of bone health in clinical research

An understanding of the assessment methods of bone strength are important, prior to discussion of skeletal development and the evidence behind the developmental origins of osteoporosis hypothesis.

1.2.1 Dual energy X-ray Absorptiometry (DXA)

DXA is considered by many clinicians to be the gold standard in bone densitometry, and as such is the most widely used technique for the measurement of bone mass and density. In DXA scanning, two beams of x-rays of different energies are directed from a radiation source towards a radiation detector, and the participant is placed on a table with the region of interest between the source (below the table) and detector (above). The principle manufacturers of DXA instruments, Hologic and GE Lunar use different technologies to generate the two energy beams. GE Lunar systems create a "K edge filter" that absorbs X rays in the middle range of energies but allows those of high and low energy to pass through to the subject, whilst Hologic instruments utilise a switching pulse system that rapidly alternates the voltage of the X-ray generator to produce the two energy levels (29). The beam attenuation is dependent on the thickness and density of the tissue- therefore, attenuation is greater when it passes through a denser tissue such as bone, than a softer tissue, such as fat.

As two different X-ray energies are used in DXA, theoretically the body is assumed to consist of two compartments – bone mineral and soft tissue. However, the DXA instrument is able to generate a pixel-by-pixel map of the X-ray attenuation coefficients. The ratios of attenuation of the two beams are compared to known constants for BMD and soft tissue to determine the content of each pixel. Bone edges are detected using computational algorithms, such that the total projected area of bone is derived by summing the pixels within the bone outline. The BMD value for the area reported is the mean BMD measured within the pixels identified as bone. The software is able to calculate the bone mineral content, from the mean BMD multiplied by the bone area. Body composition may also be assessed as the degree of attenuation of X-rays by bone mineral and fat are known constants, established by the cross-calibration with phantoms of known composition. As such, DXA reports measurements of BMC, fat mass and non-bone fat-free mass (commonly referred to as lean mass). Soft tissue composition can only be examined in pixels which do not contain bone mineral and in these pixels, the proportion of fat is linearly related to the ratio of attenuation of the two energies. Around a 40% of pixels contain bone in a whole body

scan, and therefore soft tissue composition of these pixels is estimated from the surrounding tissues (30, 31).

It is important to remember that bone mineral content (BMC) measured by DXA is purely an estimate of quantity of the bone mineral in the path of the X-ray beam (calcium hydroxyapatite only – not the protein or lipid components of bone), and is not a true measure of bone mass – rather a surrogate marker for it. The accuracy of DXA in assessing body composition has been demonstrated by comparison to chemical analysis of carcass composition in piglets and monkeys (32, 33). However, there are various limitations to DXA, particularly when used in children.

Calculation of BMC using DXA assumes a constant ratio of fat to lean mass in the overlying soft tissue, which can, of course, vary between subjects. In subjects with greater tissue depths, particularly if more than 25 cm, more of the lower energy photons will be absorbed by the soft tissue resulting in an overestimation of fat mass (34). In addition, the need for estimation of soft tissue composition in pixels which contain bone mineral can lead to under- or over-estimation of the true soft tissue composition. Intramuscular fat can also not be distinguished from lean tissue, leading to a potential underestimation of whole body fat mass. Further errors in the estimation of body composition in children can arise due to differences in the hydration of lean tissue. The attenuation coefficients for soft tissue are calibrated to assume a hydration of fat-free tissue of 73%; and, whilst dehydration, exercise and pathological processes can all result in variations in tissue hydration, variation also occurs with age. Lean tissue hydration may be up to 90% in infancy and can lead to an overestimation of fat mass, but studies have shown that in reality the magnitude of the overestimation is small and clinically irrelevant (around 1%) (35, 36). Finally, in children, bone edge detection is more difficult due to their lower BMD relative to adults, which can lead to false identification of bone as soft tissue and underestimation of the bone area. However, this issue has now largely been overcome through the use of specific paediatric software.

In the acquisition of a DXA scan, a three dimensional (3D) structure is converted into a two dimensional (2D) image, a combination of the high and low energy attenuations, therefore DXA does not provide a true measurement of volumetric BMD (vBMD). BMD assessed by DXA is therefore reported as an “areal” BMD, (aBMD, g/cm^2); hence a thin, high density bone and a thick, lower density bone could have the same aBMD. In a recent study of fractures in adults, BMC and aBMD (but not bone area) assessed by DXA similarly predict fracture risk (37), however, no similar study exists in children (this issue is discussed further in section 1.4.8). Various methods to attempt to correct for this problem have been employed, such as calculation of an approximate

volume for the projected region measured by DXA (38, 39). This allows for calculation of bone mineral apparent density (BMAD) – the ratio of the BMC to a cuboidal or cylindrical estimate of bone volume and can be used for spine and hip. A further modification of the BMAD can be made, in which bone volume is additionally adjusted for height to correct for body size. These adjustments are particularly useful in children – as children grow, so does the volume of bone (40, 41). Given a constant bone density for example, a larger vertebra (more common in people of taller stature), would typically yield higher areal BMD results than for a smaller one (shorter stature) as illustrated in Figure 1.3.

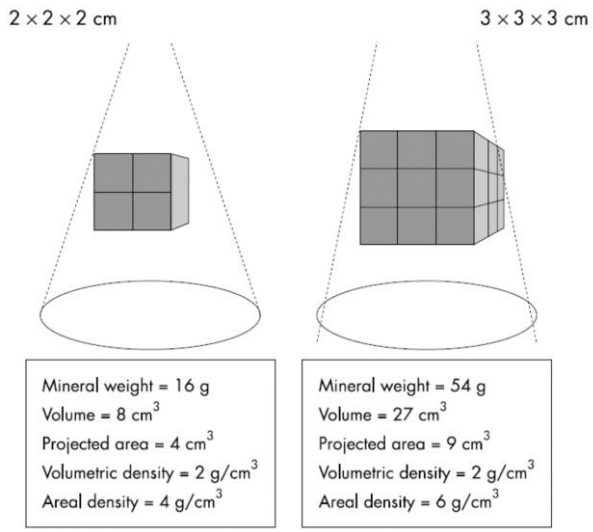


Figure 1.3 The pitfalls of areal BMD assessment in children, reproduced with permission from Fewtrell et al. (42) (adapted from Carter et al.) (39).

A further limitation in children is movement artefact - acquisition time for a whole body DXA scan in a preschool child and an adult are approximately 5 and 8 minutes, respectively – many children struggle to keep still for this duration. As the instrument is open and the radiation exposure is low, a parent can remain in the scanning room and children can watch a film to help to encourage them to keep still, as shown in Figure 1.4.

The radiation dose to which an individual is exposed in a DXA scan is low. A whole body, lumbar spine and hip DXA scan exposes a pre-school child to an effective radiation dose of approximately 21 micro Sieverts (μSv) of radiation, and an adult to 16 μSv. This is approximately equal to 2-3 days background radiation in the most parts of the UK, or one day in Cornwall where background radiation is higher (43). A transatlantic flight exposes an individual to a radiation dose of approximately 80 μSv (43).

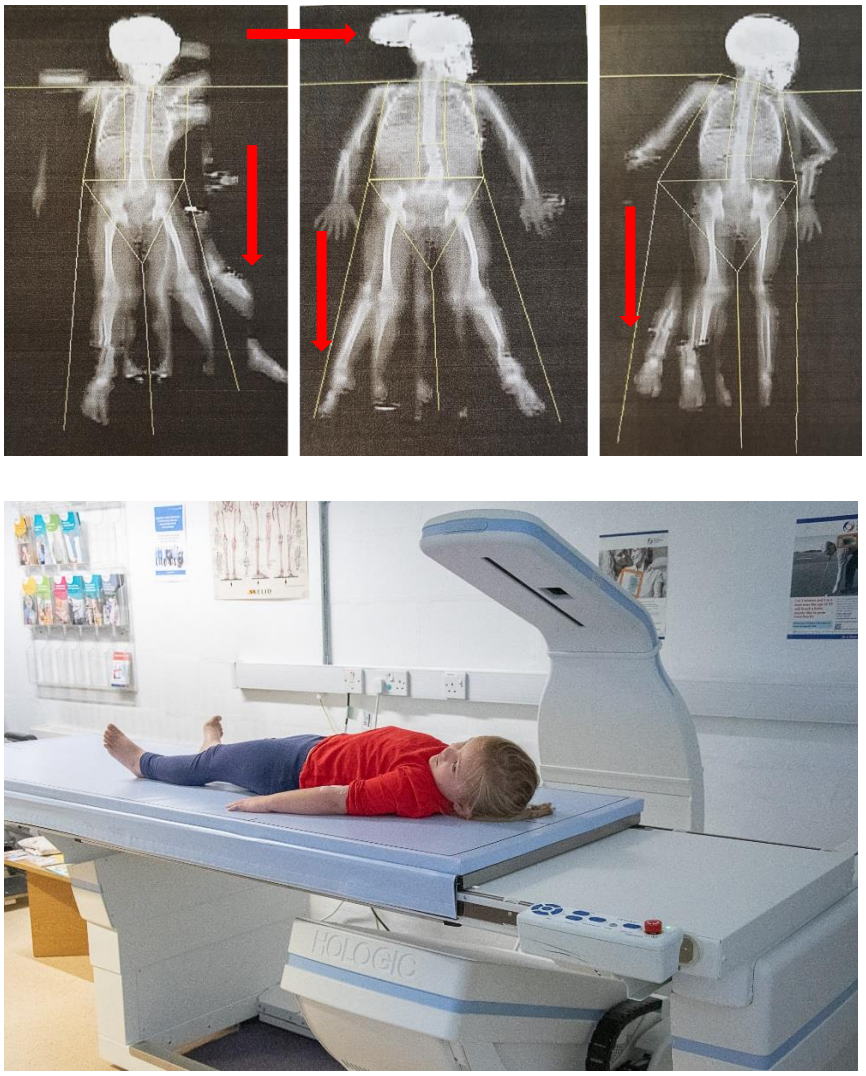


Figure 1.4 Examples of movement artefact (multiple limb images, skull images, indicated with arrows) in DXA scans collected at age 4 years in the MAVIDOS study, and a child undergoing DXA using the Hologic Horizon Dual Energy X-ray Absorptiometer
Photo credit: author's own. Parental permission was granted for publication

1.2.2 Peripheral Quantitative Computed Tomography

Computed tomography (CT) scanning was initially introduced in the 1970s, with the first CT head scan in 1973, and whole body scanning following soon after. CT, like DXA, uses the differential attenuation of X-ray by tissue types. In contrast to DXA, CT quantifies body composition at the tissue-organ level (e.g. bone, adipose tissue, skeletal muscle) rather than molecular level (44). As the X-ray beams are transmitted and detected at many points around the structure, this creates a matrix of X-ray beams of different strengths. Through computation, a cross-sectional image is formed of pixels on a grey-scale, the relative colour of each reflecting the density, and therefore composition, of the tissue. Higher atomic number materials (such as calcium hydroxyapatite) absorb more x-rays, and appear whiter on the image. To transform the radiodensity in Hounsfield

units (HU) into bone mineral equivalents (mg/cm^3), a calculation is performed by the scanner using a predetermined ratio, and is calibrated by the regular scanning of a bone mineral phantom.

Whilst CT can provide more information on tissue types than DXA, its use in clinical research is limited by a number of factors; cost, high radiation exposure and compliance with scan acquisition in young children. Peripheral quantitative computed tomography (pQCT), as shown in Figure 1.5 uses a smaller and therefore less expensive instrument with the ability to accommodate a single limb rather than the whole body. True volumetric BMD, in addition to muscle cross-sectional area and intramuscular adipose tissue can be ascertained from cross sectional images obtained through a limb. pQCT instruments are available in relatively few research units, and this technique is extremely sensitive to movement artefact, but data can be successfully obtained in children (45, 46). Another advantage is that radiation exposure is low; scanning a single limb at 4 sites exposes the child to approximately 1.5 μSv of radiation compared to 5 μSv for a whole body DXA scan.

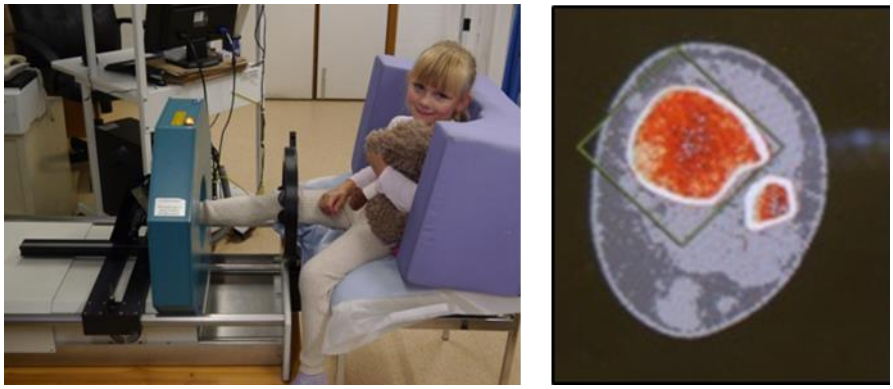


Figure 1.5 4 year old child undergoing pQCT scanning using STRATEC XCT instrument. An example of a pQCT image from the right lower leg of a child is shown (not from the photographed participant).

Photo credit: Dr Rebecca Moon, parental permission was granted for publication.

1.2.3 High Resolution Peripheral Quantitative Computed Tomography

High resolution peripheral quantitative computed tomography (HRpQCT) allows us to examine the bone at the microarchitectural level, and can be thought of as a "virtual bone biopsy". This allows us to make a more comprehensive assessment of bone strength – as, for example, the large variability in the biomechanical properties of trabecular bone cannot be explained purely by measurement of bone mass (47, 48). In effect, it may not be the mass of the bone, but rather its design, which is important in determining risk of fracture.

Bone biopsies have been used historically to assess trabecular bone morphometry, usually at the iliac crest, but these are invasive and lead to localised bone damage. This approach would

certainly not be suitable in a cohort of healthy children. Magnetic resonance imaging (MRI) has also been used to provide a non-invasive assessment of bone microstructure, but its use is challenging due to sensitivity to motion, complex image processing and operator requirements, expense and scanner availability. Its major advantage is the lack of ionising radiation exposure and also the ability to image central sites such as the proximal femur, but it often has suboptimal spatial resolution.

For many years, basic science researchers have used desktop micro-CT to investigate the microarchitecture of bone specimens, as well as small animal models of skeletal disease. More recently, a high-resolution, limited field of view CT device has become commercially available specifically for the assessment of bone microstructure in the peripheral skeleton – HRpQCT (Figure 1.6 XtremeCT, Scanco Medical AG, Brüttisellen, Switzerland). This has introduced a new dimension into the imaging of bones and joints as it provides high resolution 3D images of cortical and trabecular bone, combined with low levels of radiation exposure (5µSv) and a short scan time (< 3min).

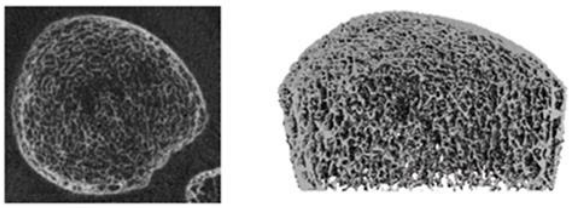


Figure 1.6 HRpQCT XtremeCT Scanner Images. Axial images of a tibia, and a 3D image demonstrating cortical and trabecular bone. *Adapted from Geusens et al. 2014 (49). Photo credit: Authors own, parental permission was granted for publication.*

HRpQCT has a nominal resolution of 82µm and can quantify trabecular and cortical bone structure by acquiring 110 consecutive slices from the distal radius or tibia, enabling the measurement of total, cortical and trabecular true volumetric BMD (vBMD). It has been shown to have a greater special resolution and signal-to-noise ratio than both pQCT and MRI techniques, and has been demonstrated to correlate well with findings from cadaveric bone biopsies (50-53).

A large number of variables can be generated from a HRpQCT of the distal radius and tibia, including the cortical and trabecular area, cortical thickness, periosteal and periosteal circumference, summarised schematically in Figure 1.7.

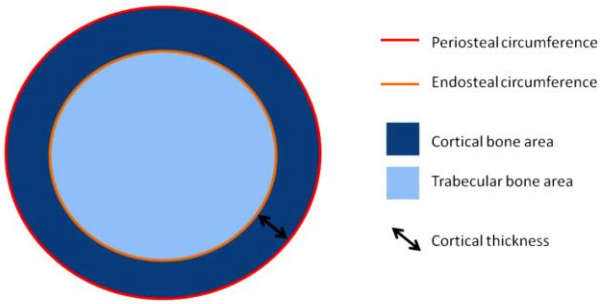


Figure 1.7 Schematic representation of bone geometry in cross section.

Image courtesy of Dr Mark Edwards.

Volumetric bone density may be calculated in the section of bone as a whole; the cortical and trabecular regions; and in the outer 40% and inner 60% of the trabecular region separately. This allows the ratio of outer trabecular density to inner trabecular density to be calculated. The ratio of trabecular volume to overall bone volume may also be calculated (bone volume/trabecular volume, BV/TV). Trabecular microarchitecture may be further assessed in terms of trabecular thickness, number and separation (although only trabecular number is measured directly) (54). An estimation is also made of the distribution (SD) of trabecular separation. The cortical region can also be assessed, both in terms of volume and porosity, using a technique developed by Burghardt et al. (55). Cortical density can be measured as cortical bone mineral density including pores, and cortical bone mineral density excluding pores.

Therefore, HRpQCT remains the most valuable non-invasive method we have for the detailed characterisation of bone properties.

1.3 Methods for assessment of muscle size and strength in clinical research

1.3.1 Anthropometric measurements and non-imaging based methods

Anthropometric measures to assess muscle and adiposity, such as arm and leg circumferences, and skinfold thicknesses, are easily obtained measurements. Mid upper arm circumferences (MUAC) provide a composite measurement of humerus cross sectional area, muscle size and subcutaneous fat. Typically, these measures have been used in the assessment of undernutrition, though their utility in assessing body composition and specifically lean mass is low (56). Some studies have used MUAC and triceps skinfold thickness to calculate an approximate arm-muscle area (AMA) (57):

AMA = (MUAC - π triceps skinfold)^2/ 4π

Methods such as bioelectrical impedance and air displacement plethysmography (enabling fat mass and fat free mass estimations from the calculation of body density) have also been used in the assessment of lean mass but have wider limits of agreement than DXA, which is considered the gold standard for assessing body composition (discussed in detail in section 1.2.1).

1.3.2 Muscle strength assessment

It is widely recognised that muscle strength and quality may not be directly proportional to muscle size. Hand grip strength is one of the simplest methods of testing isometric muscle strength and is a key part of the European Working Group on Sarcopenia in Older People (EWGSOP) definition of sarcopenia (58). Grip strength has been shown to be an important but inexpensive risk stratifying method for all-cause mortality, cardiovascular mortality and cardiovascular disease in a study of almost 143,000 adults aged 35-70 in 17 countries (59); in older adults in the Hertfordshire Cohort Study lower grip strength was associated with a greater risk of hospital admission over the following decade (60). Grip strength also has implications in child health, for example, in adolescents aged 10-16, greater grip strength has been associated with lower blood pressure and improved insulin sensitivity after adjustment for cardiorespiratory fitness (61, 62).

Reproducibility of grip strength measurement in children aged 4-11 years has been demonstrated (63) and it has also been shown to be correlated with leg muscle strength; in a study of children aged 7 to 12 years, the correlation between hand grip strength and isokinetic quadriceps strength was high (r = 0.84) (64). Other methods of assessing muscle strength such as jumping mechanography, isokinetic dynamometry and physical fitness batteries but their suitability for use in young children is limited by their complexity, duration, cost and size of equipment.

1.4 Bone biology and skeletal development throughout the lifecourse

1.4.1 Chemical structure of bone

Bones needs to be both stiff and flexible to resist fracture, which is achieved through a hierarchical structure. Collagen Type 1 fibrils are wound in a triple helical structure, linked together with non-collagenous proteins, which help to prevent shearing. Carbonated hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals deposited on the collagen structure add strength, particularly in compression. Cross- linkage between collagen fibrils with non-collagenous proteins

is also important, and is notably reduced in osteoporotic bone, leading to reduced tensile strength (65). The size of the hydroxyapatite crystals is also important - larger hydroxyapatite crystals are found in osteoporosis, making bone more brittle and prone to fracture (66).

1.4.2 Bone cells

Osteoblasts, osteocytes and osteoclasts are the three main types of bone cells. Osteoblasts are bone-forming and may become embedded within bone mineral as mature osteocytes (comprising 90-95% of the cells within bone) or remain on the surface as bone-lining cells. Osteoclasts are multinucleated cells responsible for bone resorption. Osteoblasts and osteoclasts work together in a coordinated fashion at specific sites on the surface of trabecular or cortical bone, forming "bone multicellular units". In the context of bone formation, osteoblasts lay down new osteoid collagen matrix and over a period of weeks to months, crystals of calcium hydroxyapatite form on the collagen fibrils. Bone is laid down during growth and repair and through adaptation to mechanical loading in a process known as modelling. Remodelling, in contrast, involves a cycle of resorption and formation of existing bone. Osteocytes play a key role in the regulation of modelling and remodelling. The arrangement of the osteocytes around Haversian canals acts as a mechanosensory system and allows communication both directly between neighbouring osteocytes and through the release of endocrine, paracrine and autocrine signalling factors to other bone cells. The various pathways important to the regulation of osteoblast and osteoclast activity, such as RANK-RANKL and Wnt signalling, are increasingly recognised as targets for anti-osteoporosis agents.

1.4.3 Embryological development of bone, fat and muscle

Bone, muscle and adipose tissue all have a common embryonic origin: the mesenchymal stem cell (MSC). MSCs have the potential to differentiate into a variety of different tissue types, as demonstrated in Figure 1.8. The majority of the skeleton is derived from lateral plate mesenchyme with the exception of the craniofacial bones, which are derived from neural crest (ectomesenchyme).

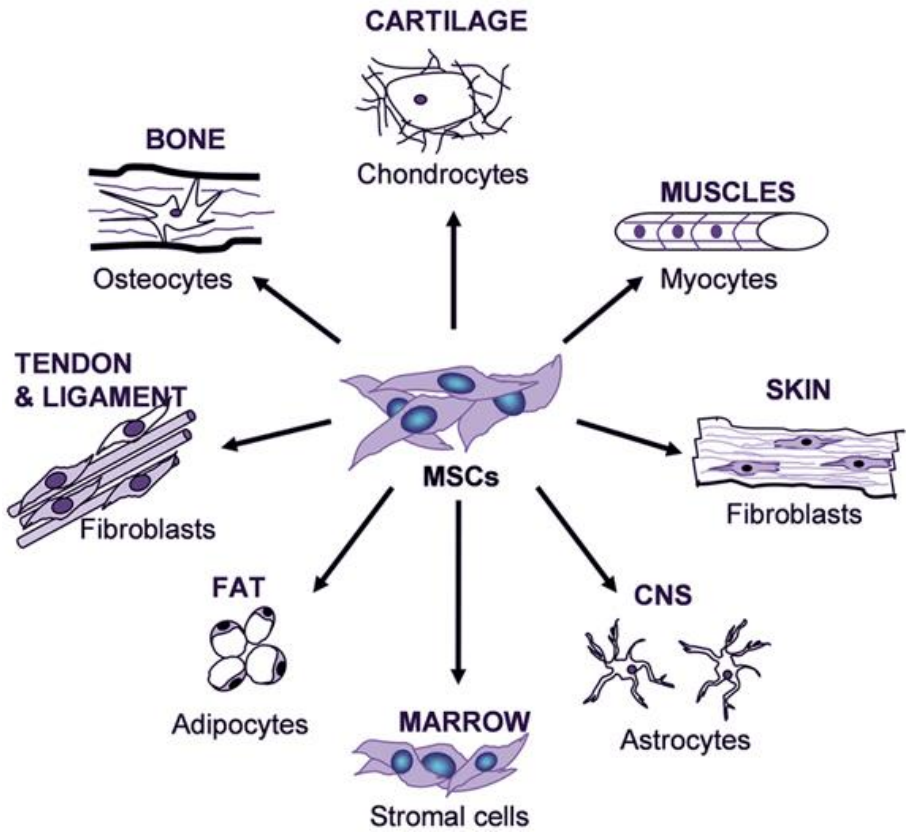


Figure 1.8 Cell types derived from the common mesenchymal stem cell. *http://www.sci-therapies.info/mesenchymal-stem-cells.jpg , and James et al., 2013, reproduced with permission (67) via Creative Commons https://creativecommons.org/licenses/by/4.0/legalcode*

A variety of signalling pathways, plus exposure to nutrients and growth factors, interact to determine the cell lineage into which MSCs differentiate. Wnt signalling, for example, stimulates myogenesis and osteogenesis whilst inhibiting the differentiation of pre-adipocytes into mature adipocytes (68). A variety of other, diverse transcriptional signals trigger embryonic adipogenesis, known to be dependent on PPARγ (69), beginning in the human fetus from around 14-16 weeks gestation. Through a complex interplay between transcription and growth factors, MSCs are stimulated to differentiate into pre-adipocytes. Hyperplasia and hypertrophy of adipocytes follows through late gestation with studies of preterm infants demonstrating that maximal gain of fat mass occurs from 30 – 36 weeks gestation (70, 71).

Myogenesis begins earlier in gestation, from 8-10 weeks, with the fusion of embryonic myoblasts, derived from the MSC, into primary muscle fibres, forming the scaffolding for the proliferation and differentiation of fetal myoblasts into secondary myofibers in mid to late gestation. In animal models, in both pigs and mice, myofiber number has been shown to be almost complete by the end of gestation. Following this, postnatal muscle growth occurs primarily by myofiber hypertrophy, accompanied by the proliferation and fusion of satellite cells with existing

myofibers. Reduced fetal skeletal muscle growth is not fully compensated after birth, as individuals who are born with low birth weight have lower muscle mass in adulthood, suggesting that disruptions in myofiber formation in fetal life may not fully recover (72).

The skeleton develops in two distinct processes, through endochondral ossification, forming the majority of the skeleton, and through intramembranous ossification, forming the skull and facial bones.

1.4.3.1 Endochondral and intramembranous ossification

Endochondral ossification requires the formation of a cartilaginous framework onto which the bone develops. This cartilage model, or anlage, begins to develop at around 5 weeks gestation with the differentiation of MSCs into chondrocytes, which secrete various components of cartilage extracellular matrix (ECM), including collagen type II and the proteoglycan, aggrecan. The cartilage model expands through rapid proliferation of the chondrocytes, forming orderly parallel columns which then accumulate a cartilaginous matrix surrounding them. Hypertrophy of the chondrocytes in the prospective mid-shaft of the bone occurs first, and recently differentiated osteoblasts deposit a periosteal bone collar around the prospective mid shaft, as shown in Figure 1.9.

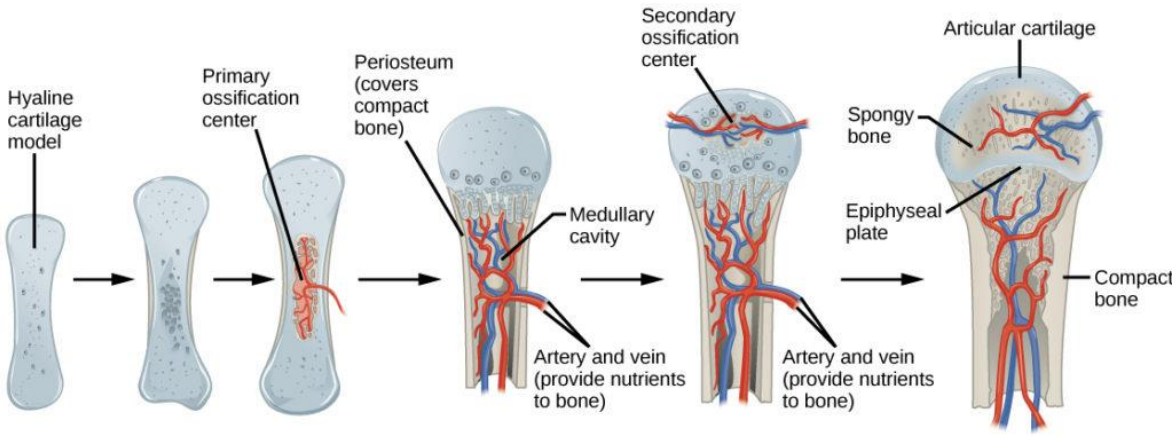


Figure 1.9 Development of a bone by endochondral ossification, showing the cartilage model of the future bone; a periosteal bone collar and formation of the primary centre of ossification; expansion of the primary ossification centre and development of vasculature; formation of secondary centres of ossification at the end of the bone, leaving a cartilaginous epiphyseal plate between primary and secondary ossification centres and articular cartilage at the end of the bone.
Reproduced with permission from https://courses.lumenlearning.com/wm-biology2/chapter/bone-growth-and-development/ via Creative Commons https://creativecommons.org/licenses/by/4.0/legalcode

Blood vessels, osteoclasts, as well as bone marrow and osteoblast precursors invade the model from the bone collar, forming the primary centre of ossification. Here, the osteoclasts remove the cartilage ECM and osteoblasts deposit osteoid (bone mineral), on the cartilage remnants. In long bones, a secondary ossification centre forms at each end of the cartilage model, which leaves a cartilaginous growth plate between the primary and secondary ossification centres, as well as the future permanent articular cartilages at the end of each bone.

The maturing chondrocytes within the growth plates are organised into zones, including a resting zone, a proliferating zone, a prehypertrophic and a hypertrophic zone, as shown in Figure 1.10 (73). Various endocrine and paracrine influences are important for chondrocyte regulation at the growth plate, including growth hormone, produced in the pituitary, which stimulates the secretion of insulin-like growth factor (*IGF1*), both by liver cells and by growth plate chondrocytes (74). Thyroid hormone, locally produced growth factors including Indian Hedgehog, WNTs, bone morphogenetic proteins and fibroblast growth factors also play a role, as do the components of the ECM secreted by the chondrocytes (including collagens, proteoglycans thrombospondins and matrilins). The chondrocytes themselves secrete factors which regulate the behaviour of invading bone cells, including vascular endothelial growth factor and receptor activator of NFκB ligand.

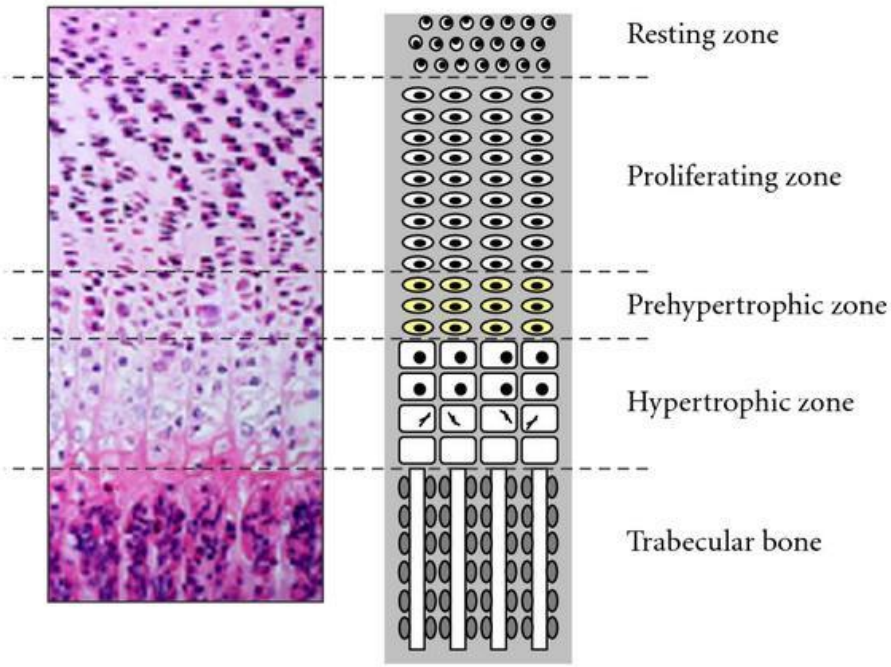


Figure 1.10 Growth plate morphology; section through the proximal growth plate of the tibia of an adult mouse. *Reproduced with permission from* *https://www.hindawi.com/journals/bmri/2010/686457/fig2/* *via Creative Commons* *https://creativecommons.org/licenses/by/4.0/legalcode*

In contrast, intramembranous ossification, responsible for the formation of the flat bones of the skull and mandible, is not preceded by a cartilaginous model. Instead, MSCs differentiate directly into osteoblasts which begin to secrete bone matrix, which becomes mineralised.

1.4.4 Mineralization of the fetal skeleton

Primary ossification centres form in the vertebrae and the long bones of the human fetal skeleton between the 8th and 12th weeks of pregnancy. However, the deposition of mineral within the bone matrix formed by both endochondral and intramembranous ossification is at its maximum in the third trimester of pregnancy (76).

Approximately 30 g of calcium are required for the development of the human fetal skeleton, and approximately 80% of this is accrued in the last trimester. From approximately 20 weeks gestation, active transport of Ca^{2+} across the placenta occurs as fetal serum Ca^{2+} is maintained at a higher concentration than maternal serum Ca^{2+} (77) . Maternal gut calcium absorption is increased during this period to meet fetal demand. Recent work has demonstrated that both sides of the placenta and its function may determine offspring bone development (78, 79). Placental Ca^{2+} transfer occurs in the syncytiotrophoblast and comprises initial apical entry through calcium transport channels, followed by diffusion through the cytosol bound to calbindin and finally basolateral extrusion through active transporters including plasma membrane calcium dependent ATPases, of which there are four isoforms (*PMCA 1-4*) (80). *PMCA* gene expression has been shown to strongly regulate Ca^{2+} transport across the placenta (81), indeed *PMCA3* gene expression has been positively correlated with whole body bone mineral content (BMC) at birth (79). The regulation of *PMCA* gene expression has not been fully characterised, though animal models suggest it may be influenced by 1,25-dihydroxyvitamin D [1,25(OH)2D] (82).

Both fetal parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP) are essential for maintaining fetal plasma Ca^{2+} concentration and transfer of Ca^{2+} across the placenta, though the exact mechanism is unknown. Fetal PTH increases Ca^{2+} resorption by the kidney and possibly from bone, whilst fetal PTHrP is elevated in response to low fetal plasma Ca^{2+} and appears to regulate calcium transport across the placenta (83). Absence of either PTH or PTHrP can lead to fetal hypocalcaemia (83). Maternal PTH appears not to cross the placenta, and is also important as it alters maternal serum Ca^{2+} concentration and therefore the supply of calcium available to the fetus (84). PTH and PTHrP also directly influence the linear growth of fetal bones, mediated through an effect on chondrocyte differentiation (85, 86). This observation is supported

by animal models of short limbed dwarfism and chondrodysplasia in which alterations in PTHrP expression or receptor function are present (85, 87).

1.4.5 The skeleton at birth, infancy and adolescence

The mean birth weight for a white male infant in the UK is 3.55 kg and for a white female infant is 3.41 kg (88). Whole body BMC in an infant with a weight of 3.0-3.5 kg is approximately 66 g, therefore representing around 2% of body weight (89). Up to 95% of the variance in whole body BMC and 86% of whole body BMD is explained by birth weight (89) (section 1.6.2). Godfrey et al. demonstrated that maternal fat stores and height are positively associated with neonatal whole body BMC and BMD, whereas smoking and vigorous physical activity displayed a negative association (90). However, it is unclear if these factors are acting through differences in birth weight or directly on bone. Furthermore, several of these maternal factors are positively associated with placental volume, which is also associated with offspring neonatal size, adiposity and bone mass (78).

The process of growth in childhood is complex, influenced by genetic predisposition, nutrition, health, illness and endocrine factors. There are linear increases in BMC throughout early childhood, followed by an accelerated increase in BMC during puberty. Concurrent changes in relative proportions of lean mass and fat mass also occur through childhood and adolescence, with marked differences between the sexes, as shown in Figure 1.11. Linear growth is completed at an earlier age in females than males, therefore, accretion of bone mass begins to plateau sooner (Figure 1.11) (91, 92). The increase in lean mass during childhood and adolescence mirrors that of BMC, whilst in prepubertal females there is a steady gain in % fat mass throughout childhood. During puberty, the accumulation of soft tissue differs markedly with a relatively greater gain in fat mass in females and lean mass in males. These findings emphasise the importance of pubertal staging in childhood assessment of the musculoskeletal system due to the well documented interactions between fat, bone and muscle.

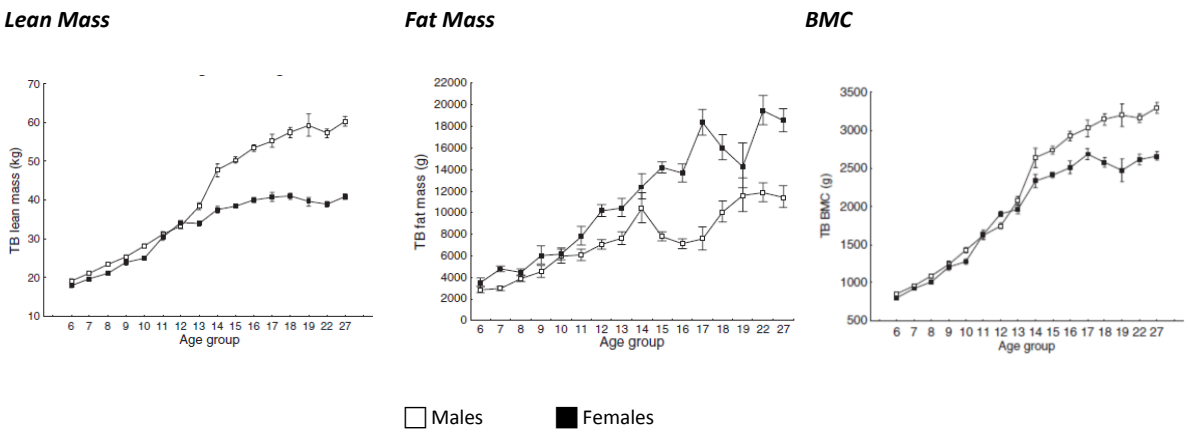


Figure 1.11 Total body (TB) Lean mass, fat mass and BMC measured by dual energy X-ray absorptiometry in males and females
Reproduced with permission from Alwis et al., 2010 (91)

1.4.6 Bone in adulthood and older age

The balance of bone formation and resorption has a critical influence on bone mass and strength throughout life. There is a positive balance during childhood until achievement of peak bone mass in the third decade of life (27), with a subsequent period of stability and then a negative balance in older age, with osteoclast activity greater than osteoblast activity, leading to bone loss. In women, this process is accelerated after the menopause. Thus, both PBM achieved and the rate of decline are important determinants of bone mass in later life. Mathematical modelling has demonstrated that a 10% increase in PBM will delay the onset of osteoporosis by 13 years (27), therefore interventions early in life to increase BMC, could reduce the incidence of osteoporotic fracture in older age.

At the level of the whole bone, cellular mechanisms and associated hormonal and genetic factors result in differences in structure between males and females, and alterations with advancing age. Females typically have a smaller bone cross sectional area than males, and in addition there is a significant reduction in cortical thickness in females following the menopause, contributing to the well-established sex differences in fracture risk as shown in Figure 1.12. The structure of the trabeculae differs between the sexes, with young women having fewer and thinner trabeculae than young men, and a greater reduction in trabecular number in women as they age (93). In addition, cortical porosity increases at a faster rate in female ageing (94).

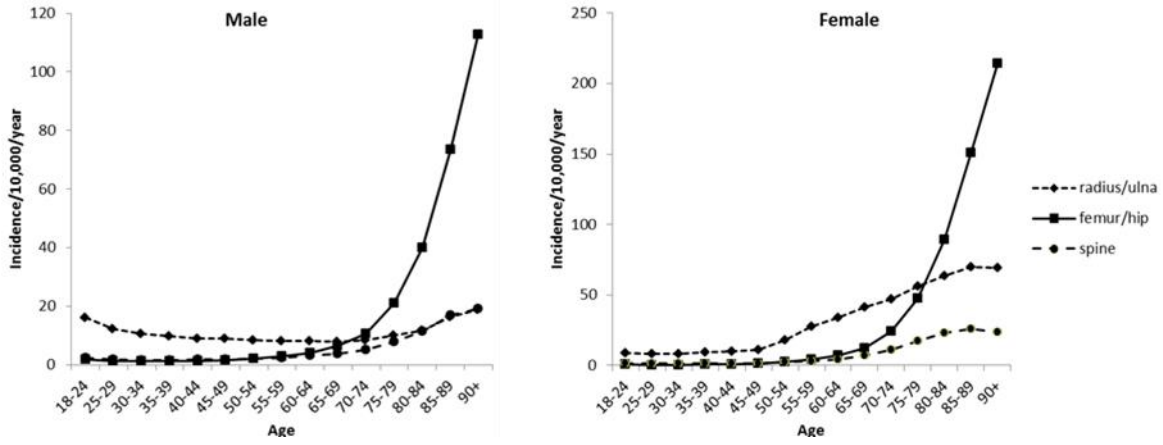


Figure 1.12 Age and sex specific incidence rates of fracture at the femur/hip, radius/ulna, and spine 1988-2012, data from the UK CPRD.

Reproduced with permission from Curtis et al., Bone 2016 (95).

1.4.7 Tracking of BMC, BMD and muscle strength throughout life

When assessing whether interventions early in life are likely to result in improvements in later life outcomes, it is important to determine whether a measurement in early life can be used to predict a future measurement in an individual. Tracking refers to the maintenance of a relative rank of an individual within a group when a measurement is repeated at two or more time intervals. As such, evidence for high levels of tracking of the measurement or characteristic would support a high likelihood of a sustained positive effect an early life intervention to improve such a measurement.

The tracking of BMD and BMC has been well described. In a large US study, 1554 children aged 6-16 years had DXA at baseline and at yearly intervals for three years (96). Measurements of bone mineral status at baseline accounted for 86-96% of the variability one year later. The correlation coefficient gradually declined with increasing time from baseline, but even at three years at least 80% (depending on sex and ethnicity) of the variation in whole body BMC z-score could be explained by the baseline measurement. Furthermore, 52% and 61% of children who had a low (z-score < -1.5) and high (z-score > 1.5) whole body BMC at baseline remained in the low or high group, respectively, at the 3 year follow-up. The study findings suggested that the timing of pubertal development might confound the tracking of bone mass, as the tracking coefficients were highest for children who were either younger (6-7 years) or older (14-16 years) at baseline, compared with the intermediate years. However smaller childhood studies, followed up after a longer time interval and spanning the peripubertal period, have similarly demonstrated moderate to high levels of tracking of BMC and aBMD (97-100).

Between 2008 and 2015, in a Danish longitudinal study, 831 children aged 8 to 17 years underwent serial DXA scanning, blood testing of luteinizing hormone levels and Tanner staging, and demonstrated that more than 80% of participants remained at the same or neighbouring quintile over the study period. Correlation coefficients between baseline and 7 year follow-up BMC, BMD and BA z-scores were high (0.80 to 0.84), irrespective of pubertal stage at baseline (101). Recently, a Tasmanian study demonstrated moderate to strong tracking of BMD between age 8 and 25 (n = 99) (correlation coefficients in males 0.59 to 0.65; females 0.70 to 0.82); increasing lean mass and participating in sports during growth were shown to be associated with improvements in BMD (102). In an older study in which radial BMC, BMD and bone area were assessed using single photon absorptiometry in 214 individuals at age 3-17 years and subsequently at a mean of 28 years later (follow up age 28-44 years), the correlation coefficients were more modest than those observed for shorter intervals, but still demonstrated moderate tracking (BMC r = 0.56, aBMD r = 0.42, bone area r = 0.58). Correlation coefficients found to be stronger for those who were older than 10 years at baseline, but were statistically significant for both age groups (103).

The tracking of lean mass and muscle function to adulthood is less well characterised. One Canadian study assessed hand grip strength at multiple timepoints – at 10, 11, 12 and 35 years of age in 106 individuals. Tracking of grip strength at yearly intervals between 10 and 12 years was high in both males (r = 0.84 to 0.92) and females (r = 0.87 to 0.91). When retested at age 35 the tracking coefficients were more modest (r = 0.45 to 0.61) and tended to be stronger in women than men (104).

Overall, these studies would therefore support the notion that children with low bone mass may be at risk of developing osteoporosis, and those with low muscle strength in childhood may go on to have weaker muscles as adults. Therefore, interventions to improve musculoskeletal health might have sustained effects into adulthood.

1.4.8 The use of bone mineral content, rather than bone mineral density in children as a marker of future fracture risk

Both the use of areal BMD and BMC measured by DXA as a descriptor of bone health in children, present problems, as they are size-dependent measures. However, it is important to report the accuracy of bone mineral content as a predictor of fracture. Areal bone mineral density (aBMD) is the basis of the WHO 1994 operational definition of osteoporosis (105). Indeed, many studies have consistently demonstrated the predictive value of aBMD for incident fracture, with an

approximate doubling of fracture risk for each standard deviation decrease in aBMD, dependent on the site of measurement and the site of fracture (106). In two prospective studies, both BMC and BMD, but not BA, at the hip, lumbar spine and whole-body sites were predictive of incident fracture (37, 107). In the data shown below from a Southampton cohort, the gradient of risk of incident fracture (hazard ratio per standard deviation decrease in the predictor) using femoral neck, lumbar spine and whole body BMC were similar (approximately a doubling of risk per SD decrease in BMC or BMD).

Table 1.2 Gradient of risk (Hazard ratio per 1SD decrease in predictor) for DXA indices and incident fracture. *Reproduced with permission from Curtis et al., Arch. Osteoporos., 2016 (37).*

Predictor	***Gradient of risk***	***95%CI***	***p-value***
Femoral neck			
Area	0.99	0.76-1.29	0.93
BMC	1.82	1.38-2.41	< 0.001
BMD	2.04	1.51-2.77	< 0.001
Lumbar spine			
Area	1.19	0.90-1.57	0.22
BMC	1.85	1.38-2.49	< 0.001
BMD	1.97	1.49-2.60	< 0.001
Whole body			
BMC	1.71	1.27-2.31	< 0.001
BMD	1.79	1.40-2.29	< 0.001

1.4.9 Bone turnover markers

As previously discussed, maternal calcium homeostasis adapts to meet the calcium demands of the developing fetus. Despite the maximal fetal demand for calcium occurring in the third trimester (108), adjustments to maternal calcium homeostasis occur in early pregnancy. Increased calcium absorption occurs, which is doubled by the second trimester (109), due in part to the elevated $1,25(OH)_2$ D concentration observed in later pregnancy (110). This physiological adaptation occurs without a significant elevation in PTH or 25(OH)D levels, and plasma calcium concentrations remain stable (111). Maternal renal calcium excretion is decreased, whilst mobilization of skeletal mineral stores increases (76). However, the relative contribution of the maternal skeleton to offspring calcium homeostasis remains unclear (112-114).

Biochemical markers of bone turnover offer a non-invasive method of monitoring changes in bone resorption or formation during pregnancy (115), and provide some insight into the impact of pregnancy on maternal bone and its contribution to the developing fetus.

Bone turnover markers (BTM) have been studied for over 30 years, though their adoption to clinical practice has been limited by their poor within-subject and between-lab reproducibility (116, 117).

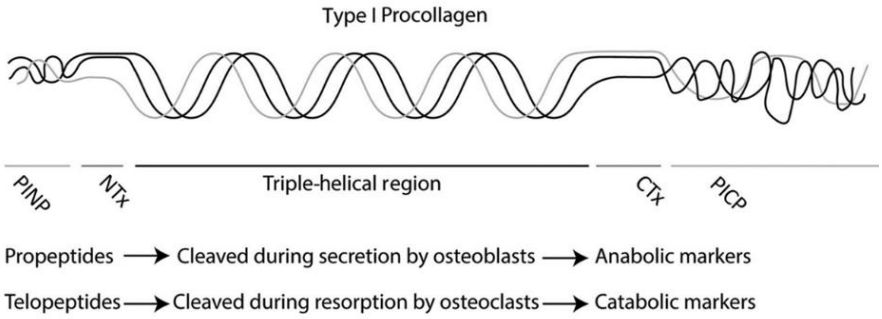


Figure 1.13 Cleavage of type 1 procollagen telopeptides in bone breakdown as a catabolic marker.
Reproduced with permission from Greenblatt et al.; Clinical Chemistry 2017.

There are two groups of bone turnover markers as illustrated in Figure 1.13 (118):

1. Markers of bone formation (chiefly N-terminal collagen extension propeptide (PINP), osteocalcin, bone alkaline phosphatase)
2. Markers of bone resorption, mainly collagen 1 degradation products (such as C-terminal cross-linking telopeptide of type 1 collagen (CTX), N-terminal telopeptide of type 1 collagen (NTX), deoxypyridinoline, hydroxyproline)

The International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry (IFCC) have recommended serum P1NP and β-CTX-I as reference bone turnover markers, and have provided recommendations for sample handling, patient preparation, and reducing pre-analytical variability (117).

To focus on C-terminal telopeptide of type 1 collagen (CTX), (the bone turnover marker measured in the MAVIDOS trial and discussed in this thesis), this is a product of the breakdown of type 1 collagen, the most abundant protein component of bone. Fragments of collagen from the C-terminus or N-terminus telopeptide region, a non-triple-helical portion near the ends of mature collagen, are released during osteoclastic reabsorption of bone (119). The process of bone breakdown in which CTX is released is mediated specifically by cathepsin K, an enzyme produced

by the osteoclast in the bone resorption process. CTX is cleared by the kidney, so is measurable in urine, as well as blood.

A variety of assays are available to measure CTX, and can display varying degrees of selectivity for the newly synthesised form, α–CTX, versus the isomerised form, β- CTX, which accumulates over time. As a result of the presence of a cross-link forming lysine in the CTX peptide, these may be present as both monomers (α-CTX and β-CTX) and dimers (α–α-CTX, α–β-CTX, and β–β–CTX). The Urinary CrossLaps® ELISA assay (Immunodiagnostic Systems, Boldon, UK), used in our study, measures both α-CTX and β-CTX (120).

In both pre- and postmenopausal women, greater urinary CTX has been shown to be significantly correlated with lower total hip BMD, distal radius BMD and lumbar spine BMD (correlation coefficients -0.14, -0.17 and -0.21, respectively), and has also been shown to increase with age (121). Cohort studies have demonstrated that bone turnover markers are predictive of fracture risk, independently of BMD, therefore might potentially be used to complement and augment fracture risk assessment by BMD, though substantial biological variability limits their use at an individual or clinical level (122). One study (in older women) showed that the risk of hip fracture increased by 1.3 (95% CI 1.0-1.6) (123) and another that the risk of any fragility fracture increased by 1.54 (1.19, 1.99) (124) for each SD increase in urinary CTX; a third study in postmenopausal healthy women showed a doubling of risk of any fracture in individuals with CTX in the highest quartile, compared to the lowest quartile (125). Others, however, whilst demonstrating an association between higher bone turnover markers and greater cortical and trabecular bone loss at the proximal femur, did not show an association with fracture risk (126). Serum CTX levels have also been correlated significantly with histomorphometric measures of bone resorption in iliac cancellous and endosteal bone (127).

Bone resorption displays significant circadian variation, with serum CTX concentrations peaking in the early morning between midnight and 8am, with a nadir in the afternoon (128). Seasonal variation in bone turnover is also seen, with a peak in remodelling occurring during the winter months (129). Vitamin D has been proposed to play a role in this seasonal variation. Several studies have demonstrated an association between greater 25(OH)D status and markers of bone resorption, in both non-pregnant and pregnant individuals (113, 129-131). A recent randomised placebo controlled trial (n = 160) of cholecalciferol supplementation 1000 IU daily in women aged 50-65 demonstrated a 24% reduction in CTX in the cholecalciferol supplemented group, though there were no between group differences in absolute CTX values at the end of the trial (132).

Bone resorption levels drop post prandially and rise on fasting, which is a major contributing factor to the rise in CTX occurring during the early morning. This postprandial drop has been shown to result from the effects of gastrointestinal hormones such as glucagon-like peptide 2, to reduce bone resorption (133). As a result, the recommended time for plasma CTX is early morning on fasting; hence fasting second void early morning urine samples, as opposed to blood samples, are simpler to obtain in practice. Various other anthropometric, hormonal and lifestyle characteristics have been shown to be associated with BTMs in premenopausal women; current smoking habit and low BMI are also associated with higher basal levels of bone turnover markers; BTMs also vary with the menstrual cycle, rising during the mid to late follicular phase and falling during the midluteal phase (134).

1.4.9.1 Bone turnover markers in pregnancy

Studies of bone turnover in pregnancy have demonstrated markers of resorption (such as CTX and NTX, pyridinoline/creatinine ratio, hydroxyproline/creatinine ratio) to be low in the first trimester but to increase steadily thereafter. They have been shown to reach levels as much as twice normal in the third trimester, whilst markers of bone formation such as osteocalcin and P1NP are low to undetectable in the first trimester, and by term have risen to normal values. A study by Naylor et al. characterised bone turnover markers and changes in BMD pre and post pregnancy. Here, α-CTX increased by 202% (SE ±45%) on average, and β-CTX by 172% (SE ±44%) from pre-pregnancy to 36 weeks gestation (135-142).

Overall, the evidence suggests that bone turnover may be relatively similar to the non-pregnant state early in pregnancy, but that it increases during the third trimester to reach a net resorptive state (though studies do not suggest that this correlates with major bone loss in pregnancy, in contrast to breastfeeding) (143). This is in keeping with the expected late-pregnancy demand for fetal calcium acquisition, and a concurrent need to resorb from the maternal skeleton, especially if intestinal absorption is not sufficient to meet the combined needs of both mother and fetus. A randomised trial found that, in pregnancy, consumption of an 1100 mg calcium supplement per day versus placebo lead to a 15% lower NTX level during the second and third trimesters (144). Twin pregnancies were shown to result in a slightly higher urinary NTX and CTX, and slightly increased bone specific alkaline phosphatase at term, in keeping with further increased resorption of maternal bone mineral to meet the demands of two fetuses.

Recent research has suggested an interaction between maternal vitamin D status, calcium intake, and markers of bone resorption. In a prospective study of 205 pregnant women, urinary NTX was shown to increase in late pregnancy, with greater increases during the winter months and in

women with lower dietary calcium intake (< 1000 mg/day). Additional dietary calcium intake was shown to be associated with reduced bone resorption in late pregnancy, with a greater effect observed in the winter (131). In a smaller study of pregnant women and non-pregnant healthy controls, greater 25(OH)D status was shown to be associated with lower CTX levels in the second and third trimesters and postpartum period; whilst no correlation between 25(OH)D and CTX was seen in the controls (130). Another recent study in which markers of both maternal and fetal bone turnover were measured in pregnant women versus non pregnant controls consuming controlled amounts of vitamin D, calcium and phosphorus, maternal free 25(OH)D and 24,25$(OH)_2$D tended to be negatively associated with maternal NTX at the end of the study, but were positively associated with fetal CTX and osteocalcin (a marker of formation), indicating promotion of fetal bone turnover (formation plus resorption) (113).

1.4.9.2 Challenges regarding the use of bone turnover markers in pregnancy

In addition to the aforementioned diurnal variation and postprandial changes in bone turnover markers, their measurement in pregnancy presents other problems. Haemodilution, increased GFR, altered creatinine excretion, placental transfer and metabolism may interfere with levels of bone turnover markers, including CTX (138). Owing to difficulties in the practicalities of obtaining fasted blood samples from pregnant women, second void urine samples provide a good alternative. Studies by Eastell et al. suggest that urinary CTX is likely to be the most appropriate measure of bone resorption in pregnancy (117).

Additionally, the developing fetal skeleton may contribute to maternal CTX excretion, and studying the different isoforms of CTX may enable this to be deciphered. The α–CTX is predominant in young bone, resulting in a higher α/β ratio in children. There is no increase in the α/β ratio in high bone turnover states, thus, an increase in the α/β ratio would indicate a fetal contribution to urinary CTX production. Naylor et al. showed that these ratios did increase from 0.71 pre-pregnancy to 0.83 at 36 weeks, but these changes were not significant. They estimated that around 9% of α–CTX and 2% of β–CTX in the mother's urine in late pregnancy may be coming from the fetal skeleton (145).

1.5 The influence of pregnancy on maternal bone health

Limited information is available regarding the changes in maternal BMD during pregnancy due to the risk of fetal radiation exposure and the difficulty of interpreting maternal BMD measurements with an overlying fetal skeleton. Of the DXA studies that exist, their interpretation is complicated by changes in body composition, weight, and skeletal volumes during pregnancy. In a 1977 study using X ray spectrophotometry of the radius and femur during the first and early second trimester, then one week postpartum, trabecular (but not cortical) bone density was shown to have decreased by the early postpartum measurement (146). Other studies measuring single or dual photon absorptiometry of the forearm or femur during and after pregnancy, found no difference in cortical or trabecular bone density (147-149).

More recently, studies have used DXA at two timepoints, within a few months of a planned pregnancy and then 1-6 weeks after delivery. In general, these showed a small (less than 5%) decrease in lumbar spine bone density between the two measurements, but very little change at other skeletal sites (135, 145, 150-152), with one study showing an increase at mainly cortical sites (arms and legs) during pregnancy and a decrease at trabecular sites (pelvis and spine) (145). In the largest of these studies, by Moller et al., 92 women had BMD measurements by DXA pre-pregnancy (hip, spine and forearm), once in each trimester (forearm only), and also in the postpartum period (hip, spine and forearm). 75 age-matched controls were followed in parallel. During pregnancy BMD was shown to decrease; postpartum it was found to differ from non-pregnant controls by around 1.8% at the lumbar spine, 3.2% at the total hip, 2.4% at the whole body and 4.2% at the ultra-distal radius. Differences were discernible from the third trimester, which would coincide with peak calcium transfer to the fetus. However, it is difficult to discern whether all of these small changes in BMD were attributable to pregnancy as bone loss is known to occur rapidly during lactation (through the action of PTHrP and falling oestrogen stimulating osteoclast mediated bone resorption (153)), and all women went on to breastfeed before the postpartum measurement (151).

Peripheral ultrasound has also been used to assess changes in bone mass during pregnancy, and both cross-sectional and longitudinal studies have demonstrated lower BMD in the os calcis or phalanges at the end of pregnancy (154, 155), however no effect was seen in one study which focussed on the mid-tibial shaft (156). It is important to note that correlations between ultrasound findings at the phalanges or os calcis and changes at the spine or hip are uncertain (143). A study of 307 pregnant women in Southampton undergoing calcaneal quantitative ultrasound (QUS) scanning were shown to have a significant decrease in calcaneal bone

measurements during pregnancy, individuals who were pregnant for the first time, had low milk intake in pregnancy and reduced measures of fat mass had the greatest decline. Seasonal effects were also apparent, as women who were pregnant over the winter months had the greatest losses in calcaneal QUS measurements supporting a role for vitamin D and calcium in maternal bone resorption (157).

In summary, available data suggest that bone turnover increases during pregnancy, especially during the third trimester, and that BMD, particularly in trabecular bone, declines during pregnancy. However, changes in bone indices in pregnancy and postpartum are poorly understood, due to a variety of challenges – in the interpretation of bone turnover and acquisition of radiological measures of bone mass, in the acquisition of samples for histomorphometry, in small study sizes and study design heterogeneity.

It is also not clear whether pregnancy has a lasting impact on skeletal health. The majority of studies have found a neutral effect of parity on BMD, with a few showing that parity confers greater BMD or that parity decreases the risk of fractures, a recent systematic review and meta-analysis has supported this assertion (mean difference in total hip BMD compared to nulliparous women = 5.98 mg/cm^2; 95% CI 1.72 to 10.24; p = 0.006) (158) . A large study of 1852 twins and their female relatives, including 83 identical twins who were discordant for ever being pregnant, found no long term detrimental effect of pregnancy or breast feeding on bone mineral measures (143, 159). Conversely, a smaller number of studies have shown that parity is associated with reduced BMD (160) or increased hip fracture risk (161), but these studies are outnumbered by studies that reported a neutral or protective effect of parity.

Few studies report the longer term effects of pregnancy on bone turnover markers. A relatively small study of 32 mothers measured bone turnover markers prospectively after delivery, and at several timepoints including 1 year after resumption of menses. They showed that subsequent to parturition, CTX decreased, whilst formation markers increased. Interestingly, higher parity and longer periods of lactation were associated with lower bone turnover markers postpartum as compared with previously nulliparous women of the same age (162).

Epidemiological studies of the association between parity and subsequent bone health have a number of limitations. It is difficult to separate the effects of parity from those of lactation, and often several decades have passed between the reproductive years and the timing of the first fracture or BMD measurement. Therefore confounding factors or events in the elapsed time could have affected the outcome. One study in the NHANES cohort avoided this lag, the investigators measured BMD in young women aged 20-25 (n = 819) and found that adolescent pregnancy did

not reduce peak bone mass, since BMD did not differ between groups of women who had experienced an adult pregnancy, an adolescent pregnancy or no prior pregnancies (163). Hence, overall it seems likely that parity does not have a long term adverse effect on fracture risk or BMD in most women, and for this reason it is not used in FRAX as a factor affecting 10 year fracture risk (164).

1.6 Determinants of peak bone mass

1.6.1 Heritability of bone mass

The inheritance of a particular genetic profile could be considered as the first stage in the determination of offspring bone health. There appears to be substantial heritability of bone mass, as determined by twin and family studies (165-168). Areal BMD appears to be characterised by high heritability (h^2), estimated to be 45% to 78% depending upon the skeletal site and age (169, 170). Initial research focused on several likely candidate genes, including the vitamin D receptor gene (VDR) (171), IL-6 (172), collagen 1α1 (173), TGFβ (174) and LDL receptor related protein 5 (LRP5) (175). Many genome wide association studies (GWAS) and their meta analyses have been conducted for BMD, and dozens of genomic loci have been identified, some of which are non-coding variants (e.g. EN1, near the WNT16 gene), and many of which occur with low minor allele frequency (MAF) (176, 177). So far, individual loci detected through GWAS studies (with the latest in UK Biobank covering almost 427,000 individuals) explain approximately 20% of the variance in BMD (178, 179), but there is increasing evidence from whole-genome sequencing that low frequency, non-coding variants can also have a large effect on BMD and fracture risk. These rare genetic variants of strong effect in combination with known variants could explain a large proportion of the "missing heritability" (176, 180).

Moreover, it has been shown that in spite of the strong heritable component of aBMD, the genes identified in genome wide association studies so far only overlap to some degree with the fracture phenotype (181). GWAS linked to HRpQCT analyses are now emerging which provide measures of trabecular and cortical traits, which may be more important predictors of fracture (182). Despite the suggestion that further variance will be explained by next-generation sequencing approaches, but there is growing evidence that some of the residual variance might be explained by interaction between genes and environment, both in utero and in early life (183). This may occur for example by epigenetic regulatory processes, which will be discussed later in this chapter.

1.6.2 Birthweight as a predictor of adult bone mass

A large volume of research has focussed on the hypothesis that osteoporosis risk might be influenced in early life by factors separate from our genetic makeup. The first study to suggest a link between weight in infancy and bone health was a small cohort study of 153 women born in Bath during 1968-69, subsequently assessed in 1990. This showed that there were significant associations between weight at one year and BMC (but not BMD) measured by DXA at the lumbar spine and femoral neck, associations which remained significant after adjusting for current weight (184). Findings from two other well characterised UK birth cohorts from Sheffield and Hertfordshire demonstrated similar relationships; low birthweight and weight at one year predicted lower BMC in later life, even after adjusting for factors known to influence bone mass (14, 185). In all cases there was a stronger correlation for BMC than BMD. A British birth cohort study of 1600 subjects with prospective DXA and pQCT measures showed that birth weight positively related to bone CSA and strength, but again, no positive association was found with cortical volumetric BMD (186). A Swedish study of 1061 women, aged 25 years, (the PEAK-25 study) presented similar findings (187); the New Delhi Birth Cohort again showed that birth length and BMI at birth were positively associated with BMC ($p < 0.01$ at lumbar spine, femoral neck and forearm) (188). A further meta-analysis of seventeen original papers based on longitudinal studies of birthweight on offspring bone mass supported positive associations between birth weight and bone mass. This relationship was clear among children, unclear among adolescents and weak among adults. The effect of birthweight was stronger on BMC than BMD regardless of age, perhaps due to corrections for body size (189). Furthermore, a recent study demonstrated that birth weight was positively associated with radial CSA and strength strain index, a measure of bone strength, at 60-64 years (190). Both smaller bone area and lower strength strain index are also associated with increased fracture risk (191, 192).

Such relationships between early growth and adult bone mass are likely to have implications for fracture risk. Indeed, in a longitudinal Finnish cohort of 3,639 men and 3,447 women born in a Helsinki hospital during 1924-33 showed that babies with low birth length, tall maternal height and low rate of childhood growth were associated with increased hip fracture risk in adulthood (193). These relationships were extended earlier into childhood growth, demonstrating that poor accrual of BMI in early childhood was again related to greater risk of hip fracture in adulthood (194). Evidence from the UK Hertfordshire Cohort Study provided a possible mechanism for this relationship beyond simple BMC: here early weight was positively associated with femoral neck cross sectional area in adult life, corresponding with greater hip strength (195). Mother offspring

cohorts have allowed a more detailed investigation of patterns of early growth (196-198) and specific maternal factors which might influence offspring development.

1.6.3 Maternal factors affecting offspring bone mass

There is also increasing evidence that maternal antenatal characteristics are associated with offspring bone development. The Southampton Women's Survey, a British prospective cohort of 12,583 initially non-pregnant women aged 20-34 years and their subsequent offspring (n = 3156) has elucidated various relationships between maternal factors and offspring bone mass. In a study of 841 mother-baby pairs from this cohort, low fat stores, first pregnancy, smoking and high levels of physical activity during late pregnancy were all associated with reduced whole body bone mineral content at birth(199), confirming findings from an earlier smaller study (200). One study suggested that the negative association between maternal smoking and offspring bone mineralisation persists into later childhood (201), although this is not consistent in all cohorts (202, 203). However, this relationship could be confounded by the greater BMI observed in children born to mothers who smoke (204), which is associated with a higher BMC and BMD.

Maternal diet during pregnancy has also been associated with offspring BMC and aBMD during later childhood. Maternal diet was assessed in 198 women participating in the Princess Anne Hospital Study using a food frequency questionnaire at 15 and 32 weeks gestation. A dietary score was calculated from this to quantify the consistency of the woman's intake with recommendations for a healthy diet. A positive association was observed between a more "prudent" diet (characterised by higher intakes of fresh fruit and vegetables and lower processed foods) in late pregnancy and offspring whole body and lumbar spine BMC and aBMD at 9 years of age (205).

In terms of the link between low maternal fat stores and low offspring BMC, there is some evidence of the effect being mediated by leptin. This was proposed through a study of umbilical cord leptin concentration and neonatal bone mass and body composition. In 117 infants, there were strong positive associations between umbilical venous leptin and neonatal fat mass and BMC, independent of serum IGF-1 levels (206). IGF-1 had been previously shown to be closely related to maternal lean and fat mass and size of the neonatal skeleton but not its mineralisation (207).

Several studies have also examined the relationships between individual dietary components, including macronutrients such as protein intake and micronutrients (e.g. phosphorus, folate, magnesium) in pregnancy and offspring bone mass. Some of the findings are inconsistent, which

could reflect the inaccuracies in estimating intake from food frequency questionnaires and differences in the populations studied (208-212).

1.6.4 Calcium intake in pregnancy and offspring bone health

Several mother-offspring birth cohort studies have investigated the associations between maternal calcium intake and offspring bone development, in general showing weak positive associations between maternal calcium intake in pregnancy and offspring bone health (209-213). In the Pune Maternal Nutrition study, India, (214) positive associations were identified between maternal calcium intake and offspring whole body BMC and BMD at age 6 years, although these associations were attenuated after full adjustment. Studies conducted in Holland (Heppe et al.) and the UK (Tobias et al.) assessed whole body less head BMC and BMD by DXA at 6 and 9 years of age, respectively, in approximately 3000 children (210, 212). In both studies greater maternal calcium intake was associated with higher offspring whole body BMC, with mixed findings for BMD and relationships were attenuated after adjustment for a range of covariates. A smaller mother-offspring birth cohort in Tasmania, Australia determined maternal calcium and milk intake using a self-administered food frequency questionnaire for the third trimester of pregnancy. Offspring BMD was assessed at 8 years (211), and again at 16 years of age (209), representing the longest follow-up of any cohort study of calcium intake in pregnancy. At 8 years, maternal milk intake during pregnancy was positively associated with femoral neck BMD after adjustment for various factors (maternal education, paternal unemployment, smoking during pregnancy and the child's sex, weight and height, sunlight exposure, sports participation, history of being breast-fed and current calcium intake) (211). However with time, the influence of maternal calcium intake on bone appeared less pronounced - at 16 years of age maternal calcium intake was positively associated with lumbar spine BMD only after adjusting for the same covariates and Tanner stage (209).

Intervention studies of calcium supplementation in pregnancy and offspring bone accrual (215-217), like the observational studies, provide inconsistent findings. An early randomised controlled trial of calcium supplementation in 87 pregnant women performed in India provided either 300 mg calcium per day, 600 mg calcium per day or placebo from 20 weeks gestation. Neonatal BMD was crudely determined by calculation of radiographic density using radiographs of the ulna, radius, tibia and fibula. Overall, the calcium supplement was associated with significant increases in offspring neonatal BMD compared to infants of unsupplemented mothers, but no difference between the two supplementation groups was noted (217). A randomised, double blind, placebo controlled trial (RCT) of calcium 1500 mg per day from 20 weeks gestation was performed in The

Gambia, a country in which calcium intake is low (typically 300-400 mg per day). Infant bone mineral status was assessed using single photon absorptiometry of the radius and whole body DXA at 2, 13 and 52 weeks post-delivery, but this study showed no significant differences between the groups in terms of BMD, or indeed breast milk calcium concentration, infant birth weight or growth during the first year of life (215). Nor did the supplement influence maternal bone health - unexpectedly, the mothers who had received calcium supplementation in pregnancy had lower BMC. The authors suggested that perhaps, in this population who are adapted to very low calcium intake, supplementation with calcium may result in detrimental disruption of calcium metabolism (218). A RCT of calcium supplementation in a developed country was performed in 256 mother-offspring pairs in Memphis, USA. Women were randomised to 2 g per day calcium or placebo from 22 weeks gestation until delivery (216), maternal dietary intake was recorded and offspring DXA measurements were performed before hospital discharge. No significant differences were found between the treatment groups overall, but when only women in the lowest quintile of calcium intake (< 600 mg per day) were considered, total body BMC was significantly greater in infants born to mothers who received the calcium supplement compared to those who received the placebo. Therefore in this study (of women adapted to a high calcium intake), in contrast to the study in Gambian women, this suggested a benefit only to offspring of mothers with low dietary calcium intake (216). It is likely that long-term intakes were lower in the Gambian than US women, and maternal bone assessment was not available in the US study. Therefore the value of calcium supplementation in pregnancy has yet to be ascertained.

1.7 The role of Vitamin D in bone and muscle health

1.7.1 Metabolism and the classical function of Vitamin D

The majority of vitamin D is formed endogenously within the skin from the action of ultraviolet B (UVB) (290-315nm wavelength), enabling the conversion of 7-dehydrocholesterol to pre-vitamin D_3. A smaller proportion of vitamin D can be derived from the diet, as ergocalciferol (vitamin D_2) from fungi sources, or cholecalciferol (vitamin D_3) from animal sources – structures of cholecalciferol and ergocalciferol are demonstrated in Figure 1.14. These pro-hormones are hydroxylated in the liver to calcidiol (25-hydroxyvitamin D [25(OH)D]) by the 25-hydroxylase enzyme, a cytochrome-p450 dependent enzyme (Figure 1.15). 25(OH) D is the main circulating form of vitamin D, and can be found bound to either vitamin D binding protein (DBP), albumin, or in the free form.

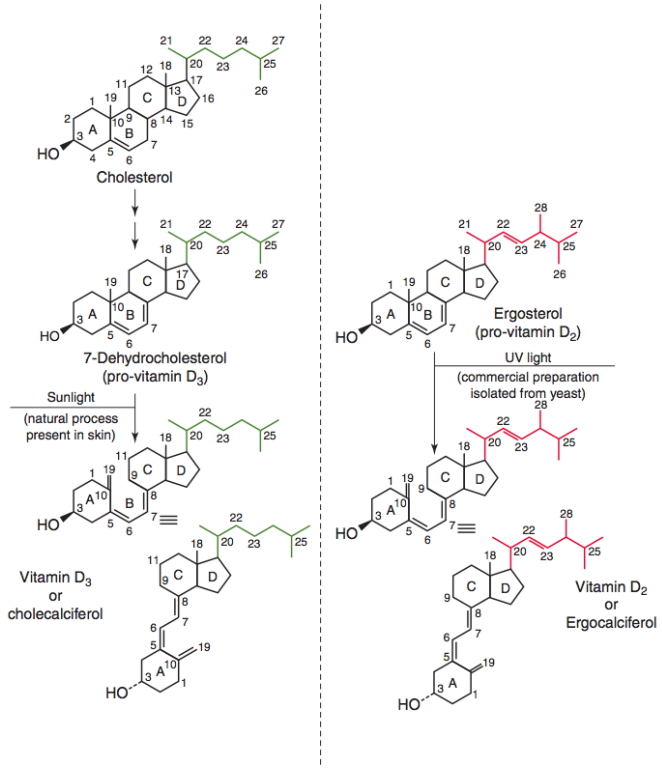


Figure 1.14 Structural relationship of the secosteroids vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) with their respective provitamins, cholesterol and ergosterol. Vitamin D_2 may be produced commercially by the irradiation of the fungi sterol ergosterol with ultraviolet light. *Reproduced with permission from Zhu et al., 1995 (219). http://vitamind.ucr.edu/about/*

Circulating 25(OH)D acts as a reservoir for conversion to the active metabolite, calcitriol (1,25-dihydroxyvitamin D [1,25$(OH)_2$D]) by the 1α-hydroxylase enzyme. This conversion occurs primarily in the renal proximal tubular cells, although additionally to a lesser degree in extra-renal tissues including bone and the parathyroid gland. Calcitriol formed in the kidney is secreted into the circulation and acts in an endocrine manner, whereas the 1,25$(OH)_2$D synthesised in bone and the parathyroid has functions on the local tissue only. The conversion of 25(OH)D to the inactive metabolite, 24,25-dihydroxyvitamin D also occurs primarily within the kidney - the first step of the degradation pathway.

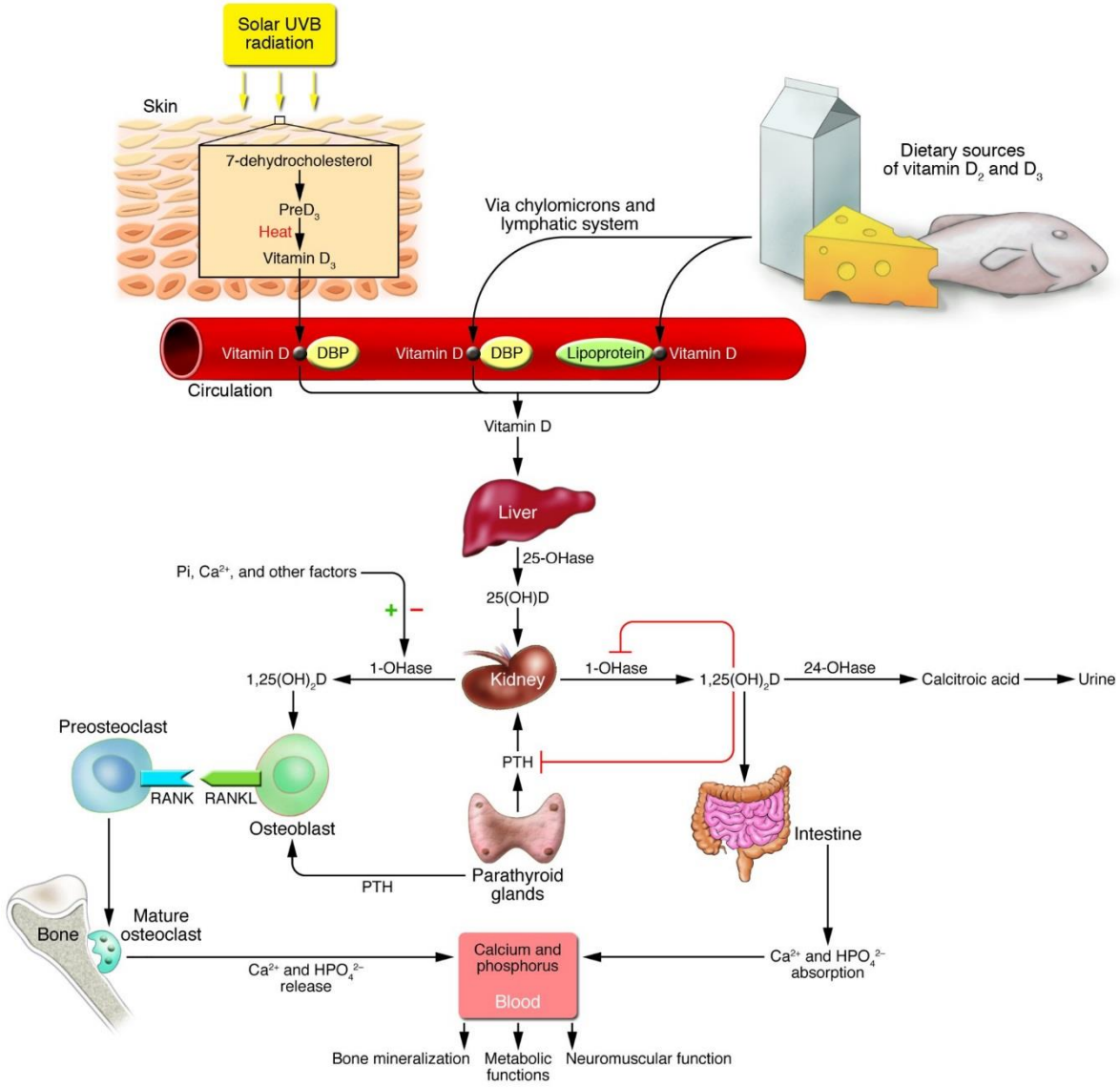


Figure 1.15 Vitamin D metabolism. The main function of the active form of vitamin D, $1,25(OH)_2D$ is to increase intestinal calcium and phosphate absorption, to facilitate bone mineralisation, metabolic functions and neuromuscular function. *Reproduced with permission from Holick, 2006 (220)*

The classically recognised functions of $1,25(OH)_2D$ are in calcium and phosphate homeostasis. Synthesis of $1,25(OH)_2D$ within the kidney is tightly regulated in response to serum ionised calcium (Ca^{2+}) levels via the actions of parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF-23). Low Ca^{2+} stimulates the release of PTH from the parathyroid gland. PTH has several actions in the kidney, simultaneously increasing renal calcium reabsorption in the distal tubule of the kidney, decreasing proximal tubule phosphate reabsorption, and upregulating 1α-hydroxylase activity to increase synthesis. The main action of $1,25(OH)_2D$ is to increase intestinal enterocyte absorption of dietary calcium. PTH also acts on bone, promoting resorption by osteoclasts,

thereby mobilising calcium and phosphate from bone mineral, an action which requires the presence of $1,25(OH)_2D$. As serum Ca^{2+} levels rise, completion of the negative feedback loop occurs, with the calcium sensing receptor in the parathyroid gland reducing release of PTH in response to increased serum Ca^{2+}, but also through FGF-23. FGF-23 production is increased by $1,25(OH)_2D$ and high circulating phosphate and it acts to increase urinary phosphate excretion and down-regulate 1α-hydroxylase activity and PTH release.

Low levels of 25(OH)D, as in vitamin D deficiency, can result in low intestinal calcium absorption, and subsequently a reduction in serum Ca^{2+}. This stimulates PTH production (secondary hyperparathyroidism), with subsequent stimulation of osteoclasts to mobilise bone mineral. PTH will also increase renal calcium re-absorption, but decrease phosphate reabsorption, leading to urinary phosphate wasting. As such, in early vitamin D deficiency, serum calcium concentration is usually maintained in the normal range, but phosphate is often low; hypocalcaemia will only result when skeletal calcium stores are depleted.

Vitamin D has both rapid non-genomic effects within the cell, such as stimulating intestinal calcium uptake, and slow genomic actions, such as transcription of intestinal calcium channels and FGF-23 (221). The genomic actions are mediated through the binding of $1,25(OH)_2D$ to the nuclear vitamin D receptor (VDR). The $1,25(OH)_2D$ –VDR complex can heterodimerise with a retinoid X receptor (RXR) (usually the RXR-alpha), which binds to vitamin D response elements in promoter regions of target genes, resulting in activation or repression of gene transcription through recruitment of complexes or either coactivators or corepressors. The mechanisms of the non-genomic effects remain to be fully elucidated, but likely involves the VDR located within the plasma membrane, via different ligand structural requirements than those utilised by the classic nuclear VDR (221).

Interestingly, although the classical function of vitamin D is in calcium and phosphate homeostasis, the nuclear VDR has been identified in a wide variety of cell types including osteoblasts, keratinocytes, macrophages, pancreatic β cells, adipose tissue and skeletal muscle (222, 223). This supports the hypothesis that vitamin D might have diverse actions apart from in bone and calcium metabolism. Consequently, research is ongoing into the role of vitamin D, not just in osteoporosis, but in cancer, type-2 diabetes, atherosclerosis, vascular disease and infection.

1.7.2 Assessment of vitamin D status

25(OH)D concentration is currently considered the best biochemical marker of vitamin D status, as hepatic 25-hydroxylation of cholecalciferol to 25(OH)D is not physiologically regulated, but is dependent on substrate availability. In contrast, the conversion of 25(OH)D to $1,25(OH)_2D$ is tightly regulated in response to serum Ca^{2+} and PTH. Also, 25(OH)D has a half-life of approximately 2-3 weeks, whereas $1,25(OH)_2D$ has a significantly shorter half-life of 4 to 6 hours (224).

The two most common methods to measure 25(OH)D concentration are liquid chromatography-mass spectrometry (LC-MS/MS), also known as tandem mass spectrometry, or immunoassay. As the analytical method used must be able to measure both $25(OH)D_2$ and $25(OH)D_3$; LC-MS/MS is considered the gold standard due to high specificity for each metabolite, combining the two to give a total 25(OH)D result. The disadvantages of LC-MS/MS are that it is time consuming, expensive and requires well-trained operators, and therefore automated immunoassays are commonly used in clinical settings. However, the pitfalls of immunoassays are their reduced ability to detect $25(OH)D_2$ compared with $25(OH)D_3$. This has implications for analysis and interpretation of immunoassay based studies on vitamin D status. For example, the Roche immunoassay does not detect $25(OH)D_2$, the Immunodiagnostic Systems (IDS) radioimmunoassay only has approximately 75% cross-reactivity for $25(OH)D_2$ and $25(OH)D_3$, whilst the Diasorin Liaison™ chemiluminescence assay is able to measure $25(OH)D_2$ and $25(OH)D_3$ equally (225). As such, care should be taken in interpreting studies using the Roche and IDS assays, particularly if high levels of dietary ergocalciferol is likely or ergocalciferol supplementation has been taken. Variability between analytical techniques has also been reported with the Diasorin Liaison platform typically reporting lower values that LC-MS/MS (225). The Vitamin D External Quality Assurance scheme (DEQAS), in which over 1200 laboratories from 54 countries participates, aims to maintain the analytical reliability of 25(OH)D and $1,25(OH)_2D$ assays (http://www.deqas.org/).

1.7.3 Epidemiology of vitamin D deficiency

The serum concentration of 25(OH)D which is felt to be sufficient is a subject of much debate, despite the fact that vitamin D status has been related to a wide range of biochemical and clinical outcomes in observational studies (226). National and expert group guidelines and consensus statements recommend varying thresholds for vitamin D deficiency, insufficiency and sufficiency. For example, the National Osteoporosis Society (UK) classifies deficiency as 25(OH)D < 30 nmol/l, insufficiency 30-50 nmol/l, and sufficiency ≥ 50 nmol/l (227), which is also in agreement with

recommendations from the Institute of Medicine (228) and the Global Consensus Recommendations on Prevention and Management of Nutritional Rickets (229). The Scientific Advisory Committee on Nutrition (SACN), and the UK Department for Health, providing population-level recommendations, classify deficiency as < 25 nmol/l, and sufficiency ≥ 25 nmol/l. However, the Endocrine Society Practice Guidelines classify deficiency as < 50 nmol/l, and sufficiency ≥ 75 nmol/l, (230), whilst the Canadian Paediatric Society set a threshold for deficiency at < 25 nmol/l, and provide a range for sufficiency as 75-225 nmol/l (231). The working groups of the Australian and New Zealand Bone and Mineral Society set an alternative threshold of deficiency as < 50 nmol/l and sufficiency as ≥ 50 nmol/l, with no "insufficiency" category (232).

The variability in these definitions between working groups and societies reflects the fact that there does not appear to be a single threshold below which secondary hyperparathyroidism or clinical outcomes, such as metabolic bone disease, always occurs in either adults (233, 234) or children (235). This is thought to be due to the interaction between vitamin D and dietary calcium intake, which will modify the association between 25(OH)D and PTH (236).

Variations in the definitions of vitamin D deficiency limit comparison of its reported prevalence between studies. Additionally, there is little data which documents the epidemiology of serum 25(OH)D concentrations across the general population, both in children and adults. Two recent large studies in the UK demonstrated that around a third of children had a serum 25(OH)D < 50 nmol/l (237, 238), and the authors identified a number of risk factors for vitamin D deficiency, linked to reduced UVB exposure and limited cutaneous synthesis of cholecalciferol.

Marked seasonal variation in the prevalence of vitamin D deficiency is noted in the UK and other high latitude countries (237-240), with 25(OH)D levels at their nadir in late winter and zenith in mid-late summer. The effect of latitude on 25(OH)D status is observed with remarkable granularity, for example, within the UK, children and post-menopausal women residing in Northern England and Scotland have been shown to have lower 25(OH)D levels than those living in the South of England even after adjustment for seasonal variation (237, 240, 241). Hence, individuals at more northerly latitudes are more reliant on dietary sources and supplementation to prevent deficiency during winter months (237, 240, 242). Skin colour also has a profound influence on serum 25(OH)D levels, with white individuals at the same latitude having consistently higher titres compared with dark skinned individuals (237, 242, 243). Clearly, greater exposure to UVB radiation through more time spent outdoors has been shown to be protective against deficiency (237, 238), whereas extensive skin covering for religious or cultural reasons and liberal use of sun protection are risk factors (244). Other less well understood, socioeconomic risk factors

for poorer vitamin D status in children have been identified, including living in rented housing, and lower parental educational achievement and income (237, 238, 242, 243). The mechanisms behind this are currently unclear, but are likely to be related to poorer dietary intake and comparatively lower durations of outdoor leisure time in individuals of lower, as compared to higher socioeconomic status.

In older people, data from three multicentre, standardised studies show that between 17% and 58% of older Europeans are vitamin D deficient (< 30 nmol/l) (245-247) , whilst in the UK, in men and women over the age of 65 years approximately 10% of free living and 40% of adults living in nursing and care homes have plasma 25(OH)D concentrations less than 25 nmol/l throughout the year (248).

1.7.4 Consequences of severe vitamin D deficiency: rickets and osteomalacia

Rickets is a childhood disorder of growth plate ossification and mineralisation, which occurs only in growing bone. Vitamin D deficiency is one of several of biochemical and/or hormonal disturbances, including calcium and phosphate deficiency which result in this clinical phenotype. Hypophosphatemia is the common end pathway in these biochemical abnormalities, and is postulated to be the cause of the hypertrophic chondrocytes which develop in the growth plate in rickets (249). Rare inborn errors of vitamin D metabolism can also lead to the rickets phenotype, and include vitamin D dependent rickets type 1, 2 and 3. The metabolic and biochemical changes in rickets and osteomalacia are described in Figure 1.16.

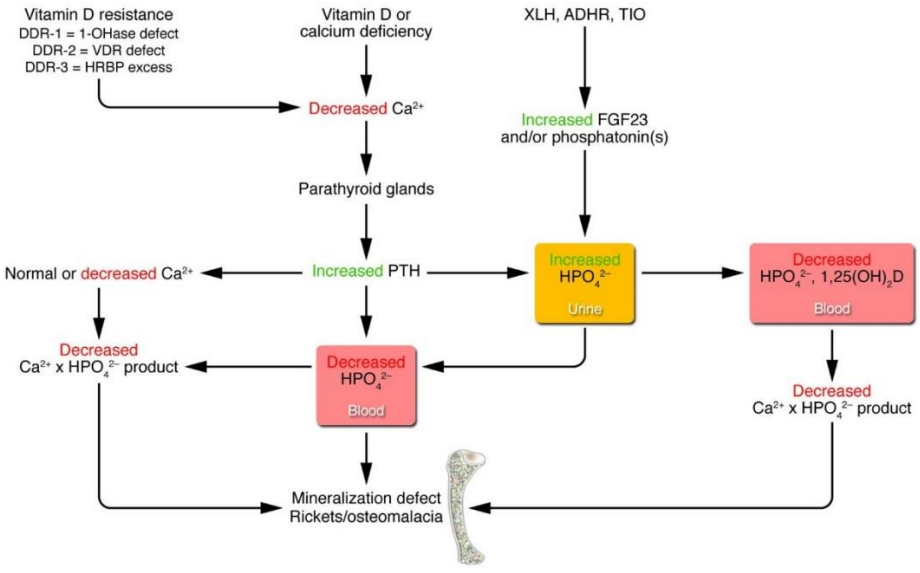


Figure 1.16 Biochemical changes in calcium and phosphorous metabolism due to vitamin D or calcium deficiency, vitamin D resistant syndromes, and hypophosphataemic syndromes leading to rickets or osteomalacia. *Reproduced with permission from Holick 2006 (220).*

Vitamin D and/or calcium deficiency leads to a decrease in the levels of Ca^{2+}, resulting in an increase in PTH, which normally acts to increase tubular reabsorption of Ca^{2+} to correct the serum level. However, in severe calcium and vitamin D deficiency, the serum Ca^{2+} is below normal. In addition, PTH causes phosphorus loss via the urine, resulting in a decrease in serum HPO_4^{2-}. An inadequate calcium-phosphorus product ($Ca^{+2} \times HPO_4^{2-}$) leads to a defect in bone mineralisation.

Various inherited and acquired disorders of calcium and phosphorus metabolism can also result in similar phenotype to rickets. These include three types of vitamin D dependent rickets (DDR), types 1-3, due to mutations in the *1α-hydroxylase* gene, *VDR* gene, and *hormone response element–binding protein* (*HRBP*), respectively (250). There are also inherited and acquired disorders that cause severe hypophosphatemia and decrease renal production of $1,25(OH)_2D$. X-linked hypophosphatemic rickets (XLH) and autosomal dominant hypophosphatemic rickets (ADHR) are caused by the increased production or decreased destruction, respectively, of phosphatonins, including FGF23. Tumour-induced osteomalacia (TIO) is caused by FGF23 production by a tumour, which results in phosphaturia and a decrease in the renal production of $1,25(OH)_2D$.

The classical skeletal features of rickets (although partly dependent on the developmental age of the child) include bony swelling at the wrists, knees, ankles and costochondral junction (rachictic rosary). In mobile children bending of the limbs is often observed, and linear growth, dentition and motor development are delayed. Hypocalcaemic seizures are sometimes the first presenting feature of rickets in the neonatal period and in adolescence, as it is at these times that calcium demands are at their highest, in order to meet the demands for rapid linear growth. Interestingly, there is currently little evidence to suggest that children with rickets have low BMD or are at increased risk of fractures compared to non rachictic children, despite the skeletal structural changes (251). Rickets is anecdotally on the increase in Western populations. However epidemiological data to support a generalised resurgence are lacking and the cases reported are predominantly in dark skinned ethnic groups (252, 253).

Following fusion of the growth plates, vitamin D deficiency can result in osteomalacia. This is a histological finding, in which the protein matrix that constitutes the basis of bone tissue is under-mineralised. Clinical features include bony pains, especially of the spine, legs and pelvis, muscle weakness and pathological fractures, but in many, fatigue is the only symptom(254). The treatment of osteomalacia is vitamin D supplementation, sometimes with calcium (255), and it has been shown that BMD can increase considerably (by 70% or more), although the evidence is based on relatively small case series (256).

1.7.5 Consequences of subclinical vitamin D deficiency and bone health in infancy, childhood and adulthood

Research is ongoing as to whether levels of serum 25(OH)D which are not low enough to cause rickets in infants and children, or osteomalacia in adults, can still have detrimental effects on bone development and BMD.

The vitamin D content of breast milk is particularly low, therefore some studies have focused on vitamin D supplementation in breast fed infants. Somewhat surprisingly, three intervention studies of vitamin D supplementation this period have not found a beneficial effect on whole body BMC, lumbar spine BMC, or aBMD at three, six, or twelve months of age (257-259). In one study, published in 1982 and using single photon absorptiometry, a significantly greater distal radius BMC was detected in the supplemented three month old infants, but the difference did not persist to six months of age (260).

In children and adolescents, various cross sectional studies have analysed the association between 25(OH)D status (as a continuous variable) and DXA indices of bone health. There is considerable heterogeneity in the study findings, and comparison is impaired by the differences in geographic location, age of the participants and other confounding factors between the studies (261-277). However, in studies which categorised 25(OH)D status, children and adolescents with the lowest levels of serum 25(OH)D had significantly lower BMC at one or more sites (261, 265, 266, 268, 278-280). One of the largest studies of adolescents, the Korean National Health and Nutrition Examination Survey, showed a significant association between serum 25(OH)D with BMC in a study of approximately 3000 adolescents and young adults, and demonstrated a supportive role of calcium on bone mass for early adolescents and young adult males, as shown in Figure 1.17. A notable exception was in studies based on ballet dancer cohorts where no associations between 25(OH)D and BMC were seen; in these individuals high levels of physical activity may have been a confounding factor (277, 281).

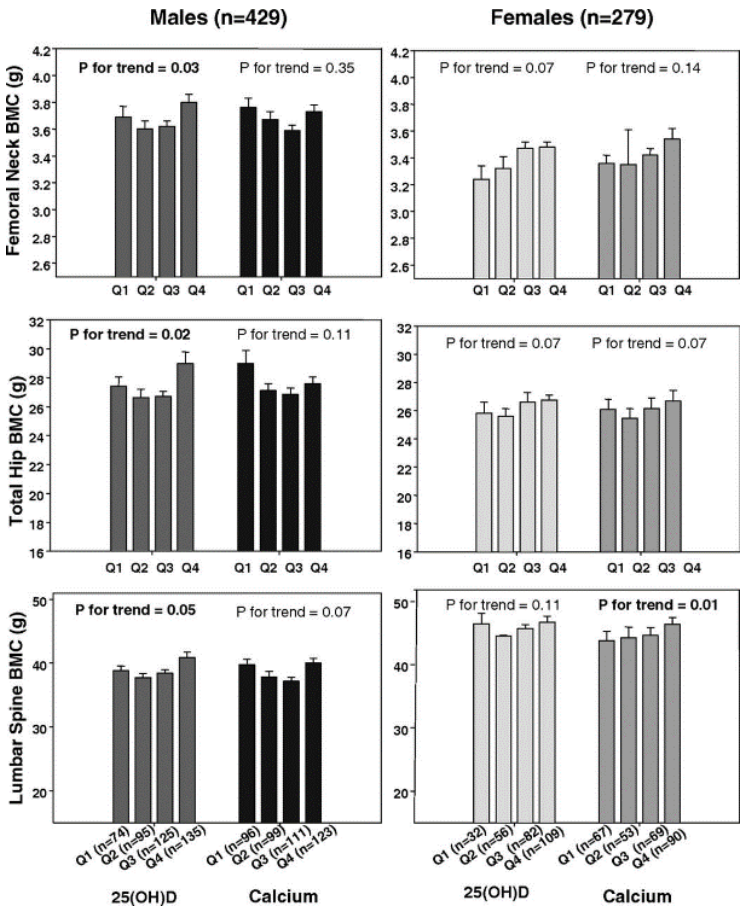


Figure 1.17 Results from the KNHANES Cohort: BMC of femoral neck, total hip, and lumbar spine by serum 25(OH)D concentration and dietary calcium intake. Four bars represent quarters (Q) of serum 25(OH)D concentrations and calcium intake for males and females. Data were adjusted for age, total calorie intake, body weight, height, calcium intake/serum 25(OH)D, age of menarche for females, and season. *Reproduced with permission from Joo et al., 2013 (280).*

A Cochrane meta-analysis of RCTs of vitamin D supplementation in childhood and adolescence published in 2010 by Winzenburg et al. concluded that it was unlikely that vitamin D supplements were beneficial in children with normal vitamin D levels, and a non-linear relationship was suggested. This meta-analysis 6 studies with a total of 343 participants randomised to placebo and 541 to cholecalciferol, with doses ranging from 132 IU/day to 14000 IU/week for one to 2 years. Whilst overall, vitamin D supplementation did not lead to significant gains in whole body BMC, forearm, hip or LS aBMD, in studies with a low baseline 25(OH)D (< 35 nmol/l), vitamin D supplementation had a significant effect on whole body BMC and LS BMD, which equated to approximately a 2.6% and 1.7% percentage change from the baseline to the supplemented group (282). The authors suggested that vitamin D supplementation of deficient children and adolescents could result in clinically useful improvements, particularly in lumbar spine total BMD and on whole body BMC, and provided support for the hypothesis that very low levels of 25(OH)D

might have subclinical effects on bone development (283). Interestingly, posthoc followup of one trial, a year after discontinuation of supplementation, demonstrated persistent benefits on hip BMC in girls (mean age 14 years), but not in boys (284). In a UK study of adolescents based in Manchester, it was shown that sun exposure alone failed to provide adequate vitamin D, with a quarter of adolescents insufficient even at the summer peak (September); this provides further support for vitamin D supplementation in this age group in the UK (285).

Further inconsistencies persist in the evidence for the role of vitamin D supplementation in bone health and fracture prevention in adults. There is epidemiological evidence that higher serum 25(OH)D levels are associated with greater BMD in both young and old populations, with a linear positive association maintained up to a serum 25(OH)D level of 75 nmol/l in white populations, but interestingly not in black or Hispanic US populations (286). However, a meta-analysis of 23 RCTs of vitamin D supplementation on aBMD assessed by DXA did not show a significant effect of supplementation on aBMD at the LS, hip, whole body or forearm, but there was some evidence of benefit in femoral neck aBMD (weighted mean difference 0.8% (95% CI 0.2-1.4%), p = 0.005) (287). However, some caution should be applied in interpretation of these findings, as there was marked heterogeneity in study design, type and dose of vitamin D supplementation, and age, sex, BMI and ethnicity of study participants. In contrast to the aforementioned childhood meta-analysis, stratification of studies by mean baseline 25(OH)D did not alter these findings, but this could reflect the higher cut-point chosen to define low 25(OH)D (50 nmol/l) (287). By comparison, one placebo-controlled study of women aged over 65 years in Aberdeen, not included in the meta-analysis by Reid and colleagues, showed that a daily dose of 1000 IU (but not 400 IU) vitamin D_3 attenuated the decline in BMD over 12 months at the total hip site(288).

The most important clinical outcome of interest in vitamin D supplementation is of course osteoporotic fracture risk reduction. Studies by Bishcoff-Ferrari et al. (2005-2012) have suggested that vitamin D supplementation in doses of at least 800–1000 IU daily are required to decrease the incidence of both falls and fractures in older women, whereas lower doses are ineffective (289-291). In contrast, a Cochrane Review (292), the DIPART (vitamin D Individual Patient Analysis of Randomized Trials) Group (293) and the Institute of Medicine (IOM) review of dietary references for vitamin D and calcium (294) found no evidence that vitamin D supplementation can prevent fractures. However, when co-prescribed with calcium, hip fracture risk, total fracture and non-vertebral fractures were shown to be reduced, particularly in individuals living in care homes or sheltered housing. For example, the Cochrane meta-analysis of low risk community dwelling post-menopausal women in whom hip fracture incidence is approximately 8 per 1000/year, vitamin D and calcium supplementation reduced this by 1 per 1000/year. In institutionalised

individuals (in whom hip fracture rate is substantially higher) vitamin D with calcium supplementation can reduce hip fracture rate by 9 per 1000/year (292).

The dose of vitamin D supplement has been shown to be an important factor in determining fracture risk – however with considerable heterogeneity between studies. Overall however, high, infrequent doses of vitamin D appears to increase the risk of falls, discussed in section 1.7.7 (295, 296).

As the effect of vitamin D dosing and interval on BMD is less clear, it is therefore possible that the reduction in fracture risk is occurring partly due to extra skeletal actions of vitamin D, or perhaps calcium metabolism. This may have lasting or transient effects on muscle function and subsequently falls incidence. It would be useful to understand more about the baseline vitamin D status in the studies in question, the dose effect on 25(OH)D levels and calcium levels at baseline and during supplementation to help interpret their effects on falls and fracture risk in the populations in question.

1.7.6 Guidance on the use of vitamin D and calcium in the clinical setting

As calcium and vitamin D metabolism are so intimately linked, various studies have investigated the role of either calcium alone or calcium in combination with vitamin D for fracture reduction, and subsequent meta-analyses aiming to elucidate the overall effectiveness of these interventions (297-299).

A large UK randomised controlled trial demonstrated that supplementation with either calcium, vitamin D or both for secondary fracture prevention at the population level appeared ineffective (300), although supplementation in high risk settings where deficiencies are expected, for example in nursing homes, may be beneficial (301). A recent individual patient data meta-analysis demonstrated that overall, there appeared to be a modest benefit for combined vitamin D and calcium supplementation for hip fractures, total fractures and probably vertebral fractures, but that there was no benefit for vitamin D alone (302). Although there has been discussion from one research group that excess calcium intake may be associated with increased cardiovascular risk (303), this has not been substantiated across many other studies. Indeed, it is reassuring to note that a recent individual-patient-data meta-analysis of the anti-fracture studies suggests that calcium and vitamin D supplementation in combination is associated with an improvement in mortality, which is not observed with vitamin D supplementation alone (304). Almost all of the randomised control trial evidence for the efficacy of anti-osteoporosis drugs comes from patients

who were prescribed commitment calcium and vitamin D supplementation; both should therefore usually be prescribed adjunctively with treatment for osteoporosis.

A recently published report by the European Society for the clinical and economic aspects of osteoporosis, osteoarthritis and musculoskeletal diseases (ESCEO) and the International Osteoporosis Foundation (IOF) provided five main conclusions:

1. Calcium and vitamin D supplementation leads to a modest reduction in fracture risk, though has not been shown to be an effective public health strategy at the population level
2. Supplementation of calcium alone for fracture reduction is not supported by the literature
3. Side effects of calcium include gastrointestinal symptoms and renal stones
4. Vitamin D supplementation, rather than calcium supplementation, may reduce falls risk
5. The assertions of increased cardiovascular risk as a consequence of calcium supplementation are not convincingly supported by current evidence.

The authors therefore recommend that calcium with concomitant vitamin D supplementation is supported for patients at high risk of calcium and vitamin D deficiency, and for those receiving treatment for osteoporosis (305).

1.7.7 Vitamin D and muscle mass and function

There are several lines of evidence in support of a role of vitamin D in muscle health (306). First, proximal muscle weakness, diffuse muscle pain and an abnormal waddling gait, and in children, developmental delay, are well known clinical symptoms of vitamin D deficiency (306, 307).

Second, the VDR is expressed in human muscle tissue (308), and VDR activation may promote de novo protein synthesis in muscle (309), with differential effects of 25(OH)D and $1,25(OH)_2D$ on gene expression and on muscle strength and lean mass (310).

Third, a range of observational studies have suggested a positive association between 25(OH)D and muscle strength or lower limb function from adolescence to older age (311).

Finally, meta-analysis of RCTs of vitamin D supplementation in adults have demonstrated a small, but statistically significant, beneficial effect of vitamin D supplementation on muscle strength (29 studies, standardised mean difference of 0.17 [95% CI 0.03, 0.31], p = 0.02) but not muscle mass (6 studies) or muscle power (5 studies) (312). It is important to note that the included studies were markedly heterogeneous in terms of population studied and degree of supplementation,

and varied in the methods of outcome assessment. When studies were stratified on participant characteristics, vitamin D supplementation had a greater effect on muscle strength in individuals with a serum 25(OH)D < 30 nmol/l, similar to studies on bone outcomes. Enhanced effects were noted in individuals aged over 65 years and female. Furthermore, when studies using grip strength and lower limb muscle strength as the outcome were considered separately, the effect was only significant for lower limb strength.

More recently however, in 2016, a trial of high dose vitamin D supplementation (Bischoff-Ferrari et al.) had no benefit on lower limb function and doses above 24,000 IU per month were associated with an increased risk of falls (296).

Though a variety of observational studies have reported positive associations between 25(OH)D status and measures of muscle mass (313, 314), muscle strength and power in adolescents (315, 316), the interpretation of these observational studies is limited by the potential for reverse causality and confounding; as more physically active individuals are probably more likely to spend greater lengths of time outdoors. Three trials of vitamin D supplementation, two in adolescents and one in children examined the effect on muscle function: Ward et al. randomised 72 ethnically diverse post-menarchal girls aged 12-14 years to 150 000 IU oral ergocalciferol or placebo every 3 months for 12 months. Improved jumping efficacy was observed in the vitamin D treatment group, but no improvements in jumping power, force or hand grip strength were observed (317). El-Hajj Fuleihan et al. randomised 179 females aged 10–17 years (mean 25(OH)D at baseline of 35 nmol/l) to receive either 1400 IU vitamin D_3 per week (low dose), 14 000 IU vitamin D_3 per week (high dose) or a placebo weekly for 1 year. Significant increases in lean mass (measured by DXA) were observed in both the low and high dose treatment groups compared to the placebo group, despite no significant change in hand grip strength between the treatment groups. The authors suggest that the changes in lean mass observed in the study may be due to a direct effect of vitamin D on muscle size; however, the lack of effect on hand grip strength may indicate that this measure is not sufficiently sensitive to detect slight changes in muscle strength in children and adolescents (318). The most recent trial in adolescents, undertaken in the USA by Wright et al., randomised 324 children aged 9-13 to cholecalciferol supplements (400, 1000, 2000, 4000 IU/day or placebo) for 12 weeks in the winter months. Changes in vitamin D metabolites (serum 25(OH)D, 1,25(OH)D, intact PTH (iPTH)) and outcomes of muscle mass, strength and body composition (using pQCT), and hand grip strength were measured. Vitamin D metabolites were not associated with muscle strength at baseline nor after the 12 week intervention, but increases in 25(OH)D were correlated with decreases in forearm intramuscular adipose tissue (319).

A trial in younger children was also published in 2018; Mortensen et al. randomised 117 Danish children aged 4-8 years to placebo or either 400 IU or 800 IU of cholecalciferol for 20 weeks (320). They showed that, although serum 25(OH)D was positively associated with muscle strength, fat free mass index and insulin like growth factor binding protein 3 (IGF-BP3) at baseline in girls only, at the end of the intervention muscle strength, fat free mass index and fat mass index did not differ between groups. In the 800 IU group, baseline-adjusted IGF-1 and IGFBP-3 were higher compared to placebo, and there was a trend to children being taller in the high- dose group. The authors suggest that, as vitamin D is suggested to stimulate liver production of IGF-1 and its binding protein, IGF-BP3 (321), vitamin D supplementation could stimulate linear growth and the hypertrophy of skeletal muscle (322); this is supported by evidence from vitamin D supplementation studies in children with nutritional rickets (323).

1.7.8 Vitamin D in pregnancy and offspring health and development

Though profound vitamin D deficiency is not commonly seen in the UK, low circulating concentrations of 25(OH)-vitamin D [25(OH)D] are highly prevalent in pregnant women.

In a cohort of 198 pregnant Caucasian women from Southampton, UK, 31% had a circulating serum 25(OH)D lower than 50 nmol/l, often considered “insufficient" and 18% were vitamin D deficient (< 25 nmol/l) (324). However, in a more ethnically diverse population in London, 36% of women had a 25(OH)D < 25 nmol/l at pregnancy booking (325). Vitamin D deficiency in pregnancy is an important consideration as the fetus is dependent on the mother for 25(OH)D, which will readily cross the placenta. Indeed, maternal and umbilical cord venous blood 25(OH)D are moderately to highly correlated (326-328). RCTs of vitamin D supplementation in pregnancy have demonstrated that supplementation can increase umbilical cord venous and neonatal serum 25(OH)D compared to placebo (327, 329-332), and that larger oral doses of cholecalciferol (1000 IU/day-4000 IU/day) result in higher umbilical cord venous or neonatal 25(OH)D when compared to supplementation with 400 IU/day (333, 334).

In order to meet the demands for fetal mineral accretion, alterations to maternal calcium and phosphate metabolism occur during pregnancy – particularly in the last trimester, when the majority of the approximately 30 g of calcium required to form the fetal skeleton is obtained (76, 335). This occurs both through increased maternal intestinal calcium absorption (136) and mobilization of the maternal skeleton (112). Maternal serum Ca^{2+} concentration is not influenced by this process, due to the role of maternal calcitropic hormones. Total $1,25(OH)_2D$ and DBP increases early in pregnancy (136, 336, 337), but unlike $1,25(OH)_2D$, DBP does not continue to

increase in late pregnancy. As a result, free $1,25(OH)_2D$ is increased in the third trimester relative to earlier in pregnancy (337). This elevation in $1,25(OH)_2D$ occurs without a rise in PTH, which remains within the normal adult range throughout pregnancy, though may occur as a result of elevated PTH related protein in the maternal circulation from early pregnancy onwards (76, 336).

Prior to recent analyses from the Southampton Women's Survey, there was little evidence on the tracking of 25(OH)D status during pregnancy, though $1,25(OH)_2D$ is known to increase. Studies produced conflicting findings, with one Irish study reporting a reduction in 25(OH)D from early to late pregnancy (though in all cases, late pregnancy coincided with winter) (338), and others reporting no significant differences in 25(OH)D status throughout pregnancy (112, 339). One study reported a significantly higher 25(OH)D in the third trimester compared to the first trimester or pre-pregnancy (136). Results from a total of 1753 women with 25(OH)D measured in both early and late pregnancy in the Southampton Women's Survey showed that there was moderate tracking of 25(OH) D status with a moderate correlation between season-corrected 25(OH)D measurements at 11 and 34 weeks of gestation ($r = 0.53$, $p < 0.0001$; $n = 1753$). This means that in general, an individual with a high 25(OH)D for the population remains within the upper end of the population distribution when 25(OH)D status is repeated at a later date) with marked seasonal fluctuation observed. Commencement and continuation of Vitamin D supplementation was associated with increases in season-corrected 25(OH)D, higher pregnancy weight gain was associated with a reduction in season-corrected 25(OH)D, whereas greater physical activity was associated with increases (340). In the MAVIDOS study, it was demonstrated that vitamin D supplementation of 1000 IU/d cholecalciferol in pregnancy led to an increased 25(OH)D at 34 weeks gestation, and that women who gained more weight during pregnancy had lower 25(OH)D in early pregnancy and delivered in winter achieved a lower 25(OH)D in late pregnancy when supplemented with 1000 IU/d cholecalciferol as shown in Figure 1.18 (341).

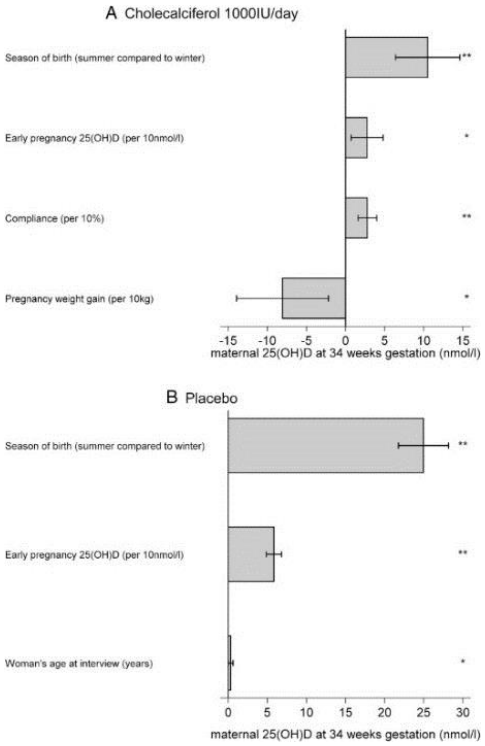


Figure 1.18 Independent determinants of maternal 25(OH)D at 34 weeks of gestation (A) after supplementation with 1000 IU cholecalciferol per day from 14 weeks of gestation until delivery and (B) receiving placebo from 14 weeks of gestation until delivery. Shown as change in 25(OH)D per unit predictor. *, $p < .05$; **, $p < .01$. A, Cholecalciferol 1000 IU/d. B, Placebo.
Reproduced with permission from Moon et al. 2016 (341)

Maternal VDD has been associated with neonatal hypocalcaemia, which can result in neonatal seizures, and has been associated with craniotabes (softening and thinning of the bones of the skull) (342), and rarely dilated cardiomyopathy (343). Symptomatic neonatal hypocalcaemia due to maternal VDD most commonly occurs in infants of mothers with dark skin pigmentation, extensive skin covering and in individuals with the most profound VDD. As such, the UK Department of Health (DoH) currently recommends supplementation with 400 IU/day cholecalciferol to all pregnant and breastfeeding women (344).

Vitamin D supplementation has been shown to consistently reduce the incidence of symptomatic hypocalcaemia in neonates (329, 345, 346). The evidence for biochemical changes to calcium homeostasis with antenatal vitamin D supplementation are variable however, with some studies demonstrating positive effects of vitamin D supplementation on umbilical venous cord blood Ca^{2+} and others a null effect (329-331, 334, 345, 347). Recent findings from the Southampton Women's Survey and others suggest that there is a variable response to supplementation according to adiposity, age , baseline 25(OH)D status and even genotypes of enzymes involved in

vitamin D metabolism (348, 349). It is therefore possible that women with certain characteristics might require higher supplement doses to achieve vitamin D replete status.

1.7.9 Maternal vitamin D and the programming of bone development

1.7.9.1 Observational studies in neonates and infants

Evidence is accruing that vitamin D may have a role, not just in the prevention of neonatal hypocalcaemia, but also in the programming of bone development. Case reports have shown that the clinical signs of rickets and other skeletal abnormalities can be present from birth in the children of mothers with VDD, and that these babies had low serum 25(OH)D (350).

From these findings, it would therefore be reasonable to suggest that subclinical maternal vitamin D insufficiency in pregnancy might also influence offspring bone mineral accrual. Early studies used season as a proxy marker of vitamin D status. Namgung et al. in 1998 demonstrated that in 71 Korean neonates, those born in summer months (July – December) had 8% higher whole body BMC after adjustment for weight than infants born in winter (January – March), and that whole body BMC was positively correlated with neonatal 25(OH)D measured at delivery (351). However, the same authors found that infants born in the USA in summer had lower whole body BMC than winter-born infants (352), proposing differences in uptake of vitamin D supplementation (higher in the USA than in Korea) as an explanation for this dichotomy.

A Canadian study (Weiler et al.) used measurements of 25(OH)D in venous cord blood and used to divide the infants into two groups using a cut point of 37.5 nmol/l. Surprisingly, the study showed that the infants in the low 25(OH)D group tended to be heavier and longer, though the authors reported that the ethnicity characteristics of the population were not balanced between the two groups and could explain these findings. However, when adjusted for body weight, whole body and femur BMC were significantly lower in the neonates in the low 25(OH)D group (353). Similarly, a small (n = 125) cross sectional Finnish study (Viljakainen et al.) used maternal serum 25(OH)D both in early pregnancy and 2 days postpartum as a marker of vitamin D status, and showed that neonatal tibial BMC and cross-sectional area measured by pQCT were 14% and 16% higher, respectively, in infants born to mothers with 25(OH)D above the median (42.6 nmol/l) for the cohort, and these differences persisted after adjustment for neonatal weight. BMD, however, did not differ between the groups (354). It should be noted that 71% of the women had serum 25(OH)D below 50 nmol/l in the first trimester. A subset of these children (n = 87) were reassessed at 14 months of age, however, in the group with lower maternal vitamin D status BMC gain was greater, catching up with the higher maternal vitamin D group so that only tibial CSA

(not BMC) remained significantly higher in those born to mothers with higher vitamin D status in pregnancy (355).

Most recently, in 2018 Boghossian et al. studied a multi-ethnic cohort of women in Memphis, USA (n = 343), analysing 25(OH)D trajectories in pregnancy and their associations with neonatal anthropometry and body composition (n = 252 with DXA). They showed that deficient maternal 25(OH)D (< 20 ng/ml, or < 50 nmol/l) was associated with lower neonatal bone mineral density (β -0.009 g/cm^2; 95% CI -0.016, -0.002). Further sex-specific differences were observed, as maternal vitamin D deficiency was also associated with lower lean mass and birthweight amongst male infants. In Caucasian women, 25(OH)D concentrations were perpetually higher throughout pregnancy than African – American women, however, the trajectory of total maternal 25(OH)D concentration in pregnancy did not differ between ethnicities (356).

In contrast, in a Gambian mother-offspring study (125 mother–offspring pairs), no significant relationships were observed between maternal 25(OH)D at either 20 or 36 weeks gestation and offspring whole body BMC or bone area at 2, 13 or 52 weeks of age (357). 25(OH)D levels in this Gambian population were much greater than in the aforementioned North American and European studies; none of the mothers in this study had a 25(OH)D below 50 nmol/l, so the lack of association between maternal vitamin D status and offspring bone indices are consistent with the hypothesis that poorer skeletal mineralisation might only occur in the fetuses of mothers with the lowest vitamin D levels.

Gestational ultrasound has also been used to study bone development in the fetus. In a subset of the Southampton Women's Survey, high resolution 3D ultrasound was used to measure fetal femur length and distal metaphyseal cross sectional area, together with the ratio of femoral metaphyseal cross-sectional area to femur length (femoral splaying index) (n = 424). Lower maternal vitamin D concentration at 19 weeks gestation was not associated with femur length, but was associated with greater metaphyseal cross sectional area and higher femoral splaying index at 19 and 34 weeks gestation (358). This observation is similar to metaphyseal splaying observed in children with post-natal rickets. In the same cohort (n = 357), femur volume measurement by 3D ultrasound was performed, and maternal 25(OH)D was significantly correlated with femur volume and proximal metaphyseal diameter. Maternal height and triceps skinfold thickness were independent predictors of femur volume with a negative effect from smoking. Adjustment for these factors attenuated the association of vitamin D with femoral volume, but the association with proximal metaphyseal diameter remained. In this subgroup, no correlation was detected between maternal 25(OH)D and BMC and BMD assessed by DXA (359).

1.7.9.2 Observational studies in older children

There is some evidence to suggest that relationships between maternal gestational 25(OH)D status and neonatal bone health persist into childhood as, in 2006, Javaid et al. published work reporting an association between maternal 25(OH)D status at 34 weeks gestation and offspring whole body and lumbar spine BMC, BMD and bone area at 9 years of age in 198 mother-offspring pairs in the Princess Anne Hospital Study, a mother-offspring cohort study in Southampton, UK (324), as shown in Figure 1.19. Maternal 25(OH)D status was strongly determined by both UVB exposure and vitamin D supplement use. Beneficial effects of vitamin D supplementation were also suggested by this study as children born to women who took vitamin D containing supplements had higher whole body BMC and bone area, but not areal BMD. Although the women who took supplements were self-selected, this finding was not changed by adjustment for socioeconomic status. Moon et al. replicated these findings in the Southampton Women's Survey (SWS) (Figure 1.20), in which 1030 mother-offspring pairs had measurement of 25(OH)D at 34 weeks gestation and whole body less head (WBLH) and LS DXA at 6-7 years. WBLH BMC, bone area, BMD and lumbar spine BMC were all significantly lower in children born to mothers with 25(OH)D < 25 nmol/l in late pregnancy, including after adjustment for maternal age, ethnicity, height, pre-pregnancy BMI, smoking in late pregnancy, social class, maternal educational attainment and duration of breast feeding (360). In terms of the mechanisms behind the associations between maternal 25(OH)D status and offspring bone development, Javaid et al. suggested that the association may be mediated by umbilical cord calcium levels, as demonstrated in Table 1.3.

The Raine Cohort in Western Australia provides one of the longest follow-up periods in mother-offspring studies of vitamin D status and offspring bone phenotype. In this study, a positive relationship was detected between maternal 25(OH)D status at 18 weeks gestation and bone mass at age 20 years, not far from the age at which peak bone mass is reached. Zhu et al. showed that after adjustment for numerous confounders (including sex, age, height, body composition at age 20 years, maternal height and pre-pregnancy weight, maternal age at delivery, parity, education, ethnicity, smoking during pregnancy and season of maternal blood sampling), whole body BMC and aBMD were 2.7% and 1.7% lower at 20 years of age in offspring of mothers with 25(OH)D < 50 nmol/l compared to those above this level (361).

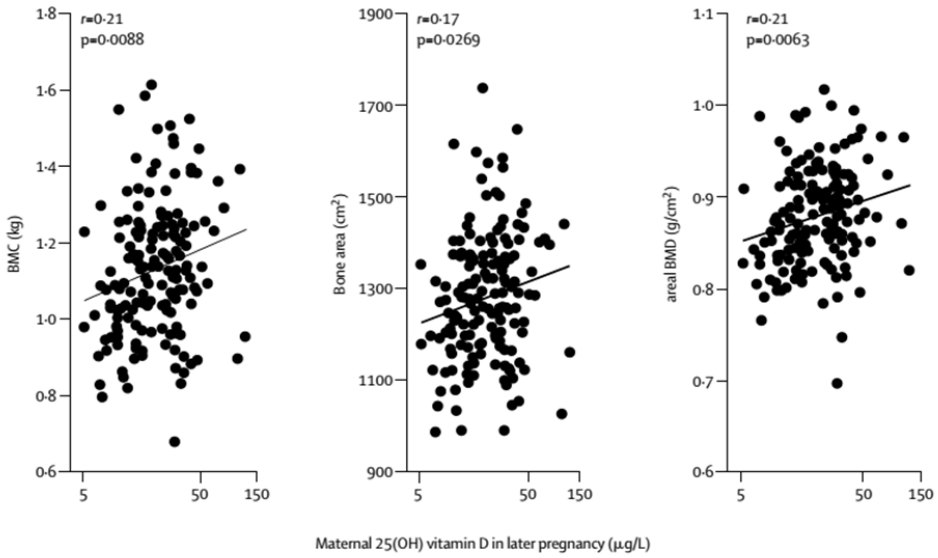


Figure 1.19 Maternal 25(OH)D is positively associated with offspring bone mineral content (BMC), bone area and areal bone mineral density (aBMD) at 9 years of age.

Reproduced with permission from Javaid et al., 2006 (324)

Table 1.3 Umbilical vein blood chemistry and whole body BMC at age 9 years.

Reproduced with permission from Javaid et al., 2006 (324)

	Relation to whole body BMC*			
	Unadjusted		***Adjusted+***	
Umbilical-venous constituent	**R^2**	**p**	**R^2**	**p**
Calcium (mmol/l)	4.7%	**0.0084**	3.4%	**0.025**
Albumin (mmol/l)	2.6%	**0.049**	0.8%	0.27
Phosphate (mmol/l)	0%	0.083	0.1%	0.71
Alkaline phosphatase (IU/l)	0.1%	0.75	0.3%	0.53
Creatinine (mmol/l)	2.0%	0.086	1.8%	0.10

*Adjusted for children's age. +Adjusted for gestational age. R^2 = proportion of variation in whole body BMC which is accounted for by the umbilical-venous measurement derived from a multiple regression model.

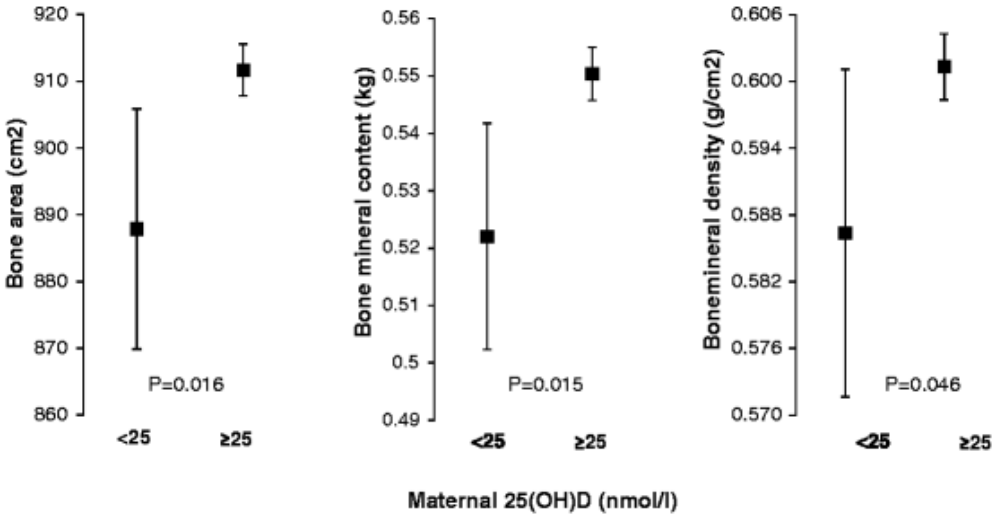


Figure 1.20 Whole body less head bone area, bone mineral content and bone mineral density at 6–7 years of age according to maternal 25(OH)D status in late pregnancy (mean ±95 % confidence interval). *Reproduced with permission from Moon et al. 2016 (360)*

Other observational studies have provided conflicting results. In a large UK based mother-offspring cohort, the Avon Longitudinal Study of Parents and Children (ALSPAC), Sayer et al. reported a positive relationship between estimated maternal UVB exposure in late pregnancy and offspring WBLH BMC, bone area and BMD at 9.9 years of age in 6995 mother-offspring pairs, initially supporting the hypothesis of an association between maternal vitamin D status and offspring bone health (362). They then carried out further analysis in a subset of 3960 children, for whom maternal serum 25(OH)D measurement was available in the first (n = 1035), second (n = 879) or third (n = 2046) trimester. However, this did not reveal any significant associations between maternal 25(OH)D and offspring bone mineralisation (363). Unfortunately, by chance, there was a strong correlation between maternal UVB exposure during pregnancy and the age at which the offspring underwent DXA assessment, which make these conflicting findings difficult to interpret with certainty, as age is a major determinant of childhood bone mass.

Another large observational study, the Generation R Study, based in Rotterdam, measured 25(OH)D concentrations either in mid-pregnancy or at birth and offspring DXA at age 6 years (n = 5294). In contrast to previous studies, inverse relationships between maternal 25(OH)D in mid pregnancy and offspring bone area, BMC and BMD were found (after adjustment for child's sex, ethnicity, age at DXA, height at DXA, sports participation, maternal alcohol consumption and smoking in pregnancy, maternal educational level, household income, maternal BMI in early pregnancy, season of blood sampling and average hours of daylight per month before maternal blood sampling). These relationships did not persist after adjustment for other potential

mediators (the aforementioned factors, plus birthweight, birth length, gestational age at birth, fat plus lean mass at DXA, and vitamin D supplementation at 1 year of age). This population had a high prevalence of maternal 25(OH)D deficiency (< 50 nmol/l) of 23.3%, and the authors acknowledge the difficulty in comparing their results with other populations with a lower prevalence of deficiency. In addition, the adjustment for season of 25(OH)D measurement and average hours of daylight per month, both predictors of 25(OH)D status but not confounders of the association between maternal 25(OH)D and offspring bone health, mean that associations would tend towards the null (364).

1.7.9.3 Maternal vitamin D status and offspring fracture rates in childhood and adulthood

Whether maternal 25(OH)D status in pregnancy bears any influence on childhood fractures rates is unclear; most of the evidence has been generated by Danish cohort studies. In the Danish National Birth Cohort, 25(OH)D concentrations were measured in 1497 pregnant women at 25 weeks gestation and were used to generate a vitamin D prediction score based on smoking, season, dietary and supplementary vitamin D intake, tanning bed use and outdoor physical activity level (n = 36,977). Forearm fracture diagnoses in their offspring were collected from the Danish National Patient register; no association between maternal 25(OH)D status and offspring forearm fractures was detected – in fact there was a trend towards increased fracture risk in the offspring of mothers taking vitamin D supplements in pregnancy (365). In contrast, in the Danish Fetal Origins Cohort, 25(OH)D sampling was undertaken in 965 pregnant women at 30 weeks gestation and related to registry data on fractures in their children up to the age of 18. 294 of their children were registered with at least one fracture diagnosis and a borderline significant inverse association (p = 0.054) between maternal 25(OH)D concentrations and forearm fractures was noted, with an increased hazard ratio for those who had maternal blood drawn Dec/Jan/Feb compared with Jun/Jul/Aug (HR 1.75, 95% CI 1.11, 2.74) (366). In a study using neonatal 25(OH)D status (measured on dried blood spot) as a proxy for intrauterine vitamin D status (again in Denmark) no evidence of an association was found between neonatal 25(OH)D status and fractures between the ages of 6-13 years (n = 1039) (367).

Currently there is only one study suggesting that vitamin D status in pregnancy might alter the risk of fragility fracture in later life. In Denmark, a country with one of the highest levels of hip fracture worldwide, and a strong seasonal variation in 25(OH)D status, Abrahamsen et al. evaluated hip fracture incidence over a 9 year period in an analysis covering 541,109 men and 691,522 women. Contrary to expectations, people born in the first quarter of the year (January to March) had the lowest risk of hip fracture. Fracture rates were approximately 4% higher in

women born in quarter 2 and 3 of the year compared to those born in quarter 1. In men, the highest hip fracture rates were seen in quarter 4 (8% higher than quarter 1), and fractures were also higher in quarter 2 and 3 compared to quarter 1 by approximately 3%, but this did not reach statistical significance (368). Although it remains possible that the effects are due to post-natal rather than in utero influences, these findings reinforce the need to evaluate the role of vitamin D supplementation in pregnancy using intervention studies.

1.7.9.4 Intervention studies of vitamin D supplementation in pregnancy

Several intervention studies of varying size and quality have assessed the effect of vitamin D supplementation in pregnancy and offspring bone mineralisation. In 1983, 64 women of Asian ethnicity living in the UK participated in a non-randomised study; 19 received a daily supplement containing 1000 IU vitamin D and calcium (of unknown strength) during the last trimester, whereas 45 received no supplementation. In this small, non-randomised study there was no significant difference in forearm BMC of the offspring at birth assessed using single photon absorptiometry (369).

Another null finding was reported in a study by Sahoo et al., in which 300 women were randomised to three groups, which received 400 IU/day cholecalciferol daily ("placebo"), 60,000 IU cholecalciferol every 4 weeks or 60,000 IU cholecalciferol every 8 weeks from the second trimester, alongside daily calcium supplementation. There was considerable loss to follow-up, with 160 women followed up until delivery, and 52 children (17% of the original cohort) undergoing DXA at 12-16 months of age. In multivariate regression analysis randomisation group was not a significant predictor of BMC or BMD, though it was noted that the children in the placebo group were significantly older at DXA scan and had higher measurements of whole body BMC and BMD, in addition to the fact that the study may have lacked power due to the small numbers (370).

Another study conducted by Vaziri et al. randomised 153 women to placebo or 2000 IU/day cholecalciferol from 26-28 weeks until delivery, but only 25 infants underwent DXA, representing 16% of the original cohort. No significant difference in whole body BMC, BMD or bone area was found (371), but again, the study was unlikely to possess sufficient power to detect a difference in the outcomes in question.

Finally, Diogenes et al. reported on the effects of calcium (600 mg per day) plus cholecalciferol (a low dose of 200 IU per day) supplementation versus placebo in a small group of adolescent Brazilian mothers (n = 56) with low calcium diets (around 600 mg per day) from 26 weeks

gestation. Unsurprisingly, no significant differences were observed between the groups in terms of infant bone indices assessed by DXA (372).

Other than the MAVIDOS trial, the only intervention study supporting a possibly beneficial effect of gestational vitamin D supplementation on offspring bone health was published recently by an Iranian group (Vafaei et al., 2019), investigating the effects on fetal skeletal development, assessed by 2D ultrasound. 140 women were randomised to 1000 IU per day cholecalciferol versus placebo from 6-8 weeks gestation. Both groups had a high prevalence of vitamin D deficiency or insufficiency (mean early pregnancy 25(OH)D 18.6 nmol/l in both groups). They found that, in the cholecalciferol supplemented group, fetal femur length and humerus length were significantly greater, when assessed in both the second and third trimester. They also demonstrated increased growth of fetal bones in the cholecalciferol supplemented group, as measurements at the femoral and humeral proximal metaphysis, midshaft and distal metaphysis were significantly greater, though exact gestation was not reported and was not corrected for in the analyses (373). A further limitation is that using 2D ultrasound, it is difficult to be certain that the femur is being measured in the correct plane, which is particularly important for diameter measurements. Unfortunately, femoral splaying index (as described by Mahon et al. (358)) could not be calculated in the Vafaei et al. study; and anthropometry or DXA characteristics of the children at birth have not been reported.

Though not describing neonatal or childhood bone or muscle outcomes, it is worth mentioning that the largest double blind RCT of gestational vitamin D supplementation, published in 2018 (Roth et al.) described a population of 1300 women in Bangladesh, randomised to weekly supplements of 4200 IU, 16800 IU, or 28000 IU cholecalciferol, plus a group who continued 28000 IU into the postpartum period. Randomisation occurred later in pregnancy than some other studies, from 17 to 24 weeks gestation, and continued until delivery. 1164 children were followed up at 1 year of age and no differences in mean length for age, weight for age, BMI for age or upper arm circumference for age z scores were observed (374). The fact that vitamin D supplementation was started fairly late in pregnancy, and additionally that this was a population in which vitamin D deficiency was common (baseline mean 25(OH)D ranging from 27.0 nmol/l to 28.7 nmol/l per group meaning it would have taken some time for the supplement to bring the women's 25(OH)D to sufficient levels), could have limited the possibility of effects being observed. These findings were not in agreement with the AVIDD Trial in Bangladesh, which showed that gestational vitamin D supplementation increases infant length– the authors suggested that in utero vitamin D exposure does affect skeletal development, though, at the very least, an increase in the size of the skeletal envelope (375).

1.7.9.5 The MAVIDOS Trial

The lack of conclusive evidence from smaller studies of maternal gestational vitamin D supplementation led to the design of the Maternal Vitamin D Osteoporosis Study (MAVIDOS). The design of this study will be discussed in more detail in the methods section. To summarise, MAVIDOS is a randomised double-blind placebo-controlled trial of antenatal vitamin D supplementation from 14 weeks gestation until delivery conducted over three centres in the UK, Southampton, Oxford and Sheffield, with neonatal bone mass as the primary outcome (376). 1134 women with a baseline 25(OH)D between 25 and 100 nmol/l were randomised to either 1000 IU/day cholecalciferol or placebo. 965 women remained in the study until delivery, and 736 infants had DXA of the whole body and/or LS.

The percentage of participants achieving a 25(OH)D > 50 nmol/l was similar at baseline ($p = 0.11$), but significantly different at 34 weeks gestation (1000 IU/day 83.3%; placebo 36.9%; $p < 0.001$). Furthermore, when the effect of vitamin D supplementation on maternal 25(OH)D status was explored by season of delivery (Figure 1.21), the reduction in 25(OH)D from 14 to 34 weeks gestation observed in placebo group women who delivered in winter and spring was not evident in the women who received the vitamin D supplement.

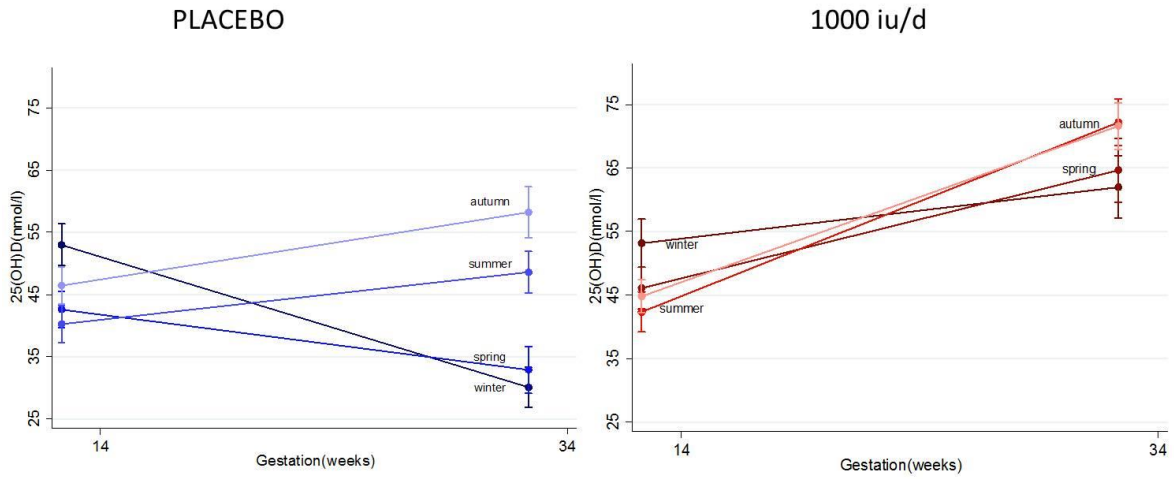


Figure 1.21 The MAVIDOS trial. Maternal 25(OH)D status at baseline (14-17weeks gestation) and 34 weeks gestation in women randomised to placebo or 1000IU/day cholecalciferol, by season of delivery. *Reproduced with permission from Cooper et al., 2016* (377) *via Creative Commons* *https://creativecommons.org/licenses/by/4.0/legalcode*.

There was no significant difference in offspring birth weight, length, head or abdominal circumference between the two treatment groups. Neonatal whole body BMC and BA were greater in infants born to mothers randomised to vitamin D supplementation, although this did

not reach statistical significance. The authors hypothesised, a priori, an interaction between treatment group and offspring season of birth. The formal interaction term between treatment group and season of birth was statistically significant (p = 0.03) and the effect of treatment was of greater magnitude (mean difference 6.3 g, p = 0.003) in winter months (December to February inclusive) than in the remaining seasons, as shown in Figure 1.22. A similar, but less marked, effect was observed for offspring whole body bone area. In a sensitivity analysis, using an alternative seasonal definition with winter as January-March, similar results were observed [BA mean difference in winter = 11.0 (95%CI: -1.2; 23.3), p = 0.08; BMC mean difference in winter = 4.1 (95%CI: 0.1; 8.0), p=0.04], overall interaction between season and treatment, p = 0.27. Furthermore, amongst infants born to mothers who received placebo, statistically significant differences in anthropometric measurements and total fat mass across the birth seasons were identified. In contrast, these differences were not observed in infants of mothers who received vitamin D supplementation.

Whole body BMC and BMD were shown to be approximately 9% and 5% higher, respectively, in the children born in winter to mothers randomised to cholecalciferol compared to those randomised to placebo. To clarify, for winter births, the difference in whole body BMC between treatment and placebo offspring was approximately 0.5 SD, which if maintained into adult life would equate to a 50% difference in fracture risk, an effect size comparable to that associated with many anti-osteoporotic medications. This effect size is substantially larger than those observed between children with and without fractures (378), and, if persisting into later childhood is likely to be clinically relevant. Findings in the pilot phase of the trial suggested that 500 IU cholecalciferol daily would be insufficient to adequately replete the majority of the population and thus the current UK recommendation of 400 IU daily may be the focus of further discussion. The MAVIDOS trial has certain limitations (most importantly that mothers with 25(OH)D concentrations < 25 nmol/l were excluded for ethical reasons). These are discussed in more detail in section 8.4.2.

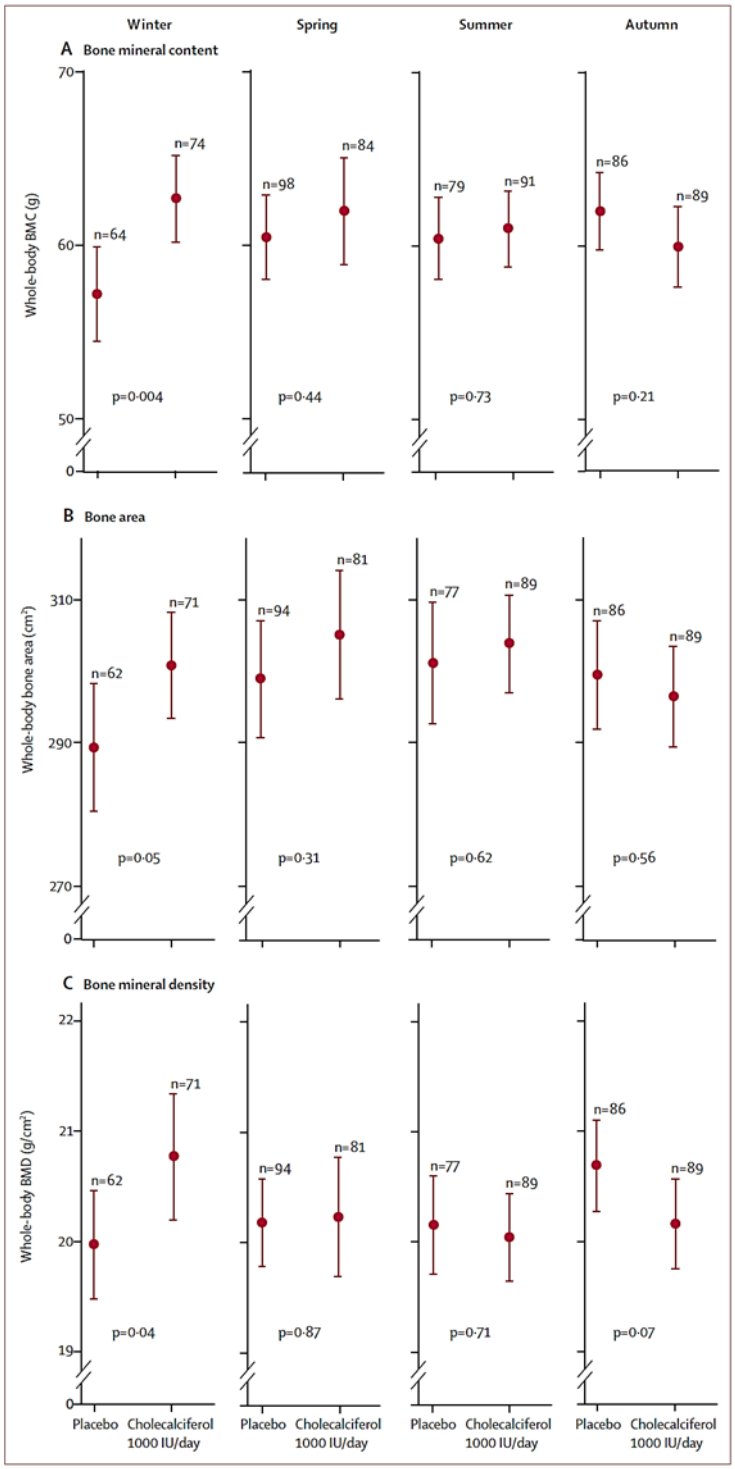


Figure 1.22 Neonatal whole body bone mineral content (BMC), bone area and bone mineral density (BMD) by intervention group and season of birth in the MAVIDOS trial. *Data are shown as mean and 95% confidence interval. Winter is December to February, spring is March to May, summer is June to August and autumn is September to November. Reproduced with permission from Cooper et al., 2016 (377) via Creative Commons https://creativecommons.org/licenses/by/4.0/legalcode*

To conclude, there is now a wealth of observational data relating vitamin D status in pregnancy to fetal growth and offspring bone development. However, the findings of these studies are inconsistent - observational data alone should not be used as a basis for population wide vitamin D supplementation in pregnancy. The variability in findings of both observational and the few intervention studies reflects the wide heterogeneity in the populations studied (including prevalence of VDD, calcium status and ethnic diversity), dose of vitamin D, timing of initiation or assessment of 25(OH)D status and the definitions used for the outcomes considered.

Initial findings from the MAVIDOS trial support a beneficial effect of maternal vitamin D supplementation during pregnancy on offspring bone health, particularly in winter births. However, further work is required to elucidate the mechanisms behind such an associations and whether skeletal effects of vitamin D supplementation in pregnancy can persist throughout childhood and influence peak bone mass obtained. Follow-up studies of the participants included in these trials, as well as the results of ongoing intervention studies, will clarify this key public health issue. Of particular importance is the need to study the influence of cholecalciferol supplementation in early gestation or ideally from the preconception period, particularly as there is evidence to suggest that the placental machinery for vitamin D metabolism and signalling are activated in the first trimester of pregnancy (379), and that environmental effects on the epigenome effects can occur before conception and in early gestation (380).

1.7.10 Maternal vitamin D status and offspring muscle development

1.7.10.1 Observational studies in children

Although a variety of studies have reported associations between vitamin D deficiency and muscle function in adulthood, few studies relate maternal 25(OH)D during pregnancy to offspring muscle development. In the Mysore Parthenon Study, maternal serum 25(OH)D concentrations were measured at 28 – 32 weeks gestation in 568 women. Their children were followed up at 5 and 9.5 years of age with measures of hand grip strength and anthropometric measures of arm muscle area, based on MUAC and triceps skinfold thickness (as described in section 1.3.1). At both 5 and 9.5 years, children of vitamin D deficient mothers (25(OH)D < 50 nmol/l) had smaller arm muscle area in comparison with children born to mothers without deficiency, but no difference in grip strength was observed. Low maternal vitamin D status was also linked to higher percentage body fat and insulin resistance in the children (57).

In the ALSPAC study, as previously described, maternal estimated UVB exposure in the third trimester of pregnancy (n = 6995) was positively related to offspring lean mass assessed by DXA at

9.9 years, [β (95% CI) = 163 g (89, 237) / 1 SD increase in UVB, p = 0.00002] (381). The relationship was reassessed using measured maternal 25(OH)D as the predictor of offspring bone indices, however the relationship with lean mass was not reported (382).

In the SWS, maternal 25(OH)D status was measured at 34 weeks of gestation, and muscle strength (hand grip strength) and offspring lean mass (measured by DXA) were assessed at age 4 years. In an analysis of 678 mother-child pairs, maternal 25(OH)D concentrations were positively associated with offspring height-adjusted hand grip strength, [β = 0.10 SD/SD, p = 0.013], and persisted after adjustment for maternal confounding factors, duration of breastfeeding and child's physical activity at age 4 years (assessed in a subset using 7-day accelerometry). Maternal 25(OH)D was also associated with offspring percent lean mass but not total lean mass – this relationship did not persist after adjustment for confounding factors (383).

It is important to mention that a number of studies have demonstrated negative associations between maternal 25(OH)D status and offspring fat mass, however thorough synthesis of the evidence is beyond the scope of this thesis (239). Because children with greater adiposity tend to have higher absolute lean mass (384), studies reporting lean mass often use a three compartment model (percentage fat mass, lean mass and bone mineral content) to provide a more favourable indication of body composition.

In the Generation R Study, maternal 25(OH)D concentrations were measured in 4903 mothers at a mean age of 20.4 weeks and at birth, and their offspring underwent lean mass assessment by DXA. Compared with children from mothers with "optimal" 25(OH)D concentrations, (≥ 75.0 nmol/l), those of severely deficient vitamin D (< 25 nmol/l) mothers had a 0.13 Standard Deviation Score (SDS) (95% CI -0.22, -0.04) lower lean mass percentage, and a 0.12 SDS (0.03, 0.21) higher fat mass percentage, associations which remained after adjustment for the child's 25(OH)D status (385). Cord blood 25(OH)D concentrations, measured in around 3000 of the offspring, were not associated with lean or fat mass percentage; however the authors suggest that the reduction in sample size may be responsible for this lack of association (385).

1.7.10.2 Animal studies

Animal studies support a role for maternal 25(OH)D to fetal muscle growth and maturation. A study tracing ^{3}H-labelled vitamin D injected into mother rats showed that 25(OH)D was transferred to the fetus and stored predominantly in fetal muscle tissue (386). In cultured chick myoblasts, vitamin D was demonstrated to have stimulatory effects on their growth and differentiation (387) and promotion of myogenesis, as indicated by an increase in specific muscle

differentiation markers and myosin expression (388). In pregnant rats fed a vitamin D deficient diet, the myoblasts of their newborns were smaller than those of vitamin D replete newborns, with evidence of differences in gene expression in the muscle transcriptome (389). Similarly, in pregnant sows fed a 25(OH)D supplemented diet, their fetuses had more muscle fibres and more myoblasts, which showed an extended proliferative phase in culture (390). Another pig study confirmed these findings, demonstrating a larger muscle cross sectional area in the piglets of mothers fed a high vitamin D diet. Reverse transcription PCR revealed that the piglets' muscles had higher levels of transcription of genes related to muscle proliferation (such as *myogenin* and *IGF-2*) than those of piglets born to vitamin D deficient sows (391). In sheep, lower fetal plasma 25(OH)D concentrations were modestly associated with lower fetal skeletal muscle fibre density (392).

1.7.11 Summary

Although low levels of 25(OH)D in pregnancy are common, it is currently not clear as to how (and whether) this affects offspring bone and muscle health. Some studies in humans and animals, observational, interventional and mechanistic, support a role for increased maternal vitamin D in increasing bone size and bone mineral content and muscle size and strength in the offspring. However, there are other studies that do not support these associations, and this inconsistency in the evidence is likely to be reflective of the wide heterogeneity in the populations studied. Variations in the prevalence of VDD, calcium intake, ethnicity, timing of assessment of 25(OH)D status, statistical methods used, factors adjusted for and method of outcome assessment make comparisons between studies difficult. The seasonal variation in vitamin D status, and the potential for confounding and reverse causality when linking a primarily environmentally determined exposure to outcomes such as bone and muscle health need to be acknowledged. In addition, many of the studies are small and thus too underpowered to demonstrate a clinically detectable effect and some intervention studies are open label or do not provide a placebo to the control group, potentially increasing the likelihood of bias.

Few high quality RCTs have been conducted to support the need for vitamin D supplementation in pregnancy (with none covering the prenatal period or early pregnancy), and reviewing this evidence simply highlights this requirement – particularly in populations of different ethnicities, and with different dietary calcium intakes.

Mendelian randomisation studies may also provide evidence of a causal or non-causal effect of 25(OH)D status in pregnancy on offspring musculoskeletal health. Genome wide association

studies have shown robust associations between genetic variants located in the genes that act in the pathway for vitamin D synthesis (*CYP2R1, DHCR7*) and metabolism (*CYP24A1* and *GC*) and 25(OH)D level (393, 394). If the maternal alleles associated with lower 25(OH)D levels in pregnancy were also associated with poorer musculoskeletal health in the offspring (after taking into account offspring genetic variation), this could point towards a direct effect. Though these analyses have not been carried out to date, the development of large scale genetic consortia of mother-offspring pairs will make this possible in the near future (395, 396), discussed in section 8.3.

1.8 Epigenetic determinants of bone health

As described in section 1.4.1, the variance in BMD is only partly explained by genetic factors(176, 178, 180), and there is increasing evidence that some of the residual variance in both BMD and fracture risk might be explained by the influence of the environment on gene expression, both in utero and in early life (183). It is widely recognised that genes effectively provide a library of information that can be read (expressed) differently in different cells and tissues according to function and need. Thus, in a single organism, although the genetic code contained in every somatic cell is the same, the genes expressed will vary widely from organ to organ and even from cell to cell, often in response to environmental cues (397).

1.8.1 Introduction to epigenetic mechanisms

As mentioned in section 1.1.1, the concept of developmental plasticity, that a single genotype may give rise to several different phenotypes in response to the prevailing environmental milieu is ubiquitous in the natural world. This process allows the next generation to be born appropriately adapted to the expected external environment, when prevailing environmental conditions act during critical period of development (397). A widely reported example of this is of the meadow vole *(microtus pennsylvanicus)*, in which the thickness of the coat in the offspring is determined by the photoperiod (number of hours of light and dark) experienced by the mother during gestation. Pups born in autumn have a thicker coat than those born in spring (398). In the meadow vole, experiments published in 1988 suggested that maternal melatonin levels during pregnancy were the most likely signal to the pup of the prevailing environmental conditions (399), allowing the pup to adopt a developmental trajectory which is appropriate to the postnatal environment to which it is likely to be exposed after birth. However, a mismatch between the expected postnatal environment and that to which the pup has been developmentally

programmed, for example due to a change in the postnatal environment or inappropriate maternal cues, would lead to a survival disadvantage.

Various experimental studies have clearly demonstrated that alterations to maternal diet during pregnancy may lead to changes in offspring phenotype and gene expression (400, 401). These effects are likely to be underpinned by epigenetic mechanisms, processes by which gene expression is modified but without changes in the DNA code itself. Such epigenetic signals are essential in determining when and where genes are expressed, and may be conserved across two generations; however true inheritance of epigenetic marks beyond the F2 generation (which has been exposed to the original environmental factor as primordial germ cells of the F1 during F0 pregnancy) remains a subject of investigation (402, 403). The epigenome can therefore be regarded as a molecular record of life events, accumulating throughout a lifetime. For example, monozygotic twins have been shown to be epigenetically most similar at birth but their epigenomes diverge with age at a rate that is lessened if the twins share a common environment (404). An understanding of these epigenetic processes has the potential to enable early intervention strategies to improve early development and later health; consequently and the study of epigenetic biomarkers is a rapidly advancing field (405).

Epigenetic mechanisms include DNA methylation, histone modification and non-coding RNA, described in Figure 1.23 (397, 406, 407). A full description of each of these processes is beyond the scope of this report, which will focus on DNA methylation which is the most stable and widely studied of these mechanisms.

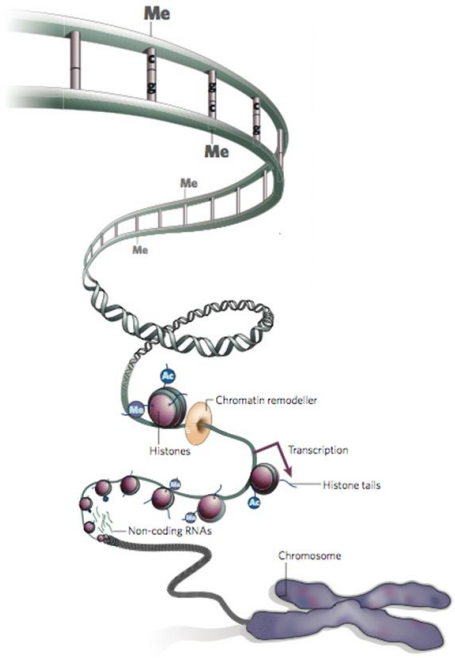


Figure 1.23 The coding and structural information superimposed the base sequence of DNA is organised in multiple epigenomes, which differ according to cell and tissue type. DNA methylation at cytosine adjacent to guanine bases (CpG sites), in addition to the covalent modifications of histone tails and histone variants can contribute information to nucleosomal remodelling machines. (Nucleosomes are a subunit of DNA packaging composed of eight histone protein cores forming a complex around which DNA is wrapped). Through nucleosome remodelling, leading to ravelling and unravelling of DNA, genes and loci encoding non-coding RNAs become susceptible to transcription. Transcription factors (not shown in this diagram) also play a major part in the competence and organisation of the genome. *Figure reproduced with permission from Jones et al., 2008 (408).*

1.8.2 DNA methylation

DNA methylation is a common modification in eukaryotic organisms, and involves the transfer of a methyl group to the 5’ carbon position of cytosine, creating 5-methylcytosine (5-mC) (409). It is a relatively stable epigenetic mark that is transmitted through mitotic DNA replication and cell division (410), though methyl marks can be added and removed throughout the lifecourse. Cytosine methylation mainly occurs within the dinucleotide sequence CpG, where a cytosine is immediately 5’ to a guanine, with a phosphate group between them denoted by “p”, although non-CpG methylation is also prevalent in embryonic stem cells (411). A CpG site can either be methylated or unmethylated in an individual cell, however, across a whole tissue where genes in

cells may be methylated or unmethylated, a range of graded gene expression from 0% to 100% is possible (397).

CpG dinucleotides are not randomly distributed throughout the genome but are clustered at the 5' end of genes in regions known as CpG islands, with hypermethylation of CpG islands generally associated with gene silencing and hypomethylation with activation as demonstrated in Figure 1.24 (412). DNA methylation can act directly to block binding of transcription factors to the DNA or by recruiting a myriad of other repressive factors, such as methyl CpG binding protein 2 (MeCP2), which in turn mediate local chromatin changes to impair transcription factor binding (413).

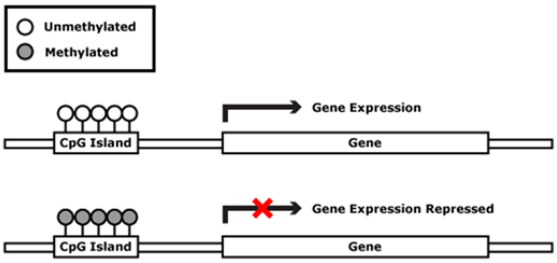


Figure 1.24 The role of CpG methylation in gene silencing and activation. Methylated CpG islands (grey markers) upstream (5') of a gene, in general, repress gene expression. *Figure courtesy of Dr Christopher Bell.*

The pattern of CpG methylation is largely established during embryogenesis and the perinatal period. DNA methylation marks on the maternal and paternal genomes are largely erased on fertilisation (with the exception of the imprinted genes and other specific genomic regions), followed by a wave of *de novo* methylation within the inner cell mass just prior to blastocyst implantation (414, 415). The *de novo* methylation of DNA is catalysed by DNA methyltransferases (DNMT) 3a and 3b (415) and is maintained through mitosis by methylation of hemi-methylated DNA by DNA methyltransferase 1 (DNMT1) (416). This enables lineage specific methylation patterns to be maintained in differentiated tissues. DNA methylation was initially thought to be relatively stable and generally maintained throughout life, but this concept has now been challenged. In 2009 the existence of another epigenetic modification, 5-hydroxymethylcytosine (5hmC), was described as present in high levels in neurons and embryonic stem (ES) cells (417). 5hmC has been shown to arise from the oxidation of 5-mC by the enzymes of the TET (Ten-Eleven-Translocation) family (418) and has been proposed to act as a specific epigenetic mark opposing DNA methylation, as well as a passive intermediate in the demethylation pathway (419, 420). The

high levels found in the brain and neurons indicate a role in the control of neuronal differentiation and neuronal plasticity (421).

1.8.3 Early life nutrition and modifications to the epigenome

Studies of the Dutch Hunger Winter of 1944-45, a famine which occurred following the German occupation of the Netherlands, provide evidence that maternal nutrition influences offspring health in later life and suggest that the timing of the nutritional restriction is important (422). Women exposed to the famine during mid- to late gestation had babies with significantly reduced birth weights. Babies whose mothers were exposed only during early gestation had normal birth weights; however, they grew up to have higher rates of obesity and cardiovascular disease than those born before and after the war and higher rates than those exposed during mid- to late gestation (423, 424). Comparable findings are now well established in a variety of animal models where nutrition can be precisely controlled. Early animal studies focussed on the effects of global maternal undernutrition or an isocaloric low protein diet, or to investigate the effect of energy rich maternal diets on the health of the offspring – particularly relevant due to the growing epidemic of maternal obesity in both industrialised and developing countries (425-429). Interestingly, offspring born to mothers fed these different diets exhibit features similar to human cardio-metabolic diseases including hypertension, dyslipidaemia, obesity and insulin resistance in later life. These animal experiments implicated altered epigenetic regulation of genes as a major mechanism through which the developmental environment induces altered phenotypes.

In humans, alterations have been reported in the methylation of a number of genes in DNA isolated from whole blood from individuals whose mothers were exposed to famine during the Dutch Hunger Winter. The timing of the nutritional constraint appeared to be important, as exposure to famine around the time of conception was associated with a small decrease in CpG methylation of the imprinted *IGF2 g*ene and an increase in methylation of leptin, *IL-10, MEG3* and *ABCA4* (430), while late gestation famine exposure had no effect on methylation. This study also provided evidence that maternal nutritional constraint induces long-term epigenetic changes in key metabolic regulatory genes, as these measurements were made 60 years after the famine exposure. Studies of folic acid supplementation by Steegers-Theunissen *et al.* have also shown altered methylation of specific CpG sites in the *IGF2* gene in the peripheral blood cells of children, dependent on whether their mothers took 400 μg of folic acid per day in the periconceptional period (431). There is also some evidence that plasticity in the human epigenome may persist into adulthood, as, for example, short-term high fat overfeeding in healthy young men was shown to

induce methylation changes in over 6,000 skeletal muscle genes, with only partial reversal after 6-8 weeks of a normo-caloric diet (432).

1.8.4 Effects of environmental insults and ageing on DNA methylation

Smoking is another well recognised exposure which is associated with methylation modifications in DNA, demonstrated in many epigenome wide association studies (EWAS) and a subsequent meta-analysis (433). Indeed DNA methylation patterns can be used as biomarkers of smoking exposure for research and clinical practice.

Age related changes in DNA methylation are well documented. Various studies have worked towards and understanding of epigenetic predictors of ageing and mortality, using methylation measures from multiple CpG sites across the genome to predict chronological age in humans (434, 435). Hannum *et al.* created an age predictor based on a single cohort in which DNA methylation was measured in whole blood (434), whilst Horvath developed an age predictor using DNA methylation data from multiple studies (including the Hannum dataset) and multiple tissues (435). In both studies, the difference between methylation-predicted age and chronological age (that is, Δ_{age}) was put forth as an index of disproportionate 'biological' ageing and was hypothesised to be associated with risk for age-related diseases and mortality. Other studies have shown that this Δ_{age}, or marker of accelerated ageing, can be used to predict mortality independently of health status, lifestyle factors and known genetic factors (436, 437).

1.8.5 Vitamin D and DNA methylation

In terms of a mechanistic link between maternal vitamin D status and offspring bone mass, data suggest that this may be mediated, at least in part, through placental calcium transport, as shown in table 1.2 (324). In the SWS, mRNA expression of an active ATP-dependent placental calcium transporter, *PMCA3*, in placental tissue, was positively associated with offspring bone area and bone mineral content of the whole body site at birth (79). More recent research, also using placental samples from the SWS, has suggested that placental amino acid transport is partly regulated by maternal vitamin D status and vitamin D binding protein levels, presenting another complementary mechanism for this association; total lean mass and bone mineral density in the offspring at age 4 years were positively associated with the expression of different amino acid transporters (438). The regulation of placental calcium transfer is poorly characterized in humans, and any mechanistic role of vitamin D remains to be elucidated, but members of the *PMCA* family appear to be regulated by 1,25$(OH)_2$-vitamin D in animal studies (82). Further insights into vitamin

D metabolism indicate that the 1α-hydroxylase gene is regulated by 1,25$(OH)_2$D, through VDR mediated transcriptional regulation. Furthermore, the ongoing regulation of vitamin D metabolism may involve methylation of sites in the 1α-hydroxylase promoter region, with the 1,25$(OH)_2$-vitamin D/VDR/RXR complex inducing DNA methylation at the 1α-hydroxylase promoter, whilst PTH signalling leads to demethylation of this region through a different pathway. This suggests a role for epigenetic processes in the vitamin D-parathyroid hormone axis as shown in Figure 1.25 (439, 440).

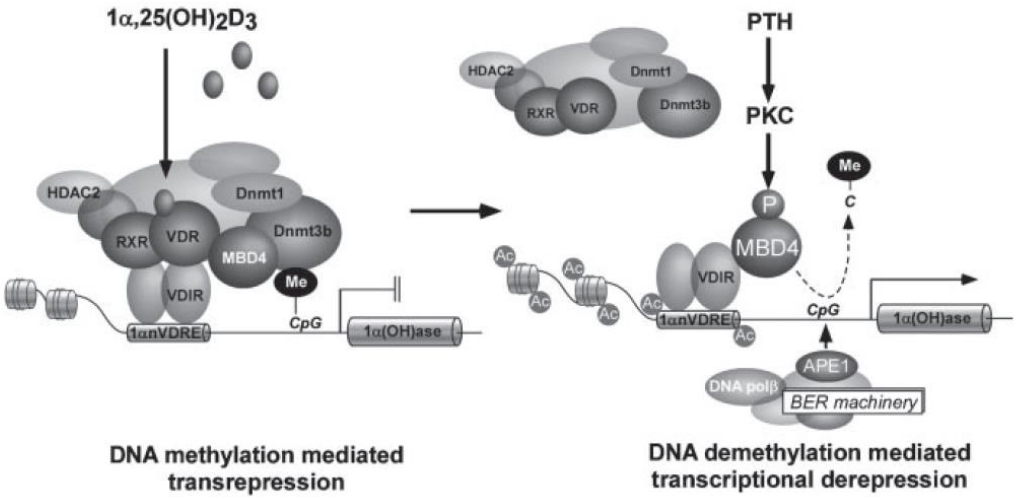


Figure 1.25 Schematic representation of transcriptional switching of the 1α-hydroxylase gene through DNA methylation (switching off) and methylation (switching on) by the actions of vitamin D and PTH. *Reproduced with permission from Takeyama et al.* *https://www.jstage.jst.go.jp/article/bbb/75/2/75_100684/_article* *(439)*

Collection of umbilical cord samples from the Princess Anne Hospital Cohort and the Southampton Women's Survey has allowed the elucidation of relationships between epigenetic marking at candidate sites, identified through array approaches (441) (including the NimbleGen Systems HG17_min_promoter array) and offspring bone size, mineralization, and density. The pipeline for identification of methyl marks from epigenome wide to candidate is summarised in Figure 1.26.

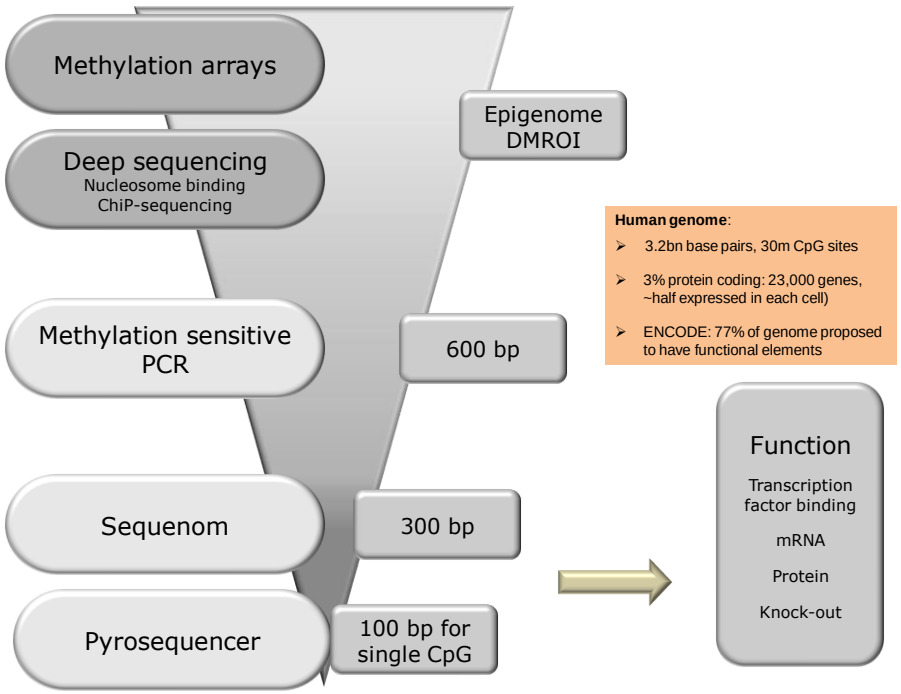


Figure 1.26 Schematic representation of the investigation of methyl marking from epigenome to candidate. *Next-generation methylation sequencing allows identification of individual methyl marks across the entire methylome. Array-based approaches permit identification of differentially methylated regions of interest (DMROI) across a wide genomic area – commonly used "450K" methylation arrays assess methylation at 450,000 CpG sites throughout the genome, but "850K" arrays offering wider coverage are now available. Refinement of candidate selection and investigation of individual CpG methylation may be obtained from techniques such as Sequenom and pyrosequencing, through which methylation can be measured at the individual CpG level. Functional significance may be elucidated from transcription factor binding, mRNA and protein expression, and further validated using knockout models in cell lines and whole animals.*

In 66 mother-offspring pairs from the Princess Anne Hospital Cohort study, percentage methylation at 2 CpG sites in the promoter region of endothelial nitric oxide synthase (eNOS) in umbilical cord was positively related to the child's whole body bone area, bone mineral content and areal bone mineral density at age nine years ($r = 0.28$ to 0.34, $p = 0.005$ to 0.02) (442).

In the Southampton Women's Survey, higher methylation at 4 out of 6 CpG sites in the promoter region of retinoid X receptor-alpha (RXRA) in umbilical cord was inversely correlated with offspring BMC corrected for body size at four years old ($\beta = -2.1$ to -3.4 g/SD, $p = 0.002$ to 0.047), with the results supported by findings from a second independent cohort, the Princess Anne Hospital Study (443). In this study, an index of maternal free vitamin D was inversely related to RXRA methylation at CpG 4/5 (chromosome 9, 136355593, 600+), as shown in Figure 1.27. As

previously stated, RXRA forms a heterodimer with several nuclear hormones known to influence bone metabolism, including 1,25(OH)$_2$-vitamin D, perhaps implying that maternal 25(OH)D status plays a permissive role in the transcriptional regulation of the RXRA gene. Evidence of functional significance was obtained through altered response to transcription factor binding and further characterization of these processes is ongoing, but clearly replication in independent cohorts will be required to validate such findings, and will form a section of this body of research.

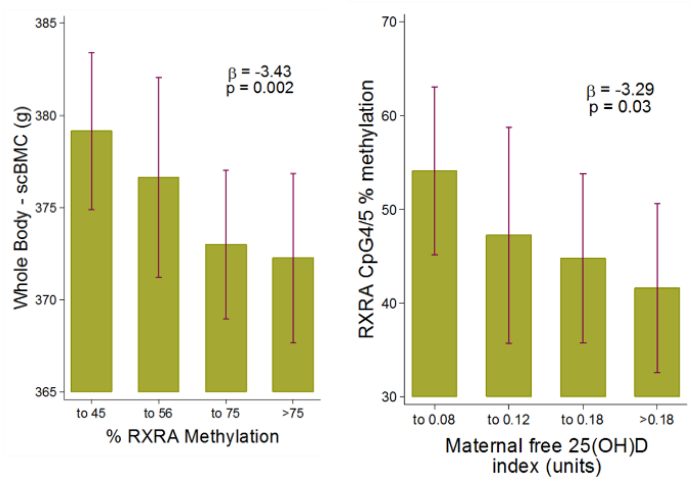


Figure 1.27 Relationships between % methylation at the RXRA promoter and BMC corrected for whole body size (left), and the association between maternal free 25(OH)D index and RXRA methylation at CpG 4/5 (right), n = 230. *Reproduced with permission from Harvey et al. 2014 (443).*

Associations between maternal 25(OH)D status and RXRA methylation could be mediated by a variety of mechanisms. Studies have shown that vitamin D may interact with the epigenome on multiple levels. The critical genes in the vitamin D signalling system, including those coding for vitamin D receptor (VDR) and the enzymes 25-hydroxylase (CYP2R1), 1α-hydroxylase (CYP27B1), and 24-hydroxylase (CYP24A1) have large CpG islands in their promoter regions and therefore can be silenced by DNA methylation. Second, the VDR/RXR heterodimer has been shown to physically interact with proteins that are able to remodel the chromatin environment through coactivator and corepressor proteins, which in turn are in contact with histone modifiers, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and with chromatin remodelers. Thirdly, a number of genes encoding for chromatin modifiers and remodelers are primary targets of VDR and its ligands, and finally, there is evidence that certain VDR ligands have DNA demethylating effects (444).

It is possible that a greater understanding of the actions of vitamin D on DNA methylation may come from EWAS studies. An EWAS study of DNA methylation in severely vitamin D deficient African Adolescents demonstrated associations between methylation in several genes, including

genes involved in vitamin D metabolism such as the 24 and 25-hydroxylase genes (445). Other studies have assessed the DNA methylation in CYP enzymes which are part of the vitamin D metabolism pathway, and found a relationship between methylation of the genes *CYP2R1* (25-hydroxylase) and *CYP24A1* (24-hydroxylase) and variations in circulating 25(OH)D levels (440). Another study, using the ALSPAC cohort and the Norwegian Mother and Child Cohort (MoBa) demonstrated no convincing associations between maternal 25(OH)D status and DNA methylation in the umbilical cord blood of 1416 newborn babies using 450k array analysis, thereby covering 473,731CpG DNA methylation sites (446). The authors suggested that to further identify associations, larger consortium studies, expanded genomic coverage, and investigation of alternative cell types or 25(OH)D status at different gestational time points was needed, which will be the focus of our planned 850k array analysis in the MAVIDOS study.

1.8.6 DNA methylation and skeletal development and homeostasis

Previous array analysis of umbilical cord samples from the Princess Anne Hospital Cohort and the Southampton Women's Survey (441) identified an association between offspring fat mass and methylation at another locus, *CDKN2A* (447-449). The CDKN2A locus encodes two cell cycle inhibitors: $p14^{ARF}$ and $P16^{INK4a}$, which play roles in cellular senescence and ageing. The CDKN2A locus also encodes the long non-coding RNA ANRIL (antisense non-coding RNA in the INK4 locus), a 3,834bp transcript which can negatively regulate p16INK4a. SNPs within the CDKN2A locus, particularly those located within ANRIL have been associated with cardiovascular disease, diabetes and frailty (450) , and DNA methylation at this locus has recently been demonstrated to vary with age (451).

Studies have demonstrated links between perinatal CDKN2A methylation and offspring fat mass, demonstrating it is a marker for later adiposity (452). The functional relationships between fat and bone are well characterised, and mediated via both mechanical and endocrine pathways (453). A section of this work will be described in chapter 4, in which we carried out a targeted approach to examine DNA methylation at CpG sites within the *CDKN2A* gene and its association with offspring bone mass at age 4 and 6 (454).

Such work emphasises the importance of DNA methylation in epigenetic processes in bone metabolism, particularly with regard to loci implicated in cellular differentiation, cell cycle regulation and bone cell function, from early in development to older age.

In bone development, DNA methylation has been shown to play an important role in osteoblast differentiation; one study demonstrated transitional hypomethylation of several genes, including

RUNX2, *osteocalcin* and *CDKN2A* in bone marrow stromal cells in their differentiation towards an osteoblastic lineage (455). Another study identified the importance of cyclin dependent kinases and their inhibitors in this process- in the osteogenic differentiation of adipose-derived mesenchymal stem cells, the promoters of *RUNX2, osteocalcin* and *osterix* genes are actively demethylated in a process dependent upon growth arrest and DNA-damage-inducible protein, GADD45, which is known to interact with both *CDK1* and *CDKN1A* (456, 457). Wnt 3a has also been shown to play a role in osteoblast differentiation, through stimulation of bone morphogenetic protein 2 (*BMP2*) and alkaline phosphatase (*ALP*) expression, in a process which appears to be regulated by *BMP2* and *ALP* promoter methylation (458).

In differentiated bone tissue of various cell types, the importance of DNA methylation marks has been demonstrated in bone remodelling and osteoclastogenesis, through regulation of the *receptor activator of nuclear factor NFκB ligand* (*RANKL*) gene and its soluble decoy receptor *osteoprotegerin* (*OPG*) (459). Finally, DNA methylation has been shown play a role in the ultimate state of osteoblast differentiation to osteocytes, embedded in mineralised bone, through the regulation of various genes including *ALP* and *sclerostin (SOST)* (23-25), and through the transduction of mechanical stimuli (460-463).

At the other end of the lifecourse, the relevance of DNA methylation has been shown in the pathogenesis of osteoporosis; it has been demonstrated that hypomethylation of *Alu* elements, (interspersed repetitive DNA elements) are associated with lower bone mineral density in postmenopausal women (464). In another study, methylation of *SOST* in blood samples was increased in osteoporotic patients, and SOST mRNA in bone cells decreased, in a suggested compensatory mechanism in osteoporosis in order to promote bone formation (465).

Genome-wide methylation profiling studies in older patients comparing individuals with low versus normal BMD have also suggested early life influences on bone quality in older age. One study, comparing bone biopsies of older people with hip fractures to osteoarthritis patients, identified differentially methylated regions (DMRs) enriched in genes associated with cell differentiation and skeletal embryogenesis, including those in the *homeobox* superfamily, which suggests the existence of a developmental component in the predisposition to osteoporosis. Genes encoding the cyclin dependent kinase inhibitor *CKDN1C* (known to be regulated by the vitamin D receptor) and cyclin dependent kinase *CDK20* were both differentially methylated in osteoporosis versus osteoarthritis samples (466). A multi-omics analysis incorporating gene expression, DNA methylation and miRNA data in high BMD versus low BMD women identified four potential regulatory patterns of gene expression to influence BMD status, two of which, the

mTOR and insulin signalling pathway, have been linked to bone cell differentiation and postnatal bone growth (467). Therefore there is evidence that DNA methylation at loci important for cell cycle regulation, differentiation and function of bone cells can have an impact on bone development and bone health throughout life.

1.8.7 Validation and relevance of epigenetic signals

From the studies described, it is apparent that epigenetic marking in early life is associated with later phenotypic variation. However, given the potential tissue specificity of epigenetic signals, the variation of such marks over time, and the difficulty in differentiating cause from effect, the exact characterization of epigenetic mechanisms in disease aetiology and pathology is a very complex process (468, 469). For this reason, epigenetic marks identified in human cohorts through array and candidate investigation must be replicated in separate independent cohorts to robustly establish associations with later disease. Experimental work using cell culture and animal models is also required to document the detailed molecular processes, regulation and functional consequences. A combination of such fundamental investigation and linkage to disease development will be essential to fully understand the role of epigenetic mechanisms in the causation of human pathology. In the meantime, whether the observed epigenetic marks are cause or consequence, if replicated, such signals may well present useful novel biomarkers for later adverse bone development.

Chapter 2: Aims and objectives

2.1 Summary of aims

The overarching aim of this work is to contribute to our understanding of the mechanisms early in life which contribute to offspring musculoskeletal development. I aim to do this through studying epigenetic change (DNA methylation) at two candidate loci of interest, studying alterations in maternal bone turnover in pregnancy, and studying the impact of gestational vitamin D supplementation on these factors. I intend to assess whether maternal gestational vitamin D supplementation is associated with musculoskeletal health in their 4 year old offspring. This will be investigated through a large prospective mother-offspring cohort, the Southampton Women's Survey (SWS) and a unique randomised, double-blind, placebo-controlled trial of vitamin D supplementation in pregnancy (MAVIDOS).

2.2 Objectives

This work will address a number of questions, as follows:

1. In a mother-offspring cohort, the SWS, what are the associations between *CDKN2A* methylation at birth (cord tissue) and bone indices (DXA) at 4 and 6 years?

2. In a randomised controlled trial of vitamin D supplementation in pregnancy, MAVIDOS, is *RXRA* methylation at birth (in umbilical cord tissue) influenced by vitamin D supplementation, and what are the associations between *RXRA* methylation and bone indices (DXA) at birth?

3. In MAVIDOS, what are the characteristics of a bone resorption marker measured in maternal urine (CTX) in early and late pregnancy, and is it associated with maternal gestational vitamin D supplementation? Are there any associations between markers of bone resorption and maternal bone outcomes, measured by DXA?

4. Does maternal gestational vitamin D supplementation influence offspring bone indices, muscle mass or strength in early childhood (in MAVIDOS, aged 4 years)?

Chapter 3: Methods

Two mother-offspring studies have been used to address the objectives of this research project; the Southampton Women's Survey (SWS) (470), an observational birth cohort study, and the Maternal Vitamin D Osteoporosis Study (MAVIDOS) (471), a randomised placebo-controlled trial of cholecalciferol supplementation in pregnancy.

3.1 The Southampton Women's Survey

The SWS is one of the few pre-conception, prospective, mother-offspring birth cohorts. It is based in Southampton, UK (Latitude 50.9^{o}N). The SWS aims primarily to examine the influence of maternal factors before and during pregnancy on offspring growth and development – an overview is shown in Figure 3.1.

3.1.1 Participant identification and data collection pre-pregnancy

Women living in Southampton, aged 20-34 years, were recruited into the study during 1998-2002 via their general practitioners (GP). Each GP provided a list of all eligible women, who were subsequently approached through postal invitation, telephone calls and home visits. Other potential participants were identified through publicity in supermarkets and at local events.

75% of those contacted regarding the study agreed to participate (12,583 women), and were interviewed by a trained research nurse between April 1998 and October 2002. Questionnaires were performed regarding their demographics (ethnicity, education, marital status, employment, housing arrangements, income and benefits), general health, medications, lifestyle (physical activity, smoking and alcohol consumption), and previous pregnancies. Dietary intake was assessed through a 100 item questionnaire (470, 472).

3.1.2 Anthropometry pre-pregnancy

Women were characterised pre-pregnancy with detailed anthropometry. Height, weight, waist, hip and mid upper-arm circumference (MUAC), plus four-site skinfold thickness (SFT) were measured by a trained research nurse.

Height measurement (without shoes) was undertaken using a portable stadiometer (Harpenden, CMS Weighing Equipment, London, UK), to the nearest 0.1 cm. Head position was standardised, through placement in the Frankfurt plane, such that an imaginary line joining the upper margin of the external auditory meatus and the lower border of the orbit of the eye was horizontal.

Calibrated electronic scales were used (Seca Ltd, Birmingham, UK), to measure weight to the nearest 0.1 kg (without shoes, heavy jewellery or heavy clothing). BMI was calculated from height and weight measurements.

MUAC was measured with cloth tape on the non-dominant side, to the nearest 0.1 cm. The tip of the acromion was palpated and marked with the participant standing with her back to the measurer and her arms by her side. The olecranon was then palpated and marked with the participant's arm flexed at the elbow to 90^{o}, and the midpoint between the acromion and olecranon was marked. The MUAC was measured at this midpoint with the participant's arm relaxed and hanging by her side.

SFT was measured using a Harpenden skinfold calliper, to the nearest 0.1mm, on the non-dominant side of the body. Three measurements were taken at each site, and to ensure independence of each measurement, the skinfold was released between measurements. The biceps and triceps SFT were measured posteriorly and anteriorly on a relaxed arm at the level at which the tape measure was placed for MUAC measurement, with the skinfold picked up vertically. The subscapular skinfold was measured at the lowest point of the scapula with the participant standing in a relaxed position, and was picked up obliquely. The upper suprailiac SFT was measured at the intersection between an imaginary horizontal line along the iliac crest and a vertical line from the mid-axillary point.

3.1.3 Assessments during pregnancy

Women were asked to contact the research centre in the event of a pregnancy. 3219 women informed the research centre of a pregnancy and underwent pregnancy assessment.

The women completed an interviewer-directed questionnaire on health, lifestyle, physical activity and diet in both early pregnancy (approximately 11 weeks of gestation) and late pregnancy (approximately 34 weeks of gestation). The same anthropometric measurements were obtained as in pre-pregnancy, and pregnancy weight gain was calculated as the difference between the weight measurements in early and late pregnancy. Details of pregnancy complications, such as gestational diabetes and pregnancy-induced hypertension, were recorded.

Blood samples were also taken at the 11 week, 14 week and 34 week assessments, and serum was stored at -70^{o}C until they were analysed. Measurement of 25(OH)D status was undertaken. Different analytical techniques were used for the early and late pregnancy samples, though they were analysed as a batch. In early pregnancy samples were analysed in 2013 using liquid

chromatography with detection by tandem mass spectrometry (Waters Corporation, Milford, MA, USA). In late pregnancy, 25(OH)D was measured in 2008 with the use of chemiluminescent assay (Diasorin Liaison, Stillwater, MN, USA), which enables the measurement of both 25(OH)D_2 and 25(OH)D_3. Total 25(OH)D was calculated from the sum of 25(OH)D_2 and 25(OH)D_3. The laboratories undertaking both analyses are members of DEQAS (Vitamin D External Quality Assessment Scheme, http://www.deqas.org/), and assays met the requirements of this scheme. The intra-assay, and inter-assay coefficients of variation (CV) were < 10%.

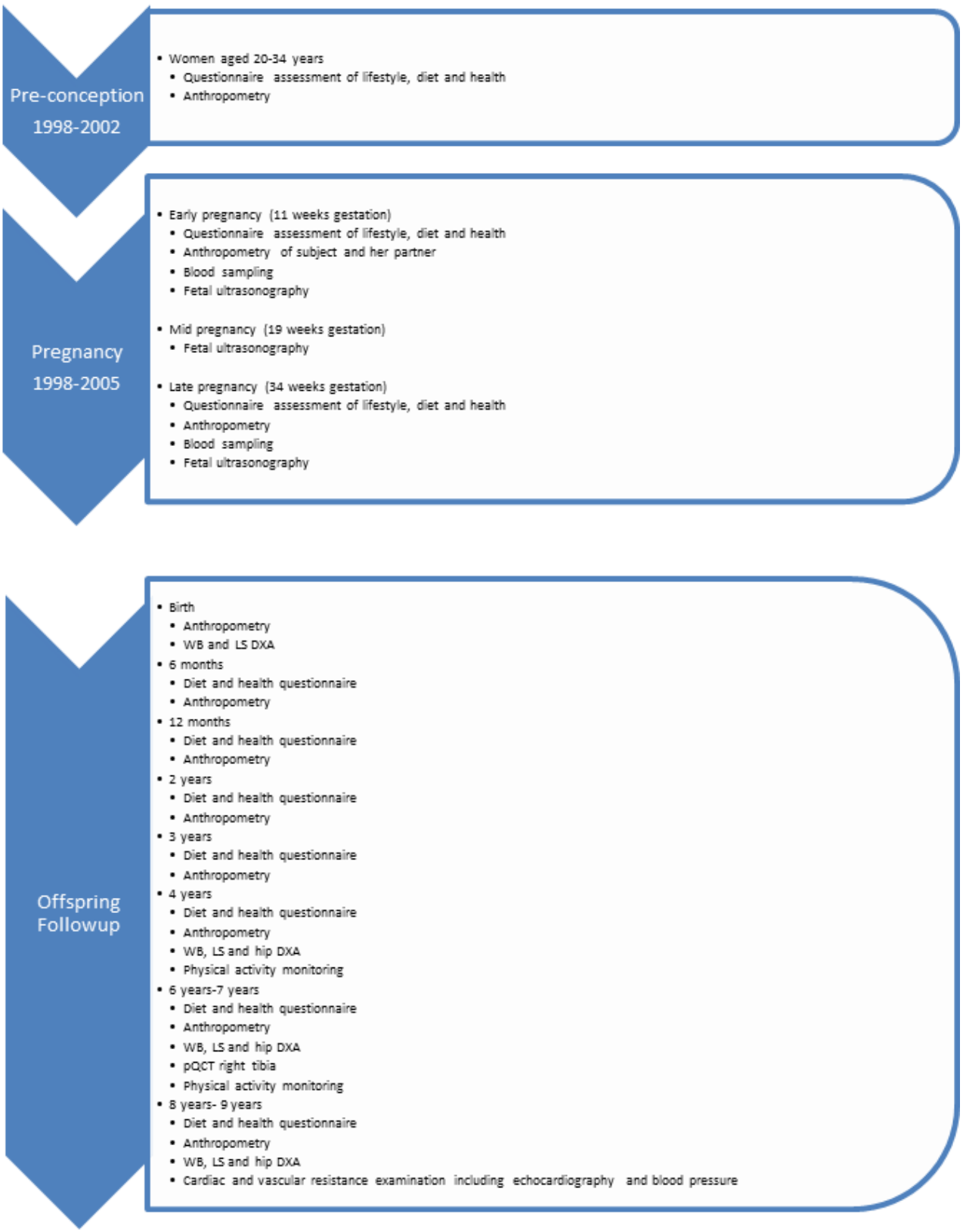


Figure 3.1 The Southampton Women's Survey Cohort Structure

(DXA, dual energy absorptiometry; LS, lumbar spine; WB, whole body)

3.1.4 Offspring assessments

3.1.4.1 At birth, and neonatal assessment

The SWS follow-up comprises 3158 women who delivered a live born singleton infant between December 1998 and December 2007. Where possible, the placenta was collected within 30 minutes of delivery. The placentas were weighed after removing any obvious blood clots, cutting the umbilical cord flush with its insertion into the placenta, trimming away surrounding membranes and removing the amnion from the basal plate. Anthropometric measurements were obtained soon after birth. Weight (naked), was measured using calibrated digital scales to the nearest 0.001 kg (Seca Ltd, Birmingham, UK). Crown-heel length (CHL) was measured using a neonatometer (Harpenden, Wrexham, UK) to the nearest 1 cm. Occipito-frontal circumference (OFC) was measured using a cloth tape positioned at the widest point of the skull anteriorly and posteriorly.

Within two weeks of birth, a subset of babies underwent a whole body and LS DXA scan (Lunar DPX, GE Corporation, Madison, Wisconsin, USA; specific neonatal software). These data have not been used in this thesis, so detailed methods have not been included.

3.1.4.2 Home visits at 6 months, 1, 2 and 3 years

A research nurse visited the participant at home at 6 months, 1, 2 and 3 years of age, at which point a questionnaire detailing the health, diet (including breastfeeding duration) and physical activity of the child was completed, alongside anthropometric measurements. The data from these visits are not used in this thesis.

3.1.4.3 Assessment at 4 years

The children were invited to attend the Osteoporosis Centre at Southampton General Hospital for assessment of bone mass and body composition at 4 years. At this visit, written informed consent for a DXA scan was obtained from the mother or father/guardian. Health, diet and lifestyle information were collected using an interviewer-administered questionnaire.

3.1.4.3.1 Anthropometry

Standing height measurement (without shoes) was undertaken using a portable stadiometer (Leicester height measurer, Seca Ltd, Birmingham, UK), to the nearest 0.1 cm. The children were measured standing with their feet together and heels in contact with the ground, and arms relaxed by their sides, with the head positioned in the Frankfurt plane. The spine was stretched by

gentle upward pressure beneath the mastoid processes. Height (standing and sitting) was measured three times and an average calculated. Weight was measured in light clothing using calibrated electronic scales (Seca Ltd, Birmingham, UK) to the nearest 0.1 kg. Skinfold thickness was measured in the triceps and subscapular regions of the non-dominant side as shown in as shown in Figure 3.2.

Figure 3.2 Measurement of subscapular skinfold thickness and sitting height.

Photographs author's own. Parental permission was granted for publication.

3.1.4.3.2 Dual energy X-ray absorptiometry

A whole body, LS and left hip DXA scan was obtained, using a Hologic Discovery instrument (Hologic Inc., Bedford, MA, USA) in paediatric scan mode. The child was asked to remove any clothing containing metal elements (e.g. zips, buttons) and metal jewellery as these would falsely elevate BMC measurement. The child laid supine on the DXA scanning table and was asked to keep as still as possible to reduce movement artefact, a suitable DVD was played to distract the child and encourage them to stay still, as shown in Figure 3.3. The duration of the scan was approximately 5 minutes for the whole body scan, 30 seconds each for the LS and left hip scans. The total radiation exposure was 18.9 µ Sv (5.2 µ Sv for the whole body scan, 8.8 µ Sv for the LS scan, 4.9 µ Sv for the hip scan).

Figure 3.3 Positioning of child for whole body and lumbar spine DXA.

Photographs author's own. Parental permission was granted for publication.

Daily calibration was carried out using a spine phantom. The manufacturer's coefficient of variation (CV) for the instrument was 0.75 % for whole body bone mineral density, and the experimental CV when a spine phantom was repeatedly scanned in the same position 16 times was 0.68%.

Two researchers reviewed all scan images, and any scans with unacceptable movement artefact (such as duplication or missing parts of limbs) or external artefact (such as a metal object) were excluded. If movement of one leg or arm was present, data from the contralateral region of interest (ROI) was imputed into this area, an example of a 4 year old DXA is shown in Figure 3.4. The ROIs were placed automatically by scanning technology but were reviewed by trained densitometry technicians and adjusted appropriately. The arm ROI was defined by a line passing through the centre of the shoulder, separating the soft tissues of the arm from that of the trunk,

the leg ROI was defined by a diagonal line passing through the femoral neck to below the pubis. When the scans were assessed by researchers, ROIs were reviewed and scan images were assessed to ensure that they contained the correct anatomical regions.

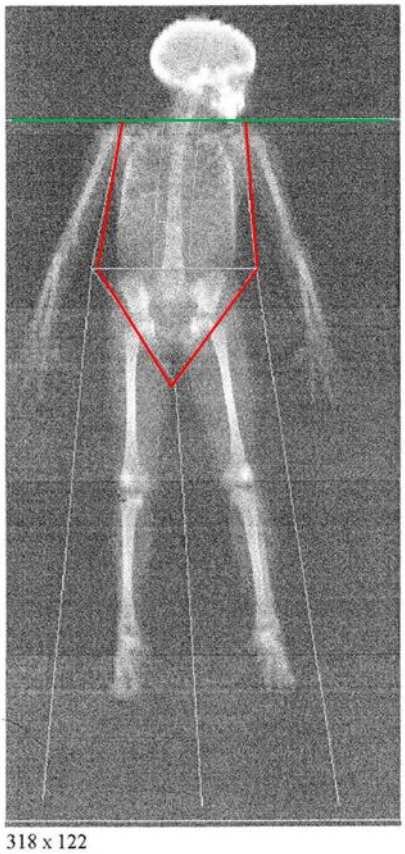


Figure 3.4 An example of a 4 year old's DXA scan, demonstrating the division of the scan into regions of interest.

3.1.4.3.3 Hand grip strength

A standardised approach was used to measure grip strength was measured using a Jamar dynamometer (Promedics, Blackburn, UK). The child was positioned seated in a standard chair with back support and a fixed flat arm. They were asked to rest their forearm on the arm of the chair with their wrist extending just beyond the end of the arm. The dynamometer was adjusted to fit the hand size of the individual, and then the researcher rested the dynamometer on the palm of their hand, to take the weight without restricting the child's movement. The child was encouraged by the researcher to squeeze as tightly as possible, alternating from one hand to the other, with three measurements on each side.

Figure 3.5 Measurement of hand grip strength with a Jamar Hydraulic Hand Dynamometer (top), in the MAVIDOS 4 year followup, and a Jamar Plus + digital hand dynamometer (bottom) in the MAVIDOS 7 year followup. *Photograph author's own. Parental permission was granted for publication.*

3.1.4.3.4 Assessment of physical activity

Habitual physical activity was assessed in a subset of children using the Actiheart monitor (Cambridge Neurotechnology, Cambridge, UK), a combined accelerometer and heart rate monitor. The device was worn continuously (except during bathing and swimming) on the chest, connected to the skin by two ECG electrodes. Data from this device have not been used in analysed in this work.

3.1.4.4 Assessment at 6 years, and 8-9 years

The assessment at age 6 followed a similar format to the 4 year visit, but in addition included peripheral computed tomography (pQCT) of the right tibia (data not used in this research).

The 8-9 year assessment included cardiac and vascular resistance examinations including echocardiography and blood pressure, in addition to the questionnaires, anthropometry and DXA.

3.1.4.5 Assessment at 12-13 years

The assessment at 12-13 years is in progress. This includes the core assessments of the health, diet and lifestyle questionnaire, anthropometry and DXA (Hologic Discovery, Hologic Inc., Bedford, MA, USA), plus high resolution peripheral computed tomography (HRpQCT) (Xtreme CT, Scanco Medical Ag, Bruettisellan, CH) for assessment of bone microarchitecture at the tibia. The total radiation dose is 36 μSv, equivalent to 5 days background exposure. At this visit, the children also undergo pubertal staging using direct examination by a trained nurse and/or self-report.

Further assessments are performed, including a buccal DNA swab, cardiorespiratory fitness test, photography of the teeth, a wellbeing assessment and an assessment of thinking skills using elements from the Wechsler Abbreviated Scale of Intelligence (WASI). In addition, having placed local anaesthetic cream on both antecubital fossae at the start of the clinic visit, venous blood sampling is performed after the other assessments are complete.

3.2 The Maternal Vitamin D Osteoporosis Study (MAVIDOS)

The aim of the MAVIDOS trial was to test, in a multi-centre double-blind, randomised, placebo-controlled setting, whether maternal supplementation with 1000 IU cholecalciferol from 14 weeks gestation until delivery of the baby would result in increased offspring bone mineral content (BMC) measured by DXA within two weeks after birth (471).

The study was approved by the Southampton and South West Hampshire Research Ethics Committee, with full approval from the UK Medicines and Healthcare Regulatory Agency (REC reference 07/H0502/113). An overview of the trial is shown in Figure 3.6.

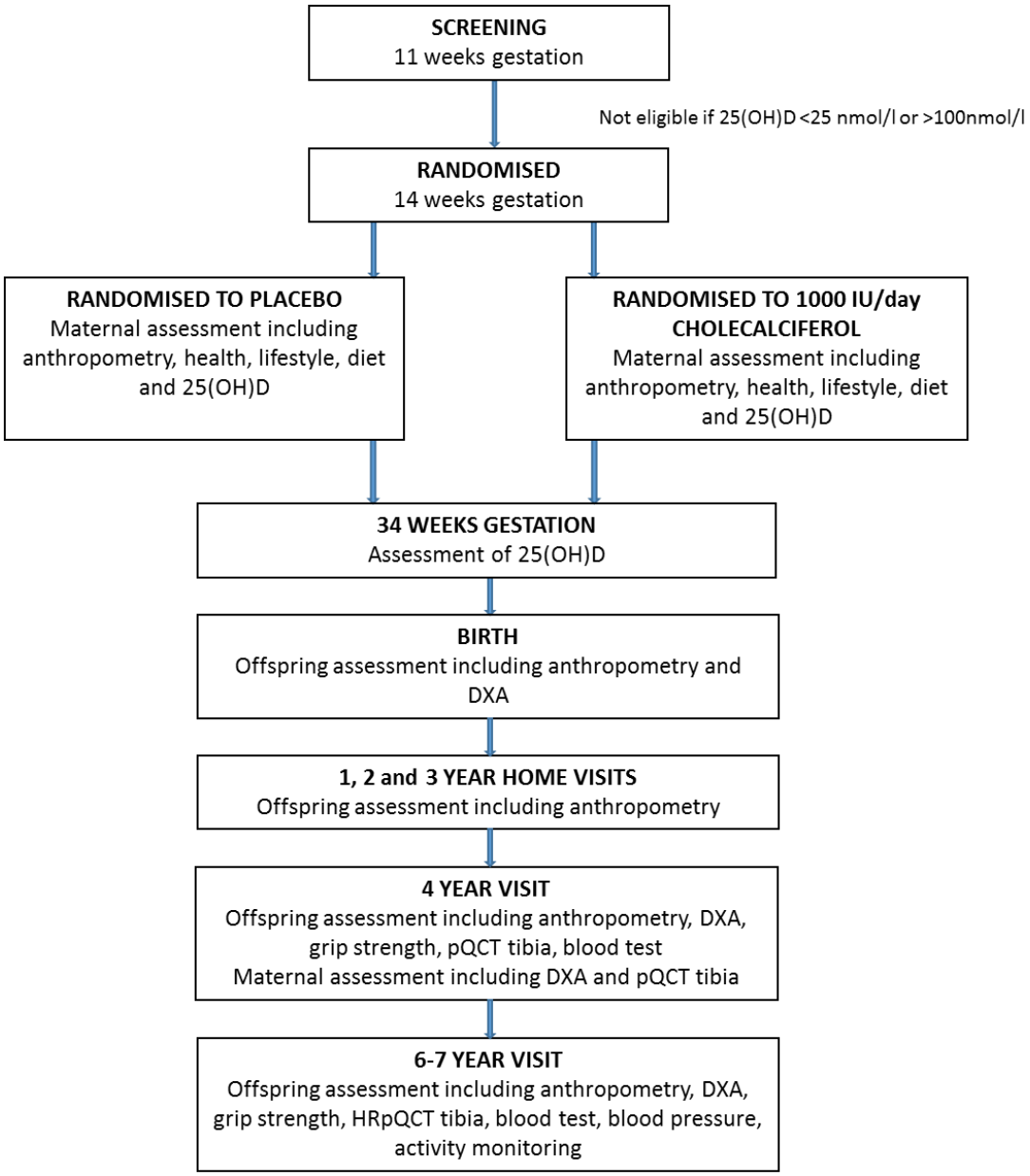


Figure 3.6 MAVIDOS Trial Overview

3.2.1 Identification and recruitment of participants

Women in early pregnancy attending one of three UK hospitals for ultrasonography at 11-12 weeks gestation were invited to participate via an information sheet sent with their ultrasound appointment (participating hospitals: Princess Anne Hospital, University Hospitals Southampton NHS Foundation Trust, Southampton [latitude 50.9°N]; the John Radcliffe Hospital, Oxford [latitude 51.8°N]; Sheffield Hospitals NHS Trust, Sheffield [latitude 53.4°N]). When attending for their scan, the women were subsequently approached by a research nurse. Informed consent was taken, and a blood sample was taken for 25(OH)D and serum calcium measurement at the local hospital laboratory; if 25(OH)D was between 25 nmol/l and 100 nmol/l women were eligible to continue.

Exclusion criteria for MAVIDOS:

- Baseline 25(OH)D less than 25 nmol/l or greater than 100 nmol/l
- Age < 18 years at recruitment
- Twin or other multiple pregnancy
- > 17 weeks gestation at recruitment
- Known metabolic bone disease, previous renal stones, hyperparathyroidism or hypercalciuria
- Medications known to interfere with fetal growth (e.g. corticosteroids, anticonvulsants, bisphosphonates)
- Current daily vitamin D supplementation > 400 IU
- Fetal anomaly on early pregnancy USS or subsequently on mid pregnancy anomaly scan
- Cancer diagnosis within the last 10 years
- Serum corrected calcium > 2.75 mmol/l

3.2.2 Investigational medicinal product and randomisation of participants

Women were randomised to receive either 1000 IU/day cholecalciferol or matched placebo (Merck KGaA, Darmstadt, Germany). Capsules were identical in appearance, and provided in a blister pack in a single box containing all medication for the whole pregnancy. The study medication was pre-randomised by the manufacturer (Sharp Clinical Services, Powys, UK) in a 1:1 ratio and was not stratified by research centre. Packs of medication were sequentially allocated to study participants and commenced at 14 weeks of gestation, or as soon as possible up to a

maximum of 17 weeks of gestation in women enrolled after 14 weeks, and continued until delivery.

3.2.3 Maternal assessments during pregnancy

3.2.3.1 Anthropometry

Immediately prior to starting the study medication (at approximately 14 weeks of gestation), and again at 34 weeks, an interviewer administered health, diet and lifestyle questionnaire was completed. Weight, height and four site SFT measurements were obtained by a trained research nurse following standard protocols identical to those used in the SWS and described in Section 3.1.2. Women attended their routine fetal anomaly ultrasound scan at 18-21 weeks gestation, in the event of significant fetal anomalies, the participant was withdrawn from further participation in the study and referred for further review in the fetal medicine department of their hospital. A measurement of the height of the baby's father was obtained at one of the pregnancy visits, where possible. If the father did not attend either visit, their reported height was documented.

3.2.3.2 Assessment of 25(OH)D in pregnancy

On the day that the study medication was collected, non-fasted venous blood samples were drawn, and again at 34 weeks of gestation. Serum was frozen at -70°C until analysis. Chemiluminescence immunoassay (Liaison automated platform, Diasorin, Minnesota, USA) was used to measure 25(OH)D concentration. All samples were analysed in a single batch at the end of the study at MRC Human Nutrition Research, Cambridge, UK. The laboratory undergoes quality control through participation in the Vitamin D External Quality Assessment Scheme (DEQAS) (224, 473).

3.2.3.3 Assessment of CTX in pregnancy

Participants were asked to provide a second-void early morning urine sample for measurement of urine β-C-terminal telopeptide of type I collagen (CTX) on the day that the study medication was started (around 14 weeks), and again at 34 weeks. All samples were stored at -70^{o}C and sent in a batch for measurement by ELISA (Ortho Clinical Diagnostics, Marlow, UK) at the Academic Unit of Bone Metabolism, University of Sheffield, Sheffield, UK.

3.2.3.4 Assessment of compliance with study medication

In order to assess compliance, participants were asked to bring any remaining study medication to each assessment. A pill count was performed and compliance calculated as number consumed divided by the expected consumption based on the number of days since the medication was dispensed, and expressed as a percentage. Ideally, compliance was calculated from the visit at 34 weeks of gestation, but the count at 18-21 weeks was used when this was not available.

3.2.3.5 Maternal DXA

Within 14 days of delivery, following informed consent, women underwent DXA assessment at whole body, lumbar spine and both hips (Hologic Discovery, Hologic Inc., Bedford, MA, USA, or GE-Lunar iDXA, GE-Lunar, Madison, WI, USA, with neonatal software, depending on the research centre). Each instrument underwent daily quality control, with cross-calibration between sites. The total radiation dose was estimated to be: whole body 4.2 µSv, lumbar spine 4.4 µSv and each hip 2.4 µSv, which is similar to 2 or 3 days background radiation. The combined time for all scans was around 10 minutes.

3.2.4 Offspring assessments

3.2.4.1 Neonatal anthropometry

Soon after birth, when possible, umbilical cord tissue and placental tissue was collected and stored at -70^{o}C. Further details of the cord tissue collection and storage process are outlined in section 3.3.1. Birth weight was extracted from hospital records and was measured with electronic scales to the nearest 0.001 kg on the day of birth. All other measurements were taken by a trained research nurse within 14 days of birth. CHL were measured using a neonatometer to the nearest 0.1 cm and following the same protocol as used in the SWS (Section 3.1.4). Crown-rump length (CRL) was also measured with the infant lying naked on the neonatometer and the legs flexed to 90° at the hips. OFC was measured using a cloth tape positioned at the widest point of the skull anteriorly and posteriorly. MUAC was measured at the point judged by eye to be the mid-point of the upper arm with the infant's arm as relaxed as possible. The unmarked tapes were marked and measured against a fixed rule. Triceps SFT was measured at the same level on the posterior aspect of the arm, subscapular SFT was measured at the angle of the scapula, as in the maternal assessment. All measurements were taken in triplicate and the average calculated.

3.2.4.2 Neonatal DXA

All neonates underwent DXA assessment at whole body and lumbar spine sites (Hologic Discovery, Hologic Inc., Bedford, MA, USA, or GE-Lunar iDXA, GE-Lunar, Madison, WI, USA, with neonatal software, depending on the research centre) within 2 weeks after birth. The infant was undressed, clothed in a standard towel, fed and pacified before the assessment. Each instrument underwent daily quality control, with cross-calibration between sites. The total radiation dose was estimated to be 0·04 mSv, equivalent to about 7 days' exposure to background radiation in the UK. All DXA images were reviewed for movement artefacts and quality by two operators who were blinded to treatment allocation.

3.2.4.3 Offspring assessments at 1, 2, and 3 years of age

Mothers recruited in the Southampton arm of the study were invited to participate in further follow-up of their children at 1, 2 and 3 years of age. The children were reviewed during a home visit, including an interviewer-led questionnaire assessment of diet, health and lifestyle and anthropometry (CHL at 1 year of age, standing height at age 2 years using the same protocol as previously described in section 3.1.4, MUAC, triceps and subscapular SFT was performed following the same protocol as used in adult study participants (section 3.1.2)).

3.2.4.4 Offspring assessment at 4 years of age

At age 4 years, the children born in Southampton were invited to attend the Osteoporosis Centre at Southampton General Hospital for a detailed assessment. A diet, health and lifestyle questionnaire was completed and anthropometric measurements were obtained including weight, height, OFC, MUAC, triceps and subscapular SFT following the previously described standard protocols. Bone indices and body composition were assessed by DXA of the whole body, lumbar spine and hip using a Hologic Discovery DXA instrument (Hologic Discovery, Hologic Inc., Bedford MA, USA). Hand grip strength was measured using a Jamar hand dynamometer (Promedics, Blackburn, UK), again using the previously described protocols. A pQCT scan of the right tibia was obtained using a Stratec XCT 2000 instrument (Stratec Inc., Pforzhein, Germany), as shown in Figure 3.7. Maternal DXA and pQCT of the tibia were also performed. Where consent was provided, a venous blood sample was collected from the child.

Figure 3.7 pQCT of the right tibia in a 4 year old child in the MAVIDOS study. Photograph author's own. Parental permission was granted for publication.

3.2.4.5 Offspring assessment at age 6-8 years

At age 6-8, the children of the MAVIDOS study have been invited for further assessment at the Osteoporosis Centre at Southampton General Hospital; these visits are ongoing. The diet, health and lifestyle questionnaire and anthropometry are performed as per protocol and the children undergo whole body DXA using Hologic Discovery DXA instrument (Hologic Discovery, Hologic Inc., Bedford MA, USA). Hand grip strength is measured using a Jamar Plus + digital hand dynamometer (Promedics, Blackburn, UK), again using the previously described protocols. HRpQCT measurements are obtained (Xtreme CT, Scanco Medical Ag, Bruettisellan, CH) of bone microarchitecture at the tibia. Blood pressure is measured, and when consent is given a venous blood sample and buccal swab is collected from the child and stored at -70°C. Physical activity is being measured in a subset of children using the GeneActiv tri-axial accelerometer (Activinsights, UK), worn on the non-dominant wrist (posted back in a reply paid envelope). The device has been validated in both paediatric and adult populations (474-478).

Figure 3.8 A 7 year old child from the MAVIDOS study undergoing HRpQCT measurement of bone microarchitecture at the right tibia.

(Xtreme CT, Scanco Medical Ag, Bruettisellan, CH). Photograph author's own. Parental permission was granted for publication.

3.3 Epigenetic analyses

3.3.1 Umbilical cord DNA extraction in the SWS and MAVIDOS studies

Immediately following delivery, a 5-10 cm segment was cut from the mid portion of each umbilical cord, flushed with saline to remove fetal blood, flash-frozen in liquid nitrogen and stored at −80°C until required for DNA isolation. Genomic DNA was isolated from frozen archived umbilical cord tissue by classical proteinase K digestion and phenol:chloroform extraction.

3.3.2 Pyrosequencing

Laboratory analyses were undertaken by Robert Murray, Eloise Cook and Nevena Krstic (Professor Karen Lillycrop's group, Institute of Developmental Sciences, University of Southampton).

Sodium bisulfite targeted pyrosequencing using the PyroMark Gold Q96 Reagent kit (Qiagen, Germany https://www.qiagen.com/fi/resources/technologies/pyrosequencing-resource-center/technology-overview/) (479)) on a PyroMark Q96 MD machine (Biotage, Uppsala, Sweden) was used to carry out in-depth analysis of the methylation status of the regions of interest.

Percentage DNA methylation was calculated using the Pyro Q CpG software (Biotage). Within assay precision is between 0.8 and 1.8 % and detection limits are 2-5 % methylation. Inter- and intra-plate controls were added to each plate as a control for inter- and intra-plate variability, and 0% and 100% methylation controls were run to ensure that the full range of methylation could be detected. To exclude the presence of SNPs at the CpGs of interest, a SNP PCR reaction may be performed. The SNP PCR reaction uses primers, with DNA amplification using HotStar Taq Plus DNA polymerase (Qiagen, Germany 203605). PCR Products were treated as for pyrosequencing. Genotyping analysis was carried out using PyroMark MD 1.0 software (Biotage).

3.4 Statistical Methods

Statistical methods will be discussed chapter by chapter. To summarise the approach, all outcomes were assessed for normality using visual inspection. Comparisons between groups were performed using t-test, Mann-Whitney U test and χ^2 test for normally distributed, non-normally distributed and categorical variables, respectively. Correlations were assessed using Pearson's correlation coefficient and Spearman's rank correlation coefficient for normally distributed and non-normally distributed variables, respectively. All women who remained in the study were included in the analysis in the group to which they were randomised. Participants who reported low compliance with the study medication were not excluded from the analysis.

In general, testing for multiple comparisons was felt to inappropriate due to substantial collinearity among the outcomes considered (480). In pyrosequencing analysis, strategies to reduce the number of statistical tests were undertaken and are described in the relevant sections. All analyses were performed in Stata v14.2 (Statacorp, College Station, Texas, USA). A p value of < 0.05 was considered statistically significant.

3.5 Author's contribution to the project

The hypotheses, analysis ideas and interpretation included in this thesis are my own, with support and statistical advice from my supervisors and statisticians. Both the SWS and MAVIDOS studies were established before I started my research at the MRC Lifecourse Epidemiology Unit, and the SWS data collection in pregnancy and at 4 years and 6 years of age had been completed. I developed and submitted the ethics application substantial amendment for the 12-13 year SWS visit, and have carried out data collection at this visit on a weekly basis.

As an NIHR academic FY2 doctor in the MRCLEU in 2011, I spent four months assisting with recruitment of women to the MAVIDOS trial. Assessments in pregnancy, at birth and 1 and 2 years were completed by trained research nurses. I developed and submitted the MAVIDOS ethics substantial amendment for the 6-8 year MAVIDOS visit, including HRpQCT and venesection. Rebecca Moon (until summer 2017) and I (to present) oversaw the running of the 4 year and 6-8 year visit, and, alongside the nursing team, assisted with the visits twice weekly. This included obtaining informed consent for participation, questionnaire administration, anthropometry, assessment of grip strength and venesection. The DXA and pQCT scans have been performed by a trained DXA technician. When abnormalities on DXA scans (such as an unexpected low Z-score) or blood tests, I liaised with the participant, GP and paediatric team to ensure participant safety.

Laboratory analyses, pyrosequencing of CDKN2A and RXRA were performed by Robert Murray, Eloise Cook and Nevena Krstic under the supervision of Professor Karen Lillycrop.

Rebecca Moon and I reviewed all DXA scan images for the MAVIDOS assessments at 4 years of age and cleaned the MAVIDOS 4 year data, with statistical guidance from Dr Sarah Crozier, Stefania D'Angelo and Dr Camille Parsons. All statistical analyses were undertaken by me in STATA 14.0 with subsequent review by a statistician from the MRCLEU.

The interpretation of the data is my own work. Two journal papers (listed in Project Outputs) that have been published with results contributing to chapters 4 and 5 were written by me primarily, though laboratory analyses were performed by Robert Murray, Eloise Cook and Nevena Krstic, hence the sharing of first authorship. Subsequent comments on the drafts of the papers were added from my co-authors and supervisors.

Chapter 4: Associations between methylation of *CDKN2A* and offspring bone health at 4 and 6 years: findings from the Southampton Women's Survey

4.1 Background and aims

As previously discussed, although there is evidence of a substantial heritable component to bone mineral density (BMD) (179, 181, 481), there is increasing evidence that interactions between environment and genotype, leading to altered gene expression, may contribute to the overall variance in BMD (28). A variety of population studies have shown that poor intrauterine and childhood growth are predictors of osteoporosis in later life and of adult hip fracture (193, 194). Maternal factors, such as diet before and during pregnancy, lifestyle (e.g. cigarette smoking and physical activity), body composition, vitamin D status during pregnancy and also paternal factors, such as skeletal size, have all been associated with offspring bone development(199, 200, 324, 377, 482).

There is accumulating evidence from the natural world and from experimental animal studies (e.g. of altered gestational diet in rat models) that associations between environmental factors at critical periods of early development and later health and disease might be mediated by epigenetic mechanisms (28, 397, 426, 483). Previously, a candidate gene approach using samples from the SWS and Princess Anne Cohort demonstrated that methylation at the Retinoid X Receptor–alpha (*RXRA*) promoter in umbilical cord DNA is associated with offspring bone mass in childhood (443). Also in the SWS, associations between offspring fat mass, perinatal *RXRA* methylation, and methylation at a further gene, Cyclin Dependent Kinase Inhibitor- 2A (*CDKN2A),* have been demonstrated (441, 448, 449).

DNA methylation at various genes, including cyclin dependent kinases and their inhibitors, such as *CDKN2A*, has been shown to play a role in skeletal development, homeostasis and bone cell activity. DNA methylation has been implicated in mechanisms of osteoblastic differentiation (455-457) and development of functional osteoclasts (459), together with the transition from osteoblast to osteocyte (460-463).

Genome-wide methylation profiling studies in older patients have complemented these experimental findings, demonstrating that differential methylation at genes such as the cyclin dependent kinase inhibitor *CKDN1C* and cyclin dependent kinase *CDK20* is associated with BMD

(466, 467). These differentially methylated loci are implicated in cellular differentiation, cell cycle regulation and bone cell function.

The *CDKN2A* locus encodes two cell cycle inhibitors: $p14^{ARF}$ and $P16^{INK4a}$, which play roles in cellular senescence and ageing. The *CDKN2A* locus also encodes the long non-coding RNA *ANRIL* (antisense non-coding RNA in the *INK4* locus), a 3,834bp transcript which can negatively regulate $p16^{INK4a}$. SNPs within the *CDKN2A* locus, particularly those located within *ANRIL* have been associated with cardiovascular disease, diabetes and frailty (450), and a recent study by Bell et al. showed that DNA methylation at this locus varies with age (451).

Both fat and bone share a common mesenchymal origin, and have strong functional relationships, mediated via both mechanical and endocrine pathways (453). This brings possible relevance to the skeletal system of the previously demonstrated links in the SWS between perinatal *CDKN2A* methylation and offspring fat mass (448). Therefore a targeted approach was used to examine DNA methylation at CpG sites within the *CDKN2A* locus and its association with bone mass in children.

DNA methylation was examined across a 300bp region within the promoter region of ANRIL that contained 9 CpG dinucleotides. The hypothesis was that DNA methylation at birth would be associated with offspring bone mass in childhood. To test this, DNA methylation levels were examined in relation to BMD in a discovery cohort, then replicated in a second separate cohort, to allow analysis of two populations. These represented two groups of approximately 400 mother-offspring pairs recruited to the SWS consecutively, analysed as separate cohorts. Collaborators in Professor Lillycrop's group at the Institute of Developmental Sciences (University of Southampton) carried out functional analysis of the region to determine its importance for local gene expression and transcription factor binding, described in the paper, published in the Journal of Bone Mineral Research in 2017 (454). In this thesis I will focus only on the epidemiology, my own component of the work.

4.2 Methods

4.2.1 The Southampton Women's Survey

The methodology for the SWS is described in detail in chapter 3. In this analysis, a subset of children are used. This group were invited to visit the Osteoporosis Centre at Southampton General Hospital for assessment of bone mass and body composition at 4 years. At this visit, written informed consent for a DXA scan was obtained from the mother, father or guardian. The

child's height and weight were measured and a whole body DXA scan was obtained, using a Hologic Discovery instrument (Hologic Inc., Bedford, MA, USA) in paediatric scan mode. Scans with unacceptable movement artefact were excluded. A similar bone assessment was undertaken at age 6 years. The whole body less head region of interest was used in the analyses.

4.2.2 Umbilical cord DNA extraction

DNA was extracted from the umbilical cord. The cords were collected immediately following delivery and stored, as described in Chapter 3. Genomic DNA was isolated from frozen archived umbilical cord tissue by classical proteinase K digestion and phenol:chloroform extraction.

4.2.3 DNA methylation analysis and pyrosequencing: Study and Replication Cohorts

The region of interest within the *CDKN2A* gene locus is located within the promoter region of the non-coding RNA ANRIL, transcribed from this gene locus, and contains 9 CpG dinucleotides (chr9: 21993583-21993721) (Figure 4.1). These CpG sites were previously identified using a whole genome methylation analysis of genomic DNA from umbilical cord in 21 children from the SWS cohort, chosen to represent a range of percentage fat between the 5th and 95th percentiles for this population measured by DXA at age 6 years. DNA methylation levels were quantified using Agilent Human Promoter Whole-Genome ChIP-on-chip array (*G4489A*), and analysed using Bayesian Tool for Methylation Analysis (BATMAN) (484) as previously described (485).

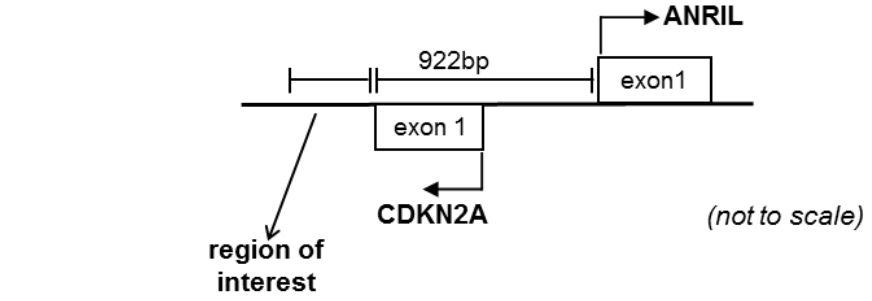


Figure 4.1 Location of CpG dinucleotides in the *CDKN2A* region. Hg19; chr9 21,993,583 - 21,993,721.

Local gene layout with relevant distances marked in base-pairs, and the CpG dinucleotides of interest marked (numbered 1-9) on an annotated primary sequence.

Sodium bisulfite targeted pyrosequencing (Pyromark MD, Qiagen, https://www.qiagen.com/fi/resources/technologies/pyrosequencing-resource-center/technology-overview/) was performed by Dr Robert Murray and other members of Professor Lillycrop's group as described in section 3.3.2 (479). This enabled in-depth analysis of the methylation status of the 9 CpGs of interest within the previously identified differentially methylated region of CDKN2A in umbilical cords.

The analysis followed a discovery/replication design, with methylation status measured in an initial consecutive series of umbilical cords from SWS deliveries (discovery cohort), on whom childhood DXA assessment had been undertaken. Subsequently, when DXA measures had become available on a further separate subset of SWS children, methylation at these CpG sites was measured in a second, consecutive and independent series of SWS deliveries (replication cohort).

To ensure that the full range of methylation could be detected, Inter- and intra-plate controls were added to each plate as a control for inter- and intra-plate variability, and 0% and 100% methylation controls were run.

The summary statistics for methylation (minimum, maximum, quartile 1, median, quartile 3), plus mean and SD, together with the genomic coordinates for the CDKN2A CpG sites are shown in Table 4.1.

Table 4.1 Percentage DNA methylation at CDKN2A in umbilical cord tissue of offspring in Combined SWS cohort. DMR: 21993583-21993721 (Human genome hg19/GRCh37 build).

CDKN2A CpG Site	*Hg19 Coordinates (Chr9)*	*N*	*Min (25th, 50th, 75th percentile) max*	*Mean (SD)*
1	21993721	592	25.2 (66.7, 72.1, 76.2) 86.2	69.9 (9.6)
2	21993697	582	23.4 (65.3, 71.0, 75.3) 92.8	68.9 (10.0)
3	21993694	496	18.3 (47.0, 53.3, 58.4) 65.6	51.8 (8.5)
4	21993654	658	20.9 (66.8, 72.7, 77.6) 94.5	71.2 (10.1)
5	21993645	643	17.6 (54.9, 60.5, 66.0) 84.2	59.3 (10.0)
6	21993638	643	25.1 (66.4, 72.5, 77.1) 90.9	70.4 (10.1)
7	21993629	601	17.1 (55.5, 61.4, 67.2) 80.3	60.5 (9.5)
8	21993603	632	23.6 (69.9, 75.0, 79.0) 99.6	73.0 (9.6)
9	21993583	546	22.0 (58.8, 65.1, 74.3) 85.6	65.3 (10.4)

4.2.4 Statistical Analysis

Statistical analysis was undertaken using Stata (Statacorp versions 14.0/14.1/14.2). The data were checked to ensure that the assumptions of linear regression were met. Regression models were built using the offspring bone indices measured by DXA (at either 4 or 6 years) as the outcome, and CpG methylation as the predictor. Results are presented as the number of observations in each regression, and regression coefficients (β) with their associated p-values and 95% confidence intervals. β coefficients represent the change in the bone outcome per 10% change in methylation at each CpG site. This analysis was initially performed on the SWS discovery cohort, repeated in the SWS replication cohort, and then analysis was performed on the two cohorts combined (SWS combined cohort), in order to maximise statistical power for further multivariate analyses. All models were adjusted for child's sex and age at DXA scan, except for at 4 years where an adjustment for age was not necessary due to the narrow age range at which the DXA scans were performed.

Where analysis was performed on the combined cohort, an indicator representing cohort was included as a covariate in the model to account for batch effect. In multivariate models potential confounding factors previously found to be associated with offspring bone development were accounted for (mother's pre-pregnancy height, maternal parity, late pregnancy walking speed, late pregnancy maternal smoking, and late pregnancy triceps skinfold thickness) (199). In further analyses, the effect of the covariates potentially on the causal pathway were explored, including offspring birthweight, and childhood height, weight, lean mass and fat mass.

Recognising that there was likely to be co-linearity between the individual exposures, and between the DXA indices, a data reduction approach was used (to reduce the number of tests) by investigating clustering of the CpG methylation. Importantly, there is evidence that where clusters of differential CpGs can be identified, they are more likely to be of functional importance than are individual CpG changes (486). Given the relatively small number of tests in our analysis, compared with larger scale genome-wide associations studies, for which methods such as Bonferroni or the Benjamini-Hochberg/ False Discovery Rate corrections for multiple testing would be appropriate (480), the clustering method was considered the most practical. By investigating the correlation between methylation at each of the individual CpG sites, and calculating the median absolute deviation (MAD) from the median for each site, CpG sites could be grouped into 4 clusters (CpGs1-2, 3, 4-7, 8-9), with each cluster represented by the site with the highest MAD score (i.e. the site with the greatest variability within the cluster), that is, CpG sites 2, 3, 7 and 9 respectively.

Table 4.2 CpG Clustering. Spearman correlation of methylation levels at CpGs 1-9 within the CDKN2A region. Four distinct clusters are defined: 1-2, 3, 4-8 and 9.

(Top) Correlations between methylation levels at CDKN2A CpGs. Red and orange colours indicate low levels of correlation (coefficients of 0.20 to 0.59), yellow indicates medium correlation (coefficients of 0.60 to 0.69), green indicates high correlation (coefficients of 0.70 and above).

(Bottom) Median absolute deviation (MAD) scores within the 4 clusters in SWS Cohort.

	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9
CpG1									
CpG2	0.73								
CpG3	0.65	0.46							
CpG4	0.61	0.70	0.47						
CpG5	0.62	0.73	0.46	0.81					
CpG6	0.66	0.71	0.43	0.79	0.81				
CpG7	0.55	0.68	0.27	0.78	0.82	0.79			
CpG8	0.58	0.70	0.39	0.68	0.70	0.74	0.69		
CpG9	0.48	0.67	0.33	0.70	0.72	0.65	0.77	0.69	

Cluster	CDKN2A CpG site	MAD score
CpG 1-2	1	4.56
	2	**5.01**
CpG 3	3	**5.59**
CpGs 4-8	4	5.30
	5	5.45
	6	5.03
	7	**5.90**
	8	4.49
CpG 9	9	**7.86**

4.3 Results

4.3.1 Characteristics of the participants

There were 332 mother and child pairs with methylation measurements at the 9 CpG sites within the CDKN2A locus and DXA data at either 4 years or 6 years of age in the SWS Discovery cohort, 374 in the SWS Replication cohort (totalling 706 when these sets were combined in the SWS Combined cohort). Table 4.3 and Table 4.4 summarise the characteristics of the mothers and children in the Discovery, Replication and Combined cohorts. Compared with mothers in the SWS as a whole, mothers in the final Combined cohort were slightly taller ($p = 0.004$), less likely to smoke during pregnancy ($p = 0.049$), and had higher educational qualifications ($p < 0.001$) and higher socioeconomic status ($p < 0.001$).

Table 4.3 Characteristics of the SWS mothers in the Discovery cohort, Replication cohort and Combined cohort. *% or median (5th, 95th percentile),*Mean (SD)*

Maternal Characteristic	*Discovery Cohort n = 332*	*Replication Cohort n = 374*	*Combined Cohort n = 706*
Woman's age at birth of child (years)*	30.44 (3.5)	31.23 (3.6)	30.86 (3.6)
BMI	24.29 (19.8, 34.6)	24.10 (19.6, 34.4)	24.19 (19.7, 34.6)
Maternal Height (cm)*	163.72 (6.7)	163.91 (6.2)	163.82 (6.4)
Maternal Weight (kg)	66.0 (52.2, 93.8)	64.8 (51.9, 96.3)	65.7 (51.9, 94.3)
Late pregnancy mid upper arm circumference (cm)*	30.04 (3.5)	30.37 (3.7)	30.22 (3.6)
Smoking (during pregnancy)	13.64%	14.21%	13.94%
Educational qualifications:			
None	0.90%	2.43%	1.71%
CSE	8.73%	8.89%	8.82%
O levels	26.81%	27.76%	27.31%
A levels	27.11%	35.04%	31.29%
HND	9.04%	6.47%	7.68%
Degree	27.41%	19.41%	23.19%
Social class:			
Professional	6.75%	4.62%	5.62%
Management and technical	42.64%	39.13%	40.78%
Skilled non-manual	32.21%	37.50%	35.01%
Skilled manual	6.75%	7.07%	6.92%
Partly skilled	10.74%	10.33%	10.52%
Unskilled	0.92%	1.36%	1.15%
Late pregnancy walking speed			
Very slow	15.26%	16.76%	16.06%
Stroll at an easy pace	53.58%	52.43%	52.97%
Normal speed	26.48%	23.78%	25.04%
Fairly brisk	4.36%	6.76%	5.64%
Fast	0.31 %	0.27 %	0.29 %

Table 4.4 Characteristics of the SWS children in the Discovery, Replication and Combined cohorts.

*[+] Whole body less head site (WBLH), adjusted for sex; [++] Whole body less head site, adjusted for sex and age. % or median (5th, 95th percentile),*Mean (SD)*

Child Characteristic	*Discovery Cohort n = 332*	*Replication Cohort n = 374*	*Combined Cohort n = 706*
Female	47.89%	51.34%	49.72%
Birth order			
1st	47.59%	51.07%	49.43%
2nd	39.46%	36.10%	37.68%
3rd or higher	12.95%	12.83%	12.89%
Birth weight, kg*	3.46 (0.5)	3.53 (0.5)	3.50 (0.5)
Gestational age, weeks	40.00 (36.6, 41.9)	40.14 (37.1, 41.9)	40.12 (37.0, 41.9)
Placental weight (g)*	467.95 (99.3)	479.09 (104.2)	473.94 (102.0)
4 year Child DXA Bone indices[+]			
4 year age at DXA scan	4.1 (4.0, 4.2)	4.1 (4.0, 4.2)	4.1 (4.0, 4.2)
WBLH BMC (g)*	374.6 (45.0)	371.8 (45.3)	373.2 (45.1)
WBLH BMD (g/cm^2)*	0.37 (0.05)	0.37 (0.05)	0.37 (0.05)
WBLH BA (m^2)*	0.49 (0.04)	0.49 (0.04)	0.49 (0.04)
4 year WBLH fat mass[+] (kg)	4.1 (2.9, 6.7)	4.1 (3.0, 6.7)	4.1 (2.9, 6.7)
4 year WBLH lean mass[+] *(kg)	9.86 (1.3)	9.73 (1.3)	9.79 (1.3)
6 year child bone DXA indices[++]			
6 year age at DXA scan	6.6 (6.3, 7.0)	7.0 (6.5, 7.5)	6.8 (6.3, 7.4)
WBLH BA (cm^2)*	908.06 (64.2)	913.18 (62.4)	910.65 (63.3)
WBLH BMC (g)*	547.0 (74.1)	551.6 (77.1)	549.3 (75.6)
WBLH BMD (g/cm^2)*	0.60 (0.05)	0.60 (0.05)	0.60 (0.05)

4.3.2 Associations between CDKN2A methylation and offspring bone size, mineralisation and areal density

Percentage methylation at the 9 CpG sites varied greatly, from 17.1% to 99.6%, as shown in

Table 4.1. There were statistically significant inverse associations between CpG methylation at sites 3, 7 and 9 (with CpGs 7 and 9 representing the clusters CpG4-7 and CpG8-9) and offspring whole body less head (WBLH) bone indices (bone area, bone mineral content and bone mineral density) at 4 years. β coefficients and 95% CIs have been multiplied by 10 and therefore represent the change associated with a 10% increase in methylation.

Table 4.5 CDKN2A CpG methylation and whole body less head bone mineral outcomes at age 4 years in the SWS Discovery Cohort, replication cohort and combined cohort. *Associations adjusted for sex. β coefficients and 95% CIs have been multiplied by 10 and therefore represent the change associated with a 10% increase in methylation. p-values < 0.05 are in bold.*

CpG cluster		*WBLH BA (cm^2)*			*WBLH BMC (g)*			*WBLH BMD (g/cm^2)*		
	n	*β*	*P-value*	*95% CI*	*β*	*P-value*	*95% CI*	*β*	*P-value*	*95% CI*
Discovery cohort										
1-2	254	-3.07	0.324	(-9.18, 3.05)	-3.06	0.297	(-8.84, 2.71)	-0.0018	0.442	(-0.0062, 0.0027)
3	229	-3.32	0.428	(-11.57, 4.92)	-4.64	0.243	(-12.46, 3.17)	-0.0037	0.236	(-0.0097, 0.0024)
4-7	288	-6.94	**0.037**	(-13.44, -0.44)	-6.23	**0.050**	(-12.44, -0.01)	-0.0033	0.195	(-0.0083, 0.0017)
8-9	262	-10.85	**0.005**	(-18.47, -3.23)	-10.27	**0.005**	(-17.47, -3.07)	-0.006	**0.045**	(-0.0118, -0.0001)
Replication cohort										
1-2	245	-2.58	0.399	(-8.61, 3.44)	-4.94	0.090	(-10.66, 0.77)	-0.0046	0.051	(-0.0092, 0.000002)
3	193	-9.8	**0.016**	(-17.74, -1.86)	-10.22	**0.008**	(-17.76, -2.69)	-0.007	**0.027**	(-0.0131, -0.0008)
4-7	267	-6.93	**0.025**	(-12.96, -0.89)	-9.9	**0.001**	(-15.67, -4.14)	-0.0081	**0.001**	(-0.0128, -0.0035)
8-9	216	-6.39	0.056	(-12.95, 0.17)	-8.79	**0.008**	(-15.24, -2.35)	-0.007	**0.009**	(-0.0122, -0.0018)
Combined cohort										
1-2	499	-2.83	0.195	(-7.11, 1.45)	-3.99	0.054	(-8.04, 0.07)	-0.0031	0.054	(-0.0064, 0.0001)
3	422	-6.52	**0.026**	(-12.25, -0.80)	-7.4	**0.008**	(-12.83, -1.98)	-0.0053	**0.016**	(-0.0096, -0.0010)
4-7	555	-6.93	**0.002**	(-11.34, -2.52)	-8.18	**< 0.001**	(-12.40, -3.96)	-0.0059	**0.001**	(-0.0093, -0.0025)
8-9	478	-8.28	**0.001**	(-13.23, -3.32)	-9.42	**< 0.001**	(-14.18, -4.65)	-0.0066	**0.001**	(-0.0104, -0.0027)

Table 4.5 shows the associations between *CDKN2A* methylation and WBLH DXA outcomes at age 4 years in the discovery cohort (n = 288), the replication cohort (n = 267), and the cohorts combined (n = 555). The relationships were similar in both cohorts, therefore the combined cohort was used for all remaining analyses.

Table 4.6 shows the combined cohort adjusted for pyrosequencing batch effects and maternal factors previously identified as being associated with offspring bone indices [mother's late pregnancy walking speed, late pregnancy smoking, pre-pregnancy height, late pregnancy triceps skinfold thickness and parity (model 1)]. There were strongly statistically significant inverse associations between CpG methylation at CpG sites 3, 4-7 and 8-9 with the WBLH bone area, BMC and BMD at 4 years. Additional adjustment for child's height (model 2) led to a reduction in magnitude of the associations. After this size adjustment, statistically significant inverse associations remained between CpG sites 3, 4-7 and 8-9 and WBLH BMC, CpG 4-7 and 8-9 and WBLH BMD, and CpG 8-9 and WBLH bone area. Additional adjustment for child's weight (but not height, model 3) markedly attenuated associations between *CDKN2A* methylation and childhood bone indices at the age of 4 years below statistical significance.

In further analyses, aimed at identifying potential mediators of the methylation-bone relationship by adjusting for childhood factors previously shown to be associated with bone mass, the CDKN2A methylation-bone associations remained robust after inclusion of child's whole body lean mass or fat mass, or child's birthweight, in the multivariate models as shown in Table 4.7.

Similar inverse associations between *CDKN2A* methylation and bone indices were seen at age 6 years, and the effect sizes were similar across different CpG sites at the two different ages (as shown in Table 4.8). For example, for every 10% increase in methylation at CpG 9, there was a 9.1 g decrease in whole body minus head BMC at 4 years, and a 10.2 g decrease at 6 years.

The associations between *CDKN2A* methylation at CpG 6 and CpG 9 and offspring bone area, bone mineral content and bone mineral density at age 4 years are graphically represented in Figure 4.2.

Table 4.6 CDKN2A CpG methylation and WBLH bone outcomes at age 4 years accounting for maternal factors in the Combined SWS cohort.

Model 1: Adjusted for batch, child's sex, mother's LP walking speed, LP smoking, pre-pregnancy height, LP triceps skinfold thickness and parity. Model 2: Model 1, plus child's height. Model 3: Model 1, plus child's weight.

CpG cluster		*WBLH BA (cm^2)*			*WBLH BMC (g)*			*WBLH BMD (g/cm^2)*		
	n	*β*	*P-value*	*95% CI*	*β*	*P-value*	*95% CI*	*β*	*P-value*	*95% CI*
Model 1: Batch, child's sex, mother's LP walking speed, LP smoking, pre-pregnancy height, LP triceps skinfold thickness and parity										
1-2	484	-3.00	0.163	(-7.21, 1.22)	-4.11	**0.041**	(-8.05, -0.17)	-0.0032	**0.043**	(-0.0064, -0.0001)
3	408	-5.93	**0.043**	(-11.68, -0.18)	-7.01	**0.010**	(-12.33, -1.69)	-0.0052	**0.015**	(-0.0094, -0.0010)
4-7	538	-5.85	**0.008**	(-10.20, 1.51)	-7.17	**0.001**	(-11.30, -3.04)	-0.0053	**0.002**	(-0.0086, -0.0019)
8-9	461	-8.26	**0.001**	(-13.11, -3.41)	-9.11	**< 0.001**	(-13.79, -4.43)	-0.0062	**0.002**	(-0.0100, -0.0024)
Model 2: Model 1, plus child's height at age 4 years										
1-2	484	-1.23	0.428	(-4.27, 1.81)	-2.31	0.079	(-4.89, 0.27)	-0.002	0.100	(-0.0044, 0.0004)
3	408	-2.59	0.220	(-6.73, 1.55)	-3.67	**0.041**	(-7.19, -0.15)	-0.003	0.074	(-0.0063, 0.0003)
4-7	538	-2.75	0.089	(-5.92, 0.42)	-3.9	**0.005**	(-6.60, -1.20)	-0.003	**0.021**	(-0.0055, -0.0004)
8-9	461	-5.2	**0.003**	(-8.67, -1.73)	-4.02	**< 0.001**	(-6.48, -1.56)	-0.0039	**0.008**	(-0.0069, -0.0010)
Model 3: Model 1, plus child's weight at age 4 years										
1-2	482	1.05	0.536	(-2.27, 4.37)	0.77	0.539	(-1.69, 3.24)	0.0005	0.632	(-0.0016, 0.0026)
3	407	-0.29	0.900	(-4.86, 4.27)	-0.49	0.778	(-3.87, 2.90)	-0.0003	0.818	(-0.0032, 0.0026)
4-7	536	-1.24	0.485	(-4.73, 2.25)	-1.54	0.257	(-4.20, 1.13)	-0.0009	0.448	(-0.0032, 0.0014)
8-9	459	-2.89	0.144	(-6.78, 0.99)	-2.54	0.103	(-5.59, 0.51)	-0.0011	0.429	(-0.0038, 0.0016)

Table 4.7 Associations between *CDKN2A* methylation and offspring WBLH BMC at 4 years in the Combined cohort, adjusted for child's sex, batch effect and additionally either whole body lean mass, fat mass, or birthweight.

β coefficients and 95% CIs have been multiplied by 10 and therefore represent the change associated with a 10% increase in methylation. p-values < 0.05 are in bold.

CpG Cluster	*n*	*WBLH BMC (g), adjusted for sex and batch effect*								
		β	*P-value*	*95% CI*	*β*	*P-value*	*95% CI*	*β*	*P-value*	*95% CI*
		Additionally adjusted for lean mass			Additionally adjusted for fat mass			Additionally adjusted for birthweight		
1-2	420	-2.04	0.129	(-4.67, 0.59)	-0.04	0.985	(-3.93, 3.85)	-2.14	0.282	(-6.05, 1.77)
3	352	-3.03	0.097	(-6.60, 0.55)	-3.09	0.244	(-8.29, 2.12)	-5.8	**0.030**	(-11.01, -0.58)
4-7	471	-4.01	**0.005**	(-6.80, -1.22)	-6.37	**0.003**	(-10.50, -2.23)	-7.44	**< 0.001**	(-11.55, -3.33)
8-9	405	-4.85	**0.003**	(-8.02, -1.69)	-7.53	**0.002**	(-12.18, -2.89)	-8.29	**0.001**	(-12.91, -3.67)

Table 4.8 Age 6 years: Relationships between methylation at CpG sites within the *CDKN2A* region of interest and total bone area, bone mineral content and bone mineral density in the 6 year old children (whole body minus head).

Adjusted for batch effect, sex and age. β coefficients and 95% CIs have been multiplied by 10 and therefore represent the change associated with a 10% increase in methylation. p-values < 0.05 are in bold.

CpG cluster	*n*	*Age 6 years: WBLH BA (cm^2)*			*WBLH BMC (g)*			*WBLH BMD (g/cm^2)*		
		β	*P-value*	*95% CI*	*β*	*P-value*	*95% CI*	*β*	*P-value*	*95% CI*
Adjusted for batch effect, sex and age										
1-2	438	-0.4251	0.195	-1.1000, 0.2179	-6.46	0.094	(-14.02, 1.11)	-0.0004	0.098	-0.0009, 0.0001
3	371	-0.1912	0.648	-1.0000, 0.6312	-4.36	0.375	(-14.02, 5.30)	-0.0003	0.283	-0.0009, 0.0003
4-7	444	-0.7167	**0.036**	-1.4000, -0.0465	-11.45	**0.005**	(-19.42, -3.49)	-0.0007	**0.005**	-0.0012, -0.0002
8-9	398	-0.5898	0.142	-1.4000, 0.1983	-10.21	**0.034**	(-19.66, -0.77)	-0.0007	**0.025**	-0.0013, -0.0001

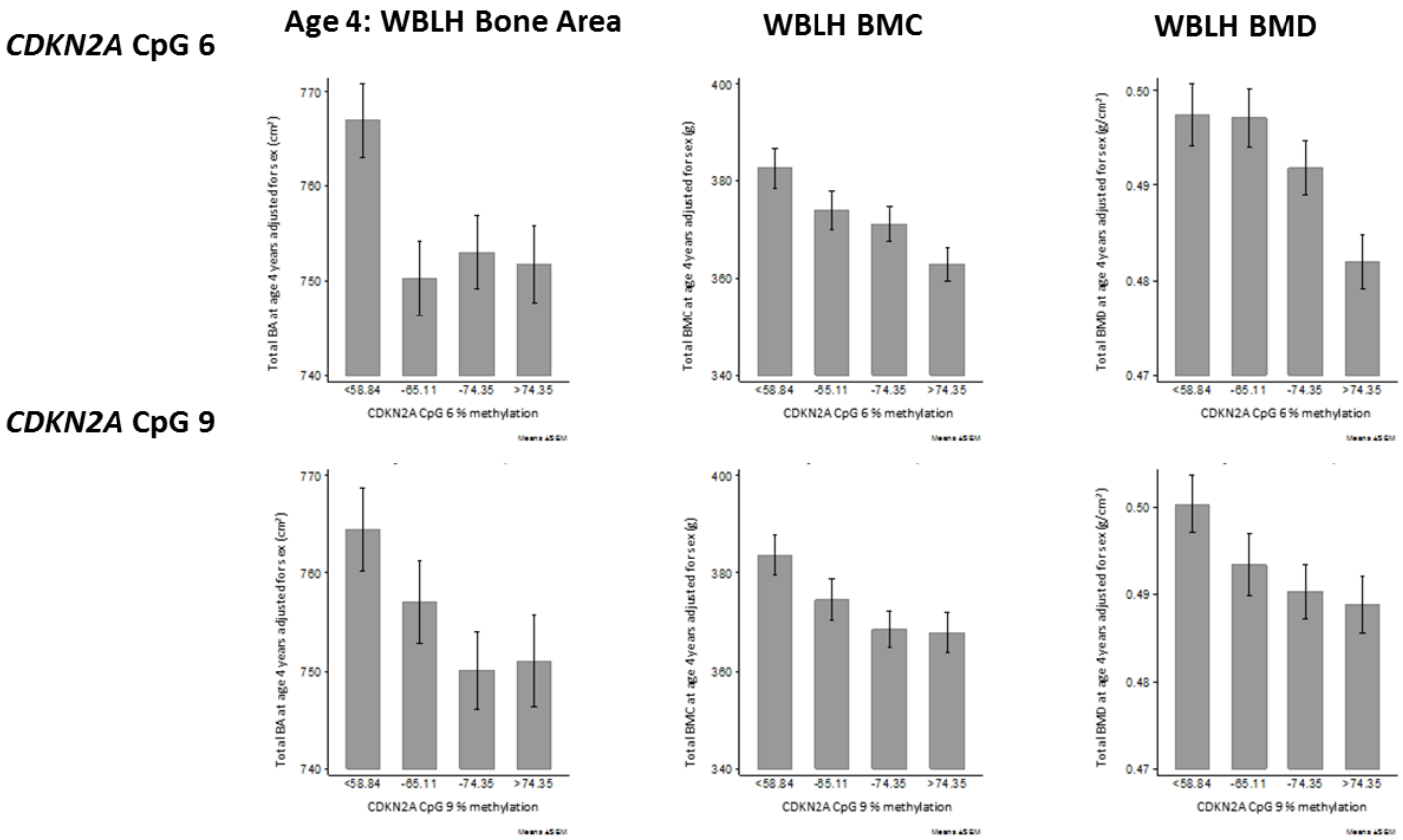


Figure 4.2 *CDKN2A* CpG methylation in relation to bone mineral outcomes. Mean +/- SEM

Percentage methylation in umbilical cord tissue at CDKN2A CpG 6 (top row) and CpG 9 (bottom), expressed in quarters of the distribution and offspring total bone area (whole body minus head) cm^2), bone mineral content (g) and areal bone mineral density (g/cm^2) at age 4 years.

4.3.3 *In silico* analysis of the CDKN2A region

In silico analysis of ENCODE ChromHMM data (487) () revealed that this region is enriched for both promoter and enhancer activity across multiple cell types (red, orange or yellow in chromatin state segmentation) , as well as overlapping DNase I hypersensitive sites, suggesting that the CpG sites examined lie within a key regulatory region of CDKN2A.

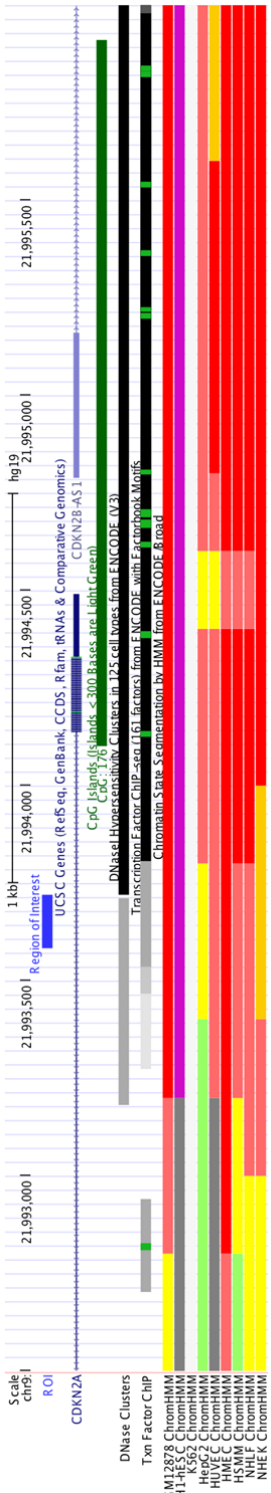


Figure 4.3 Location of CpG dinucleotides in relation to the known genes in the CDKN2A region. Region of interest: 21993583-21993721. (Human genome hg19/GRCh37 build). *Figure shows the UCSC-genome annotation for this region; outlining the region of interest in association with CpG islands, DNase1 hypersensitivity clusters, transcription factor binding sites and chromatin state across a variety of cell types. Cell types include GM12878 (B cell derived cell line), H1-hESC (human embryonic cell line), K562 (human chronic myeloid leukaemia), HepG2 (human epithelial cell line), HUVEC (human endothelial cell derived cell line), HMEC (human mammary gland cell line), HSMM (human skeletal muscle myoblast), NHLF (human lung fibroblast) and NHEK (human epidermal keratinocyte cell line).*

4.4 Summary of findings and discussion

Lower perinatal methylation of specific CpG dinucleotides within the *CDKN2A* gene locus was shown to be associated with greater total whole body minus head BA, BMC and areal BMD at 4 and 6 years of age in the SWS.

Methylation in the CDKN2A region: Functional experimental findings in an osteosarcoma cell line

Functional experimental work performed by Professor Lillycrop's group, published alongside these epidemiological findings (454) demonstrated the importance of these CpG sites in the expression of ANRIL in osteosarcoma cell lines (SaOS2). To summarise, mutation of CpGs 2-8 led to a decrease in ANRIL promoter activity. Electrophoretic mobility shift assays, in which nuclear extracts from SaOS 2 cells were incubated with oligonucleotides containing CpG1, CpG 2-3, CpGs 4-7 and CpG 8-9 demonstrated strong specific binding of protein complexes, particularly to the oligonucleotides containing CpG1 and CpGs 8-9. This suggests possible functional relevance in terms of binding of transcription factors to the region, with evidence of stronger protein binding when CpGs 8-9 were unmethylated. Binding of transcription factor sequences (488) for Interferon-gamma Activated Site (GAS) and SMAD3/4 competed-out binding of a radiolabelled probe to the CpG8-9 region in an osteosarcoma cell line extract. In order to understand the role of ANRIL in SaOS2 cells, silencing RNAs against ANRIL (450) led to a decrease in ANRIL expression and a reduction in the number of live cells, with a concurrent increase in the number of cells undergoing apoptosis. In summary, these experiments suggest that these CpG sites may play a role in modulating the level of expression of the long non-coding RNA ANRIL, which regulates cell survival, suggesting that these relationships have functional relevance.

Findings in relation to the known function of the CDKN2A locus

To my knowledge, this is the first time that associations between *CDKN2A* methylation and childhood bone development, and the related consequences for cell survival and gene expression have been demonstrated. In terms of the known function of the C*DKN2A* (*INK4A-ARF*) gene locus, we know that it encodes two potent inhibitors of cell growth: $p14^{ARF}$ (alternative reading frame relative to p16) and $p16^{INK4a}$ (inhibitor of cyclin-dependent kinase 4), both of which have been shown to play a role in driving cellular senescence and ageing (489, 490). GWAS studies have shown that single nucleotide polymorphisms (SNPs) in a region spanning 160 kb around the *CDKN2A* locus, with the majority located within *ANRIL*, were associated with increased susceptibility to frailty and a variety of ageing related diseases such as coronary artery disease, myocardial infarction, type 2 diabetes, and late onset Alzheimer's disease (450, 491-497). Whilst

the majority of these SNPs are located towards the 3' end of the *ANRIL* coding region, over 100Kb from the DMR identified in this study, these SNP associations, and recent evidence linking methylation at *CDKN2A* with ageing (451), do highlight the importance of the *CDKN2A* locus in general, and *ANRIL* in particular in altered susceptibility to ageing-related diseases.
Evidence in the literature for the specific involvement of methylation of the *CDKN2A* locus in bone metabolism is limited, although $p16^{INK4a}$ expression has been linked to altered osteoblast morphology and senescence in animal models (498, 499), with another study demonstrating transitional hypomethylation of *CDKN2A* in human bone marrow stromal cells in their differentiation towards an osteoblastic lineage (455). Active demethylation of the promoters of *RUNX2*, *osteocalcin* and *osterix* genes has been demonstrated in the osteogenic differentiation of adipose-derived mesenchymal stem cells, in a process dependent upon growth arrest and DNA-damage-inducible protein, GADD45, which is known to interact with both *CDK1* and *CDKN1A* (456, 457).

In addition, genome-wide methylation profiling studies in older patients comparing individuals with low versus normal BMD have also suggested early life influences on bone quality in older age (467). In a genome-wide association study, genes encoding cyclin dependent kinase inhibitor *CKDN1C* and cyclin dependent kinase *CDK20* have been found to be differentially methylated in histological bone samples from patients who have experienced a low trauma hip fracture compared with those from patients undergoing elective arthroplasty for osteoarthritis (466). In this study, DMRs enriched in genes associated with cell differentiation and skeletal embryogenesis, including those in the homeobox superfamily, were identified, supporting a developmental component to these conditions. In another GWAS of heel ultrasound eBMD in the UK Biobank, SNPs in other genes encoding proteins similar to, or known to interact with, products of the CDKN2A gene were shown to be important (*CKD2AP1*, which regulates $p14^{ARF}$ levels (500), and *CDK-5*, a member of the cyclin family important in cell cycle regulation (501)) providing support for a role for the *CDKN2A* region in bone health.

There are several possible explanations for our findings demonstrating inverse associations between *CDKN2A* methylation in umbilical cord tissue and bone outcomes in childhood. Firstly, given that inverse associations between *CDKN2A* methylation and offspring adiposity have previously been observed in the SWS (502), the relationships with bone might have been mediated through fat or lean mass. However, when we adjusted for DXA whole body fat or lean mass in the multivariate models, the *CDKN2A*-bone relationships remained robust. A second explanation could be that *CDKN2A* methylation and childhood bone mass are both influenced by a common factor during intrauterine life. In this situation, one might expect that the *CDKN2A*-bone

relationships would be attenuated through adjustment for birthweight, but we found that statistically significant relationships remained, although were markedly attenuated by inclusion of current weight in the model. It is difficult to interpret this latter observation owing to the high correlation between body weight and bone indices, but the persisting associations between perinatal CDKN2A methylation and bone indices after adjustment for childhood height suggests that they are not mediated by skeletal size alone. It is, of course, possible that *CDKN2A* methylation in umbilical cord tissue does have a truly causal relationship with bone development, particularly given the common mesenchymal origin of elements of both tissues. This could mean that the alterations to methylation detected in umbilical cord tissue are consistent with changes in bone tissue, or that the umbilical cord methylation patterns influence other processes which in turn influence bone development. It may be intuitively reasonable to assume, therefore, that a resetting of gene expression via epigenetic marking, might have a long-term influence on skeletal growth.

In vitro investigations, performed by Professor Lillycrop's group, were supportive of the epidemiological findings. They showed the functional importance of these CpG dinucleotides for ANRIL expression, and that DNA methylation affects protein binding in the region, outlining possible roles for specific transcription factors. Elucidation of the causal (or not) nature of the associations is of course essential for mechanistic understanding and identification of potential therapeutic targets. However, even without this information, our findings may be of future importance in terms of risk stratification for future bone health, and identification of those individuals potentially at increased risk of osteoporosis in old age.

4.4.1 Limitations

The data presented are from a well characterised cohort, using the gold standards of pyrosequencing (to determine CpG site-specific DNA methylation) and DXA (to assess bone mass). However, the limitations of the association of pyrosequencing data from umbilical cord to phenotypic outcomes must be considered. These are discussed in detail in section 8.3. To briefly summarise, when analysing pyrosequencing data it is important to consider the possibility of genetic trans- effects due to SNPs in regions other than the region of interest (SNPs at the CpG sites of interest were excluded previously by sequencing (449)). These may influence both DNA methylation and phenotype.

Other limitations include the variety of cell types in umbilical cord samples which may vary between individuals, leading to different measures of methylation driven solely by cell

composition. Clearly, it was not possible to measure methylation levels in children's bone biopsies, due to ethical considerations, therefore umbilical cord is being used as a proxy for bone tissue. It is important to note that both bone and cord tissue are derived from the mesoderm and share mesenchymal cell origins; mesenchymal stem cells differentiate into osteoblasts, playing a role in bone formation both in the embryo (503), child and adult (504). In this study, it was also not possible to examine levels of gene expression in the umbilical cord tissue – however, altered gene expression patterns are likely to be cell type dependent, so the ability to extrapolate this data to the behaviour of cells in bone tissue would be limited.

There are also challenges associated with the interpretation of DXA data in children, both due to movement artefact and due to the fact that measurement of bone mineral is hampered by their low absolute BMC – specific paediatric software was used to limit this. DXA studies compared to the ashed mineral content in piglets has confirmed the accuracy of DXA in children (505). Due to the inability of DXA to measure true volumetric bone density, it is difficult to be certain whether associations with BMD are due to skeletal size, or true differences in volumetric density.

Finally, the cohort used was a subset of the SWS (although mothers whose children underwent DXA and those who did not were broadly similar, albeit slightly older and less likely to smoke). There is no known reason to suppose, however, that relationships between methylation at the *CDKN2A* promoter in umbilical cord and childhood bone mineral accrual would differ between these two groups. Still, it would be advisable to replicate these findings in a geographically separate and ethnically diverse cohort, as the SWS is of mainly Caucasian ethnicity.

4.4.2 Conclusion

This research has demonstrated that increasing methylation at CpG sites within CDKN2A in umbilical cord is associated with decreased bone size, mineral content and mineral density in childhood. Laboratory studies have also shown that these CpG sites are functionally important for local gene expression, and that DNA methylation alters transcription factor binding within the region. These findings yield mechanistic insights into the early determinants of skeletal growth, and may identify novel biomarkers for future adverse bone development.

Chapter 5: The influence of Vitamin D supplementation on RXRA methylation and associations with bone outcomes at birth

5.1 Background and aims

As discussed in Chapter 4 and Chapter 1, studies from the MRCLEU have previously shown that methylation at the cyclin-dependent kinase inhibitor 2A (*CDKN2A)* (506) and retinoid-X-receptor-alpha (*RXRA*) (443) loci in umbilical cord DNA was associated with offspring bone mass in childhood in the Southampton Women's Survey (SWS) mother-offspring cohort. RXRA is an essential part of vitamin D signalling, forming a heterodimer with the vitamin D receptor (VDR) in the nuclear action of 1,25$(OH)_2$-vitamin D. It is therefore possible that this latter observation might be of key relevance to previous demonstrations of associations between maternal 25(OH)-vitamin D status in pregnancy and offspring bone mass (324, 360, 507) together with the finding from the MAVIDOS trial of a positive effect of maternal vitamin D supplementation during pregnancy on neonatal bone mass for winter births (when background 25(OH)-vitamin D concentrations are lowest) (377). In the SWS, methylation at one CpG site upstream of the *RXRA* promoter was associated with a marker of maternal pregnancy 25(OH)-vitamin D status (443). Clearly, causation cannot be concluded from an observational study. Therefore, the setting of the MAVIDOS randomised, double-blind, placebo-controlled trial of vitamin D supplementation in pregnancy (377) provided the opportunity to test the hypothesis that maternal vitamin D supplementation would lead to altered *RXRA* DNA methylation in umbilical cord tissue at birth compared with placebo.

5.2 Methods

5.2.1 The MAVIDOS Study

The MAVIDOS study methodology is described in detail in Chapter 3 and the primary findings have been published previously (376, 377). To briefly summarise, *RXRA* DNA methylation data was analysed from a multicentre, double-blind, randomised, placebo-controlled trial of vitamin D supplementation in pregnancy, in which the primary outcome was neonatal bone mass. Women attending University Hospital Southampton NHS Foundation Trust, Southampton; Oxford University Hospitals NHS Foundation Trust, Oxford or Sheffield Hospitals NHS Trust, Sheffield for

early pregnancy ultrasound screening (11-14 weeks' gestation) between 6th October 2008 and 11th February 2014 were invited to participate in the study. Inclusion and exclusion criterial are summarised in Chapter 3. A screening blood sample was obtained; women with 25(OH)D between 25 and 100 nmol/l and serum calcium < 2.75 mmol/l were eligible to enrol fully in the study.

Participants were randomised to receive either cholecalciferol 1000 IU/day or matched placebo from before 17 weeks' gestation until delivery; randomisation was performed via computer generation sequence. The participants, antenatal caregivers and researchers were blinded to the intervention. All participants received standard antenatal care, and could continue self-administration of dietary supplements containing up to 400 IU/day vitamin D.

As described in Chapter 3, the participants attended the research centre for a detailed assessment of diet, lifestyle and health at 14 and 34 weeks. Ethnicity was reported by the participant and categorized as 'white' or 'non-white'. A non-fasted venous blood sample was obtained on the day that the study medication was dispensed and at 34 weeks' gestation, and serum stored at -80°C. 25(OH)D was assessed by chemiluminescent assay (Liaison automated platform, Diasorin, Minnesota, USA).

All neonates underwent DXA assessment at whole body and lumbar spine sites within 2 weeks of birth. All DXA images were reviewed for movement artefacts and quality by two operators (Professor Nicholas Harvey and Dr Rebecca Moon), who were blinded to treatment allocation.

Umbilical cord was collected (as a 5-10 cm portion from the midsection), frozen, and genomic DNA extracted, as described in Chapter 3.

5.2.2 Region of interest

The region of interest (previously identified and studied in umbilical cord tissue in the Southampton Women's Survey (443)) is in close proximity of the *RXRA* gene locus, 2,252 base pairs upstream from the transcriptional start site. It contains 12 CpG dinucleotides (chr9: 137215735- 137216064, Human genome hg19/GRCh37 build) as shown in Figure 5.1.

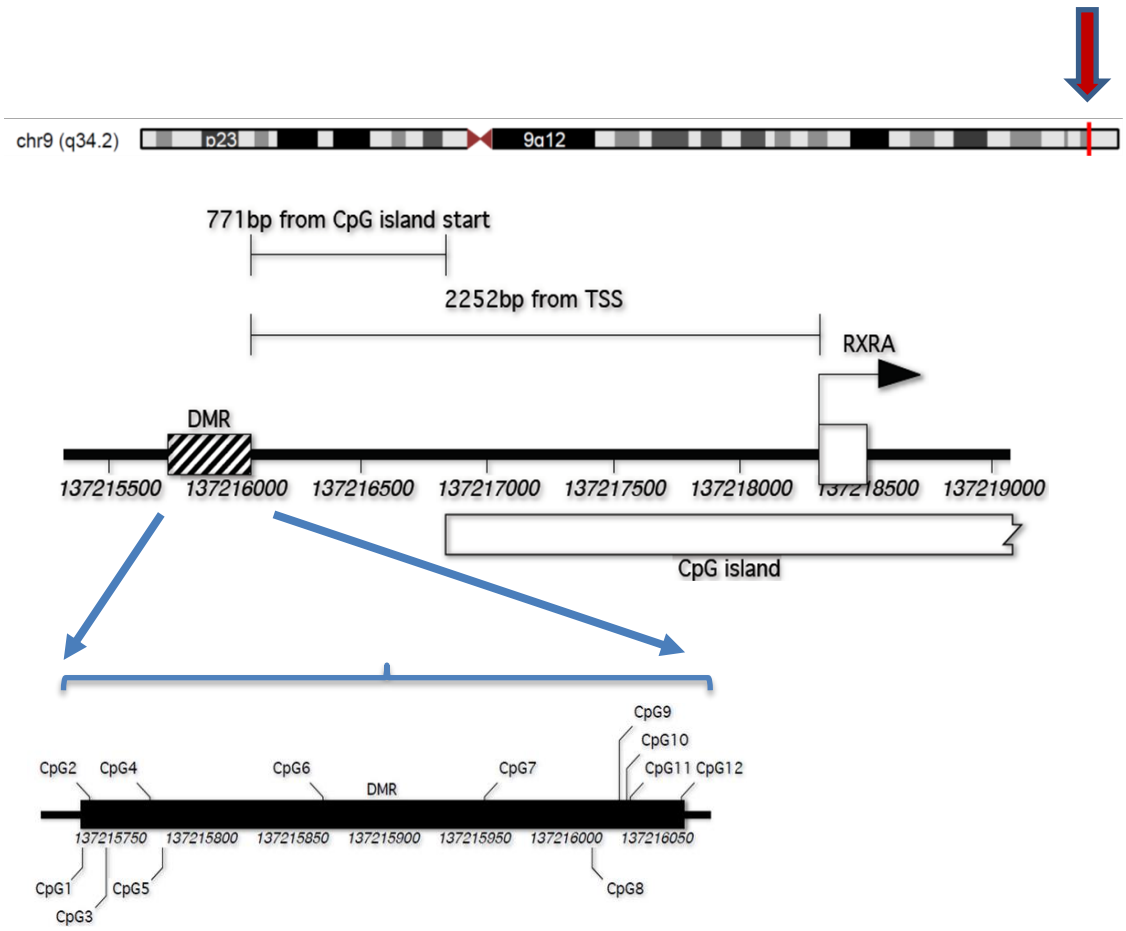


Figure 5.1 Location of CpG dinucleotides in the *RXRA* region. Region of interest: (chr9: 137215735- 137216064). (Human genome hg19/GRCh37 build).

Local gene layout is shown with relevant distances marked in base-pairs (bp) from the transcriptional start site (TSS) of RXRA. The CpG dinucleotides of interest are marked on an annotated primary sequence.

Nevena Krstic and Eloise Cook performed sodium bisulfite targeted pyrosequencing (479) to carry out in-depth analysis of the methylation status of 10 out of 12 CpGs within the previously identified differentially methylated region of *RXRA* in umbilical cords. Pyrosequencing was not performed on CpGs 6 and 7 (at genomic coordinates (hg19) chr9 137,215,867 and 137215956 respectively) for sample conservation purposes due to their distance from other CpGs (therefore requiring separate amplicons), as shown in Figure 5.1. Inter- and intra-plate controls were added to each plate as a control for inter- and intra-plate variability, and 0% and 100% methylation controls were run to ensure that the full range of methylation could be detected. The genomic coordinates for the *RXRA* CpG sites are shown in Table 5.1.

Table 5.1 Percentage DNA methylation at *RXRA* in umbilical cord tissue of offspring. DMR: Chromosome 9: 37215735- 137216064, Human genome hg19/GRCh37 build.

Pyrosequencing was not performed at CpG 6 and 7.

CpG position	Distance from transcriptional start site (bases)	Human genome 19 coordinates	N	Min (25^{th}, 50^{th}, 75^{th} percentile) Max	Mean (SD)
1	-2686	137215735	446	16.9 (27.5, 31.7, 38.6) 70.6	34.0 (9.7)
2	-2682	137215739	449	37.2 (50.0, 54.9, 59.5) 89.5	55.5 (8.0)
3	-2673	137215748	450	21.0 (30.3, 34.8, 41.4) 70.0	36.6 (9.1)
4	-2649	137215772	444	32.5 (44.7, 49.3, 55.2) 80.4	50.7 (8.7)
5	-2642	137215779	447	29.0 (41.5, 46.5, 52.5) 81.4	47.7 (9.0)
6	-2554	137215867			
7	-2465	137215956			
8	-2406	137216015	449	44.6 (58.9, 63.0, 67.2) 84.5	63.1 (6.2)
9	-2391	137216030	448	29.4 (46.4, 50.5, 55.6) 80.6	51.5 (8.0)
10	-2387	137216034	447	46.9 (60.7, 64.7, 69.6) 88.9	65.5 (7.1)
11	-2385	137216036	446	37.8 (53.6, 57.5, 63.4) 84.9	58.6 (7.8)
12	-2357	137216064	446	46.1 (62.5, 66.1, 70.7) 86.1	66.5 (6.2)

5.2.3 Statistical Analysis

Women and babies who had umbilical cord *RXRA* pyrosequencing analysis and had delivered a liveborn infant, were included in the analysis. All outcomes were assessed for normality using visual inspection of histograms. Percentage DNA methylation at all *RXRA* CpGs analysed was normally distributed, except at CpGs 1 and 3. Characteristics of the women in the two treatment arms were compared using t-tests, Mann-Whitney U and χ^2 tests for normally distributed, non-normally distributed and categorical variables, respectively. Characteristics of the MAVIDOS babies (boys versus girls) for whom neonatal DXA and *RXRA* methylation data were available were also compared. Neonatal DXA indices were whole-body bone area (BA), bone mineral content (BMC), and bone mineral density (BMD). Continuous child characteristics were summarised using mean (SD) or median (interquartile range [IQR]), depending on normality. Categorical variables were summarised using percentages. Differences in continuous variables between boys and girls were tested using t-tests and Mann-Whitney U-tests where appropriate. *RXRA* methylation was Fisher-Yates transformed to standard deviations. Separate linear regression analyses were carried out to analyse the difference in methylation between the treatment groups. The interaction between treatment group, *RXRA* methylation and season of birth was analysed, due to previously described seasonal variations in 25(OH)D concentrations reported in many previous studies. To

ensure adequate sample sizes, we defined season of birth as a binary variable using the UK Meteorological Office classification, combining winter (December to February) with spring (March to May), to give an overall "winter/spring" variable (December-May), and summer (June to August), with autumn (September to November), to give an overall "summer/autumn" variable (June-November). To explore associations between *RXRA* methylation and bone outcomes, linear regression analyses were carried out, adjusted for treatment group and sex.

Based on previous findings detailed in Chapter 4, it was likely that there would be co-linearity between the individual exposures and outcomes (443, 506) so, in keeping with the previous *CDKN2A* analyses, a data reduction approach was undertaken by investigating clustering of the CpG methylation (480, 506, 508). This approach was considered appropriate given the relatively small number of tests in the analysis, compared with larger scale genome-wide associations studies, for which methods such as Bonferroni or the Benjamini-Hochberg/ False Discovery Rate corrections for multiple testing would be appropriate (480).

Previous studies have shown that where clusters of differential CpGs can be identified, they are more likely to be of functional relevance than individual CpG changes. The correlations between methylation at each of the individual CpG sites was tested (Table 5.2) and this was used to calculate the median absolute deviation (MAD) from the median for each site. Then the CpG sites were grouped into 3 clusters (CpGs 1-5, 8-11, 12), with each cluster represented by the site with the highest MAD score (i.e. the site with the greatest variability within the cluster), that is, CpG sites 5, 11, and 12 respectively. Further biological support for this clustering was provided by exploration of the ENCODE data (509), demonstrating distinct DNase I hypersensitivity sites at either end of the differentially methylated region, and discrete grouping of transcription factor binding. For completeness, the Simes modification of the Bonferroni method was used to undertake a p-value correction on the analyses, using the Stata "qqvalue" command (508). These are presented as q-values in the relevant results tables. All analyses were performed in Stata v14 (Statacorp, College Station, Texas, USA). A p-value of < 0.05 was considered statistically significant.

Table 5.2 CpG Clustering. Pearson's / Spearman's correlation of methylation levels at CpGs 1-9 within the *RXRA* region.

3 distinct clusters are defined (dark green R ≥ 0.90, pale green R ≥ 0.80, yellow R ≥ 0.70, orange R ≥ 0.60, red R ≥ 0.50).

	RXRA CpG 1†	RXRA CpG 2	RXRA CpG 3†	RXRA CpG 4	RXRA CpG 5	RXRA CpG 8	RXRA CpG 9	RXRA CpG 10	RXRA CpG 11
RXRA CpG 1†	1								
RXRA CpG 2	0.89	1							
RXRA CpG 3†	0.93	0.92	1						
RXRA CpG 4	0.91	0.94	0.91	1					
RXRA CpG 5	0.90	0.90	0.89	0.93	1				
RXRA CpG 8	0.69	0.76	0.70	0.76	0.75	1			
RXRA CpG 9	0.78	0.77	0.77	0.78	0.78	0.77	1		
RXRA CpG 10	0.78	0.79	0.77	0.81	0.80	0.86	0.88	1	
RXRA CpG 11	0.79	0.79	0.78	0.82	0.81	0.82	0.86	0.92	1
RXRA CpG 12	0.57	0.59	0.54	0.63	0.61	0.81	0.58	0.71	0.69

† Spearman correlation used for CpG1 and CpG3

Table 5.3 Median absolute deviation (MAD) scores within the 3 clusters of RXRA CpGs.

Cluster	CpG site	MAD score
CpG 1-5	1	5.26
	2	4.92
	3	5.35
	4	5.03
	5	**5.41**
CpG 8-11	8	4.16
	9	4.58
	10	4.25
	11	**4.66**
CpG 12	**12**	**3.98**

5.3 Results

5.3.1 Characteristics of participants

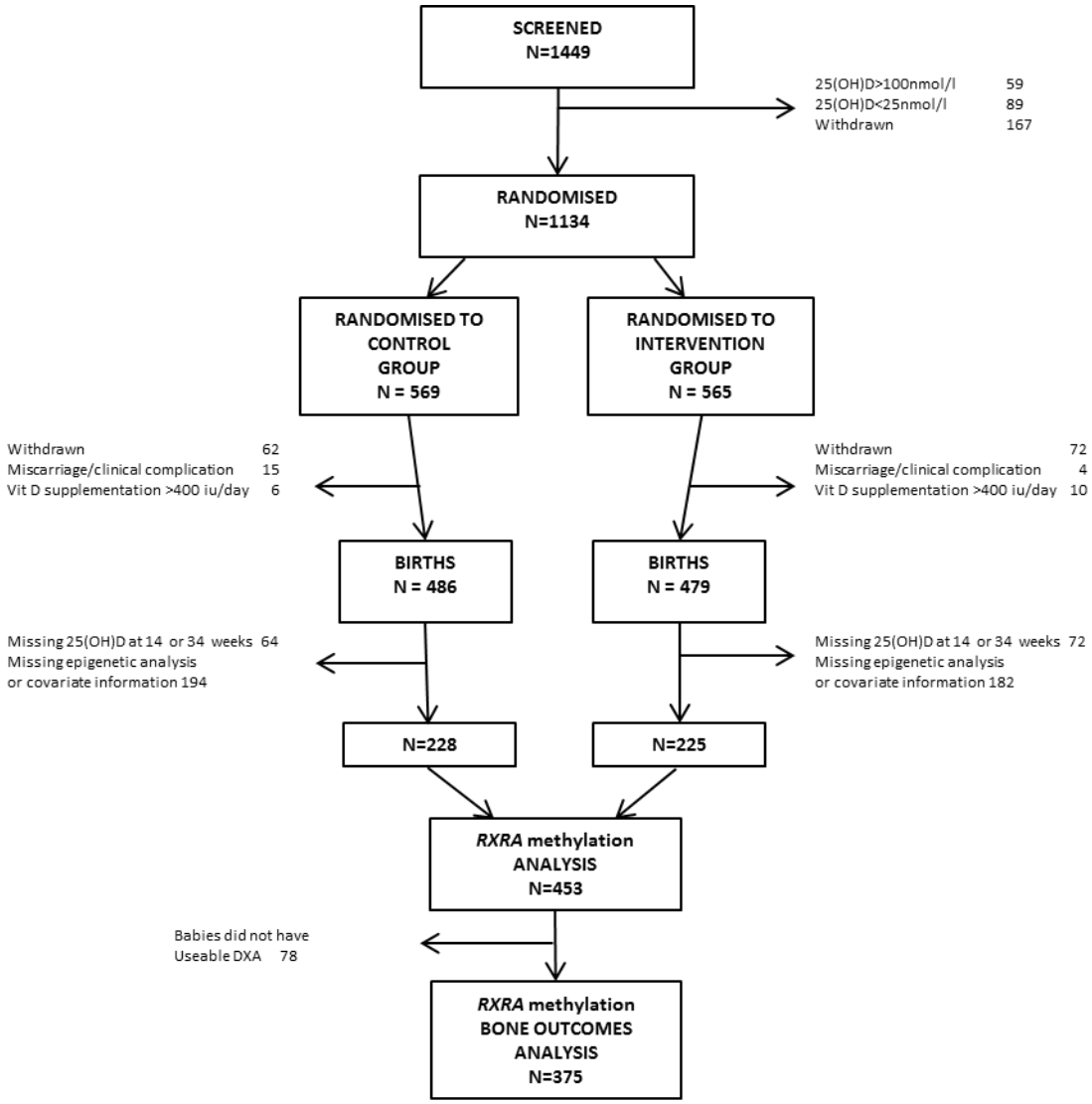


Figure 5.2 MAVIDOS Trial Consort Diagram with numbers used in *RXRA* analyses.

965 women (85%) remained in the study until delivery as shown in Figure 5.2 (377). There were 486 live births in the control group, and 479 in the cholecalciferol group, of which 228 and 225 umbilical cords respectively underwent pyrosequencing of the *RXRA* region of interest. 78 babies for whom pyrosequencing results were available did not have a useable DXA scan, leaving a remaining 375 babies with *RXRA* methylation analysis, DXA outcomes and the relevant maternal information. Of the 453 women included in the initial analysis, the mean (SD) age at delivery was 30.9 (5.2) years in the placebo group and 30.7 (5.1) years in the cholecalciferol supplemented group. Baseline characteristics of women in the placebo and cholecalciferol groups at

randomisation were similar, as shown in Table 5.4. 25(OH)D in late pregnancy (34 weeks) was significantly higher in the cholecalciferol supplemented group (p difference < 0.001).

Table 5.4 Baseline characteristics of the randomly assigned pregnant women included in the analysis. *Values are n (%), mean (SD) or median (IQR) †.*

	n	*Placebo (n = 228)*	*Cholecalciferol 1000 IU/day (n = 225)*	*P difference*
mean (SD) or median (IQR)				
Age (years)	427	30.9 (5.2)	30.7 (5.1)	0.767
Height (cm)	423	166.4 (6.3)	165.3 (6.1)	0.085
Weight (kg)	427	73.6 (13.1)	71.6 (14.1)	0.127
Pregnancy weight gain (kg)	415	9.4 (3.6)	9.7 (3.5)	0.410
BMI, (kg/m^2) †	423	25.7 (23.0,29.7)	24.9 (22.4,28.8)	0.145
Sum of skinfold thickness (mm)	360	81.9 (27.0)	78.3 (29.1)	0.223
25(OH)D at 14 weeks, (nmol/l)	445	45.1 (16.2)	44.4 (15.2)	0.630
25(OH)D at 34 weeks, (nmol/l)	432	42.8 (20.0)	66.3 (19.8)	**< 0.001**
n(%)				
Nulliparous	427	99 (46.3)	91 (42.7)	0.462
Educational qualification > A level	423	156 (74.3)	163 (76.5)	0.593
Current smoker	426	16 (7.5)	12 (5.7)	0.449
Strenuous exercise ≥ once a week	390	22 (11.3)	32 (16.4)	0.143

Neonatal DXA whole body less head bone measurements were available for 202 boys (91 born to mothers randomised to placebo, 111 to cholecalciferol) and 173 girls (94 born to mothers randomised to placebo, 79 to cholecalciferol) (Table 5.5). A Chi-Squared test demonstrated no difference in sex distribution or gestational age between the boys and girls. As would be expected, boys had a greater average bone mineral content (BMC, g) and bone area (BA, cm^2) than girls. In this subset of the MAVIDOS trial population, no differences in gestational age or whole body minus head DXA outcomes were observed in the babies by maternal randomisation group to placebo or 1000 IU cholecalciferol.

Table 5.5 DXA characteristics of the MAVIDOS babies for whom DXA and *RXRA* methylation data are available.

Characteristics by sex and by maternal randomisation group are displayed. BA: bone area; BMC: bone mineral content; aBMD: areal bone mineral density.

	Boys (n =202)	*Girls (n = 173)*	*p difference between boys and girls*
Gestational age (weeks)	40.2 (1.3)	40.1 (1.4)	0.22
BA (cm^2)	306.5 (34.8)	295.7 (33.8)	**0.003**
BMC (g)	63.1 (10.7)	59.9 (10.5)	**0.004**
aBMD (g/cm^2)	0.205 (0.018)	0.202 (0.020)	0.100
	Placebo (n = 185)	*Cholecalciferol 1000 IU/day (n = 190)*	*p difference between maternal randomisation group*
Gestational age (weeks)	40.1 (1.4)	40.2 (1.2)	0.23
BA (cm^2)	299.4 (36.7)	303.6 (32.6)	0.24
BMC (g)	61.2 (10.9)	62.0 (10.6)	0.46
aBMD (g/cm^2)	0.204 (0.019)	0.203 (0.019)	0.93

5.3.2 Cholecalciferol supplementation and *RXRA* methylation

Percentage methylation at the RXRA Differentially Methylated Region (DMR) varied greatly across the ten CpG sites measured, for example ranging from 29.0% to 81.4% at CpG 5 (mean 47.7%, SD 9.0%) (Table 5.1). However, percentage methylation tended to be lower in the cholecalciferol supplemented group than in the placebo group (Figure 5.3 and Table 5.6). At CpG 5 (representing CpGs1-5), mean (SD) percentage methylation was 46.7 (8.2)% in the cholecalciferol group and 48.7 (9.7)% in the placebo group, difference -1.98% percentage points, p = 0.02. Whilst percentage methylation at both CpGs 11 (representing CpGs 8-11) and 12 was lower in cholecalciferol than placebo group births, these differences were not statistically significant.

No consistent associations between maternal 25(OH)D status at 34 weeks, or change in 25(OH)D from early to late pregnancy, and RXRA methylation in umbilical cord tissue were found, when combining cholecalciferol and placebo groups. However, there was evidence of an interaction

between change in 25(OH)D between 14 and 34 weeks gestation 25(OH)D and treatment allocation to cholecalciferol or placebo on RXRA methylation at CpG 5 (p = 0.022).

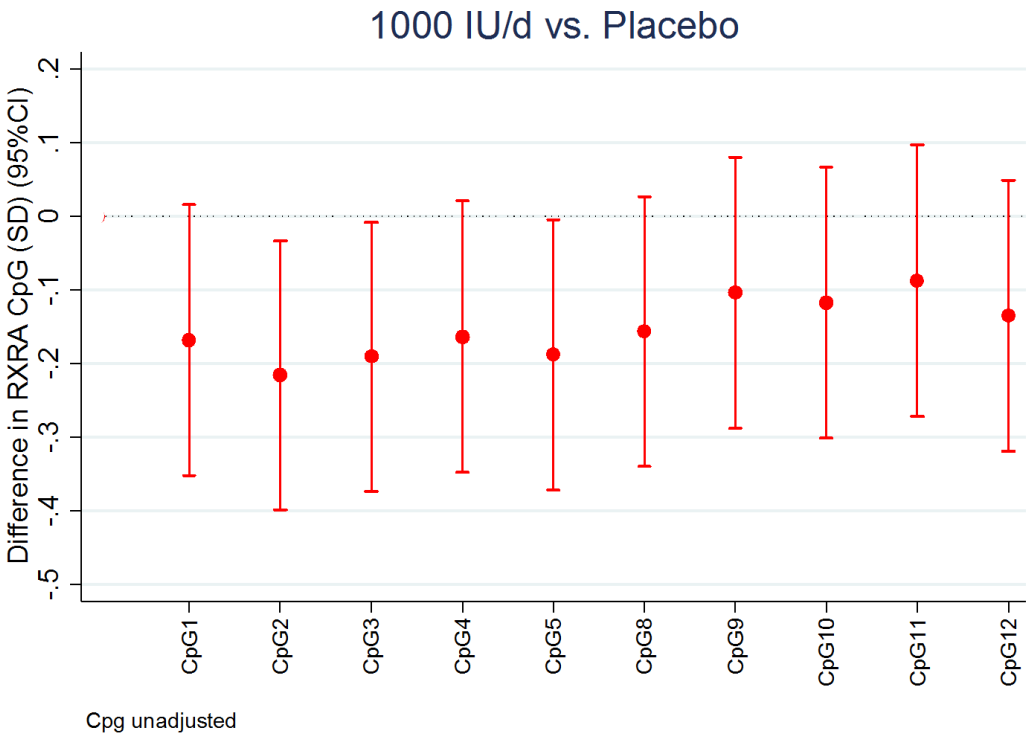


Figure 5.3 Difference in RXRA methylation at each CpG site between Cholecalciferol 1000 IU/day supplemented group and placebo group (expressed as Standard Deviations).

Each bar is the outcome of a separate linear regression (mean difference and 95%CI).

Table 5.6 *RXRA* DNA methylation in cholecalciferol 1000 IU/day supplemented[a] and placebo[b] groups. CpG 5 represents CpGs 1-5; CpG 11 represents CpGs 8-11. p-values < 0.05 are in bold. q-values were obtained using the Simes method. *CpG sites are shown clustered (top) and all CpGs (bottom).*

CpG	*n*	*% methylation Cholecalciferol 1000 IU/day[a]*	*% methylation Placebo[b]*	*Mean difference % methylation [(a-b)]*	*95%CI*	*p difference*	*q difference*
Methylation results from clustered RXRA CpGs only							
RXRA CpG 5	447	46.7 (8.2)	48.7 (9.7)	**-1.98**	**-3.65, -0.32**	**0.02**	**0.06**
RXRA CpG 11	446	58.3 (7.5)	58.9 (8.1)	-0.67	-2.12, 0.78	0.36	0.36
RXRA CpG 12	446	66.1 (5.8)	66.9 (6.6)	-0.84	-1.99, 0.31	0.15	0.23
Methylation results from all RXRA CpG sites							
RXRA CpG 1†	446	31.4 (27.1,37.8)	32.3 (27.9,40.6)	-0.17	-0.35, 0.02	0.1	0.183
RXRA CpG 2	449	54.6 (7.5)	56.5 (8.4)	**-1.86**	**-3.34, -0.37**	**0.01**	0.100
RXRA CpG 3†	450	33.9 (29.7,40.7)	35.8 (31.0,42.5)	**-0.19**	**-0.37, -0.007**	**0.04**	0.100
RXRA CpG 4	444	49.8 (8.0)	51.5 (9.3)	**-1.70**	**-3.32, -0.08**	**0.04**	0.100
RXRA CpG 5	447	46.7 (8.2)	48.7 (9.7)	**-1.98**	**-3.65, -0.32**	**0.02**	0.100
RXRA CpG 8	449	62.7 (5.9)	63.6 (6.5)	-0.92	-2.07, 0.22	0.11	0.183
RXRA CpG 9	448	51.1 (7.5)	52.0 (8.5)	-0.95	-2.44, 0.53	0.21	0.233
RXRA CpG 10	447	65.1 (6.8)	66.0 (7.5)	-0.88	-2.20, 0.45	0.19	0.233
RXRA CpG 11	446	58.3 (7.5)	58.9 (8.1)	-0.67	-2.12, 0.78	0.36	0.360
RXRA CpG 12	446	66.1 (5.8)	66.9 (6.6)	-0.84	-1.99, 0.31	0.15	0.214

5.3.3 Interactions between season of birth, treatment group and DNA methylation at *RXRA*

Greater increases in maternal 25(OH)D status were seen in summer (June-November) than winter (December-May) births, with the increase in 25(OH)D during pregnancy being more than double in the women giving birth in summer. In summer births, mean (SD) change in 25(OH)D between 14 and 34 weeks gestation was 8.1 (16.0) nmol/l in the placebo group (n = 124), and 28.0 (19.8) nmol/l in the vitamin D supplemented group (n = 127). In winter births, mean (SD) change in 25(OH)D between 14 and 34 weeks gestation was -15.0 (17.6) nmol/l in the placebo group (n = 103), and 13.6 (22.6) nmol/l in the vitamin D supplemented group (n = 95). There was evidence of statistically significant interactions for the outcome of *RXRA* methylation between treatment allocation (cholecalciferol vs placebo) and season of birth (at all three representative CpGs: CpG 5, p = 0.02; CpG 11, p = 0.009; and CpG 12 p = 0.01). The effect of treatment group on *RXRA* methylation appeared greater in summer than winter births, as shown in Table 5.7. In summer births there was a difference in percentage methylation at *RXRA* CpG 5, 11 and 12 between treatment groups ranging from -3.69% at CpG 5 (p = 0.001), -2.38% at CpG 11 (p = 0.02), and -2.13% at CpG 12 (p = 0.005), which persisted after adjustment for multiple testing. The differences between groups were non-significant for winter births. This interaction persisted after adjustment for potential differences in maternal characteristics between the season groups (maternal BMI and skinfold thickness), and for other factors known to influence methylation (offspring sex and maternal smoking).

Table 5.7 RXRA DNA methylation in cholecalciferol 1000 IU/day supplemented and placebo groups, stratified by season - winter births (December-May) and summer births (June-November).
To assess differences in % methylation between two groups, t-test used (variables normally distributed). p-values < 0.05 are in bold. q-values were obtained using the Simes method.

CpG	*Winter births (Dec-May)*				*Summer births (June-Nov)*			
	Mean diff.% methylation	95%CI	p	q	Mean diff.% methylation	95%CI	P	q
RXRA CpG 5	0.27	(-2.27,2.82)	0.83	0.83	**-3.69**	**(-5.92,-1.45)**	**0.001**	**0.004**
RXRA CpG 11	1.51	(-0.73,3.76)	0.18	0.55	**-2.38**	**(-4.29,-0.47)**	**0.02**	**0.01**
RXRA CpG 12	0.79	(-1.00,2.58)	0.38	0.58	**-2.13**	**(-3.60,-0.65)**	**0.005**	**0.007**

5.3.4 Associations between *RXRA* methylation and neonatal bone indices

In the population as a whole, there were modest positive associations between *RXRA* methylation at CpG 5 and offspring BA, BMC and aBMD (Table 5.8). However, on stratification according to treatment allocation, associations were noted in the placebo but not the cholecalciferol supplemented groups, as documented in Table 5.9 and Figure 5.4. In the placebo group (red bars), *RXRA* methylation at CpG 11 was positively associated with BA (β = 6.96 cm^2 per 10% increase in methylation, p = 0.05). There was also a tendency towards positive associations between methylation and BA at CpG 5 and CpG 12. Furthermore, again in the placebo group, methylation at CpGs 5 and 11 was positively associated with offspring BMC (at CpG 5, β = 1.75 g per 10% increase in methylation, p = 0.03; at CpG 11, β = 2.34 g per 10% increase in methylation, p = 0.02). Conversely, in the cholecalciferol supplemented group (blue bars), no statistically significant associations were found between methylation at CpGs 5, 11 and 12 and offspring neonatal DXA bone outcomes.

Table 5.8 Relationships between perinatal methylation in umbilical cord at CpG sites within the RXRA region of interest and bone outcomes at birth (measured by DXA, WBLH), placebo and 1000 IU / day cholecalciferol supplemented groups combined. *Associations are adjusted for sex. β coefficients and 95% CIs have been multiplied by 10 and therefore represent the change associated with a 10% increase in methylation. p-values < 0.05 are in bold.*

RXRA CpG	*WBLH BA, (cm^2)*		*WBLH BMC, (g)*		*WBLH aBMD, (g/cm^2)*	
	β (95% CI)	**p**	**β (95% CI)**	**p**	**β (95% CI)**	**p**
CpG 5	**4.18 (0.16, 8.20)**	**0.04**	**1.50 (0.27, 2.74)**	**0.02**	**0.002 (0.000, 0.004)**	**0.05**
CpG 11	2.77 (-1.93, 7.48)	0.25	0.84 (-0.61, 2.30)	0.26	0.001 (-0.002, 0.004)	0.47
CpG 12	2.85 (-3.19, 8.88)	0.35	0.62 (-1.25, 2.49)	0.52	0.000 (-0.003, 0.003)	0.91

Table 5.9 Relationships between perinatal methylation in umbilical cord at CpG sites within the RXRA region of interest and bone outcomes at birth (WBLH bone area (BA), bone mineral content (BMC) and areal bone mineral density (aBMD)), stratified by placebo and 1000 IU / day cholecalciferol supplemented groups. *Associations are adjusted for sex. β coefficients and 95% CIs have been multiplied by 10 and therefore represent the change associated with a 10% increase in methylation. p-values < 0.05 are in bold.*

	WBLH BA, (cm²)				*WBLH BMC, (g)*				*WBLH aBMD, (g/cm²)*			
	Placebo		*1000 IU/d*		*Placebo*		*1000 IU/d*		*Placebo*		*1000 IU/d*	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
CpG 5	5.22 (-0.18, 10.62)	0.06	2.48(-3.71,8.67)	0.43	**1.75 (0.17, 3.33)**	**0.03**	1.10(-0.91, 3.10)	0.28	0.00(-0.00, 0.01)	0.10	0.00(-0.00, 0.01)	0.26
CpG 11	**6.96 (0.09, 13.82)**	**0.05**	-1.66(-8.10, 4.77)	0.61	**2.34 (0.32, 4.37)**	**0.02**	-0.76(-2.85, 1.34)	0.48	0.00(-0.00, 0.01)	0.08	-0.00(-0.01, 0.00)	0.47
CpG 12	7.76 (-0.77, 16.28)	0.07	-3.16(-11.75, 5.43)	0.47	2.05(-0.47, 4.57)	0.11	-1.13(-3.92, 1.65)	0.42	0.00(-0.00, 0.01)	0.46	-0.00(-0.01, 0.00)	0.52

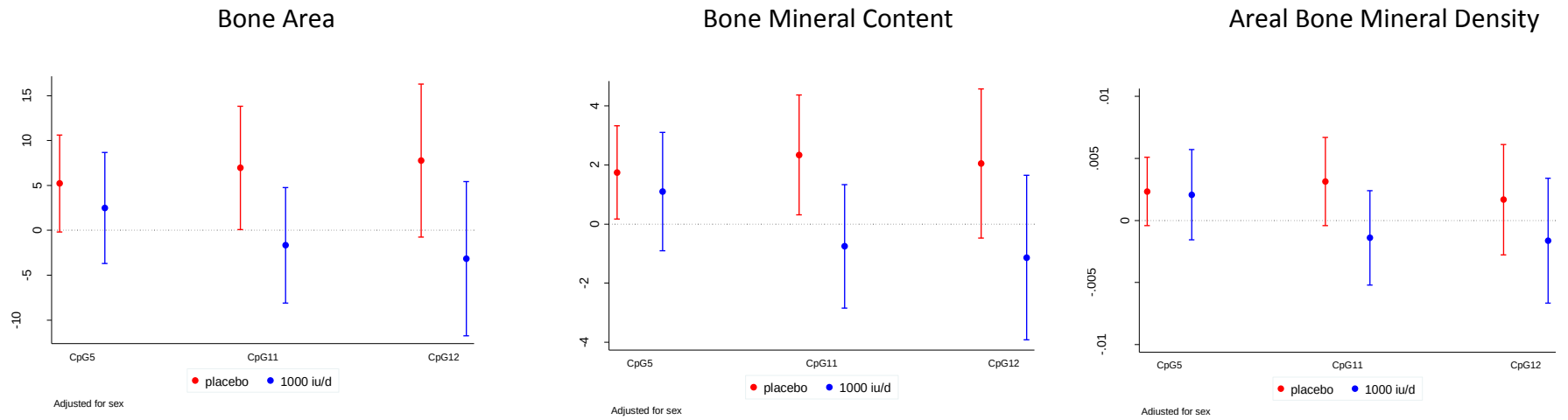


Figure 5.4 Associations between *RXRA* methylation at CpG5, CpG 11 and CpG 12 and bone area (cm^2), bone mineral content (g), bone mineral density (g/cm^2), adjusted for sex, by treatment group (placebo [red bars] or 1000 IU cholecalciferol [blue bars] daily). Outcomes expressed per 10% increase in methylation. In the vitamin D supplemented group, no significant associations were observed between RXRA methylation and bone indices at the three CpGs of interest. In the placebo group, positive associations were observed between methylation at CpG 5 and BMC, and CpG 11 and BA and BA in addition to BMC.

5.3.5 ENCODE functional analysis

The differentially methylated region resides within the upstream CpG island shore region (within 2kb) of the *RXRA* 5'CpG Island. Encyclopedia of DNA elements (ENCODE) consortium data were interrogated for evidence of function within this location (509). This region of interest within the *RXRA* locus contains a cluster of DNase I hypersensitive sites (DHS), identified in 84 cell lines out of 125 (ENCODE V3). DNase I hypersensitive regions are a general regulatory marker, often found within regulatory elements such as promoters and enhancers, (510) as they represent open chromatin structure susceptible to cleavage by the DNase I enzyme. These loci, therefore, enable transcription factor and RNA polymerase binding, and as RXRA is a partner for many nuclear hormone receptors, it is perhaps unsurprising that a DHS is found in the region. An overview of DNase I hypersensitivity clusters is given in Figure 5.5), with examples from the chorion and osteoblast cell lines highlighted. It may be of relevance that the osteoblast DNase I hypersensitivity clusters form two distinct groups covering either end of the *RXRA* DMR, indicating potential differences in function at either end of the region.

Furthermore, significant enrichment of the enhancer-related H3K4me1 histone mark across a range of tissue types was found both across and within 250bp of the DMR. Enhancer loci may show dynamic DNA methylation indicative of transcription factor interaction within these functional regions (511). Consistent with this, genome segmentations from ENCODE which display chromatin state segmentations from 6 cell lines, predict weak enhancer activity or an open chromatin *cis* regulatory element at the RXRA DMR (yellow region, bottom row, in Figure 5.5).

Finally, ENCODE transcription factor (TF) binding data demonstrate significant binding within the *RXRA* DMR. The numbers of transcription factor binding sites found at the RXRA DMR in the ENCODE database vary between cell types, for example in the cell line, MCF-7, highly responsive to oestrogen and TSH (512), three transcription factors bind with high affinity at the *RXRA* DMR (MYC, CTCF and POL2RA). In summary, these findings suggest the *RXRA* DMR is a region of significant functional activity across a range of cell types, with evidence of strong DNAse I hypersensitivity sites, weak enhancer or cis regulatory element activity, and transcription factor binding.

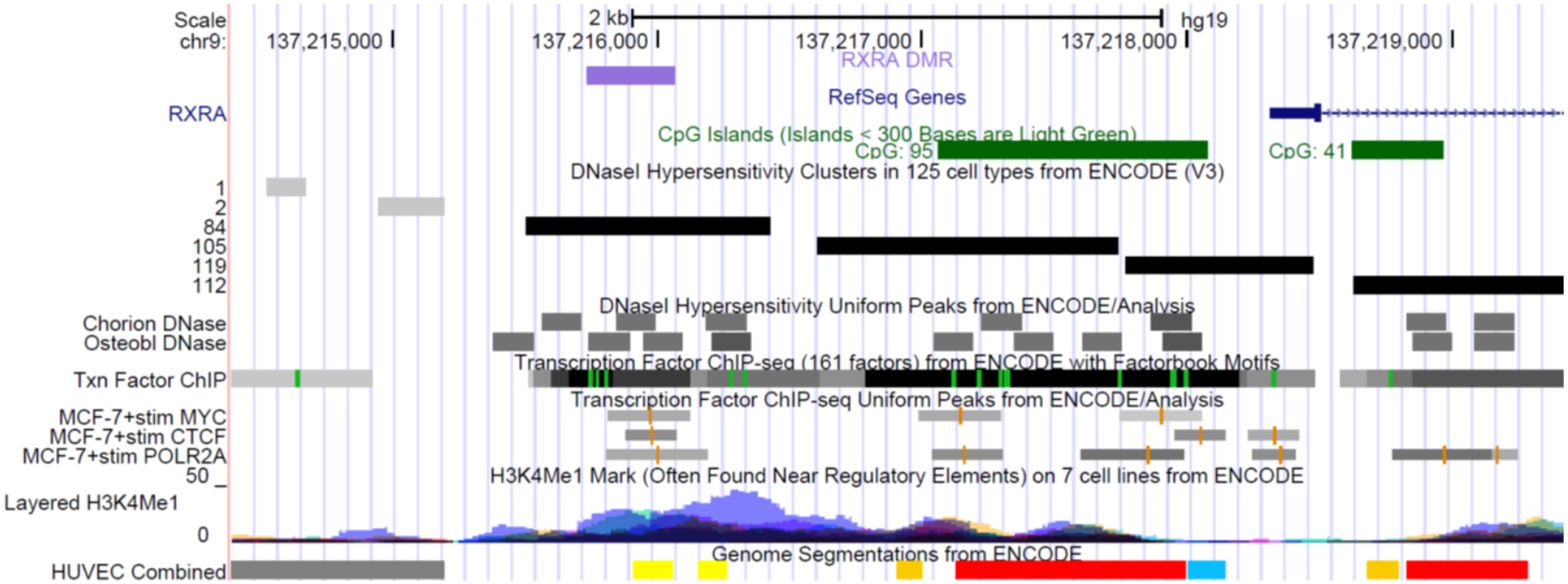


Figure 5.5 UCSC- Human Genome Reference Sequence annotation for the RXRA region on chr9, Feb. 2009 (GRCh37/hg19) Assembly.

The RXRA DMR is annotated in purple. DNase1 hypersensitivity (HS) clusters are displayed across 125 cell types, with examples of DNase1 HS peaks demonstrated in chorion and osteoblast cells. Enrichment of Transcription Factor binding sites (Transcription Factor CHIP-seq (161 factors) are outlined, with an example of peaks overlying the RXRA DMR in MCF-7 cells. Enhancer related mono-methylation of lysine 4 of the H3 histone protein (H3K4me1) are demonstrated. Genome segmentations from ENCODE across a variety of cells demonstrate the following predicted functional regions: in red, promoter regions; orange, enhancers; yellow, weak enhancers or open chromatin cis regulatory elements; blue, CTCF transcriptional repressor enriched element;grey, predicted repressed regions. A yellow weak enhancer region is shown in the region of the RXRA DMR.

5.4 Discussion

This study demonstrated, in a randomised controlled trial setting, that supplementation with cholecalciferol in pregnancy is associated with altered methylation at specific regions near to the *RXRA* promoter in fetal DNA derived from the umbilical cord of the offspring. Percentage methylation levels measured by pyrosequencing were lower in the cholecalciferol supplemented group than the placebo group (statistically significantly at the cluster of CpG sites represented by CpG 5), raising the possibility of site-specificity for a molecular interaction between 25(OH)D in pregnancy and DNA methylation (513).

These results are consistent with previous observational findings in the Southampton Women's Survey, in which a negative association was found between an estimate of maternal free 25(OH)-vitamin D and *RXRA* methylation at CpG 4/5, (443) measured using the Sequenom MassARRAY EPITYPER. Additionally, the associations between *RXRA* methylation and neonatal bone indices in the placebo group replicated those observed previously in the SWS; conversely, in the present study, the direction of the association appeared to be reversed (albeit not reaching statistical significance) in the group whose mothers were supplemented with cholecalciferol. It is interesting that the methylation difference between treatment and placebo groups in the present study was greater in summer than winter births. In the supplemented group, the increase in 25(OH)D from baseline to 34 weeks was markedly higher for summer than winter deliveries, although the absolute difference between groups at 34 weeks was less in summer than winter (as shown in Figure 1.21). This suggests that greater increases in 25(OH)D across pregnancy might facilitate methylation differences consequent to vitamin D supplementation. However, given that *RXRA* interacts with several different nuclear hormone receptors, such as thyroid hormone receptor and PPAR-gamma, activation of which tends to have detrimental effects on bone, it is possible that we are seeing the net result of a complex series of inter-relationships at this molecular level, with exogenous vitamin D perhaps modifying the balance in RXRA interaction between receptor types, resulting in heterogeneous associations between *RXRA* methylation and bone indices. Such considerations may also be relevant to the seasonal differences we observed, although ultimately these questions must remain the focus of future research.

Interestingly, we observed no consistent associations between maternal serum 25(OH)D in late pregnancy and umbilical cord *RXRA* methylation, but these measures were in different tissues, 6 weeks apart. In studies of placental villous fragments, cultured with and without 20μM 25(OH)D, in which an Illumina EPIC 850k methylation array and RNA sequencing was performed, *RXRA*

methylation was lowered. *RXRA* expression was shown to be upregulated in those treated with 25(OH)D, which suggests a specific role for the vitamin D-*RXRA* interaction (514).

Although the exact nature of the mechanistic underpinnings of our findings remains to be elucidated, there are several routes by which maternal 25(OH)D status might influence perinatal *RXRA* methylation. As previously stated, RXRA forms a heterodimer with several nuclear hormones known to influence bone metabolism, including 1,25$(OH)_2$-vitamin D, perhaps implying that maternal 25(OH)D status plays a permissive role in the transcriptional regulation of the *RXRA* gene. Studies have shown that vitamin D may interact with the epigenome on multiple levels, (439, 444, 509, 515, 516) and our evaluation of public data from ENCODE suggest that methylation at the studied CpG sites is likely to have functional relevance with evidence for DNase I hypersensitive regions, enhancer activity and transcription factor binding. Furthermore, ENCODE data suggested function within the DMR, which itself resides within the shore region of the 5' CpG island; this location has been associated with influences on gene expression (517).

Epigenome-wide association studies (EWAS) have also provided some insight into the actions of vitamin D on DNA methylation. A small EWAS of DNA methylation in severely vitamin D deficient African-American adolescents demonstrated associations between vitamin D status and methylation in several genes, including genes involved in vitamin D metabolism such as the 24 and 25-hydroxylase genes. In the context of low serum vitamin D levels, the promoter of *CYP2R1* may become methylated, and this is reversible on exposure to vitamin D (445). Other studies have assessed the DNA methylation in CYP enzymes which are part of the vitamin D metabolism pathway, and found a relationship between methylation of the genes *CYP2R1* (25-hydroxylase) and *CYP24A1* (24-hydroxylase) and variations in circulating 25(OH)D levels (440). However, a study using the ALSPAC cohort and the Norwegian Mother and Child Cohort (MoBa) in which maternal 25(OH)D was measured in mid-pregnancy, demonstrated no convincing associations between maternal 25(OH)D status and DNA methylation in the umbilical cord blood (as opposed to umbilical cord tissue in our study) of 1,416 newborn babies using Illumina 450k DNA methylation array analysis, thereby covering 473,731 CpG DNA methylation sites (446). The authors suggested that to further identify associations, larger consortium studies, expanded genomic coverage, and investigation of alternative cell types or 25(OH)D status at different gestational time points would be needed.

5.4.1 Limitations

The data presented are from a placebo-controlled, double-blind, randomised trial, using the gold standard of pyrosequencing to determine CpG site-specific DNA methylation and DXA to assess bone mass. However, the limitations of our study must be considered and are discussed in detail in section 8.3. To briefly summarise, the limitations of the use of pyrosequencing are those of tissue specificity (methylation was measured in cells from whole umbilical cord, comprising different component cells, e.g. fibroblasts and epithelial cells). It is possible that the differential methylation we observed arose from different component cells in individual samples, so differences in DNA methylation between treatment and control groups may thus partly reflect different proportions of cells and their cell-specific DNA methylation. However, any unaccounted cell-heterogeneity may represent proportional differences that are related to the observed phenotypic outcomes (518, 519) and so could potentially be on the causal pathway. Secondly, though we have previously excluded the presence of any SNPs at the CpG sites of interest at the *RXRA* locus by sequencing, we did not have information permitting exclusion of a genetic *cis* or *trans*-effect of local or distant SNPs, respectively. These could influence either associations between vitamin D supplementation and *RXRA* methylation, or influence both *RXRA* methylation and the child's bone phenotype.

Third, owing to stipulations made during the ethics approval process, participants with baseline 25(OH)D concentrations less than 25 nmol/l or greater than 100 nmol/l could not be included. The supplement dose of 1000 IU was modest in comparison with a variety of other trials of cholecalciferol supplementation in pregnancy. In addition, the study population did not include many women who were of non-white ethnicity, who are more likely to have low 25(OH)D concentrations; this affects the generalisability of our findings to a multi-ethnic populations. Fourth, some participants were taking vitamin D supplements in addition to the study drug, though supplement use was recorded at interview and did not differ between the treatment groups. Fifth, we did not have measurements of 25(OH)D in umbilical cord blood and thus were not able to directly assess a potential mediating role for 25(OH)D for *RXRA* methylation in the same organ at the same time. Sixth, DXA assessment in neonates presents some difficulties, both due to the low absolute BMC of newborn babies and their tendency to move. However, the validity of DXA in small animals, of comparable size to neonates, has been documented (520) and appropriate DXA software was used. Finally, it should be noted that the analysis is *post hoc* and that methylation outcomes were not pre-specified in the original analysis plan, and so will require replication in further intervention studies.

5.4.2 Conclusion

These findings show, in a randomised controlled trial, that maternal supplementation with cholecalciferol from 14 weeks gestation to delivery leads to lower levels of DNA methylation at the *RXRA* promoter in umbilical cord. This informs our understanding of early life mechanisms underpinning maternal vitamin D status, epigenetic change and bone development, and may suggest a novel biomarker for a child's future bone health.

Chapter 6: Maternal urine C-terminal telopeptide of type I collagen (CTX) in pregnancy: descriptive characteristics, influence of cholecalciferol supplementation and associations with maternal bone health

6.1 Background and aims

The human fetal skeleton begins to mineralise between the 8th and 12th weeks of pregnancy when primary ossification centres form in the vertebrae and the long bones (76). Around 30 g of calcium are required in total for its development, 80% of which is accrued in the third trimester of pregnancy. Maternal calcium homeostasis adapts to meet the calcium demands of the developing fetus (76, 108, 110). The relative contribution of the maternal skeleton to offspring calcium homeostasis and bone development remains unclear (112-114). Biochemical markers of bone turnover offer a non-invasive method of monitoring changes in bone resorption or formation during pregnancy (115) and provide some insight into the impact of pregnancy on maternal bone.

There are two groups of bone turnover markers, markers of bone formation (such as N-terminal collagen extension propeptide (P1NP), osteocalcin, bone alkaline phosphatase) and markers of resorption (such as C-terminal cross-linking telopeptide of type 1 collagen (CTX), N-terminal telopeptide of type 1 collagen (NTX), deoxypyridinoline, hydroxyproline) as described in section 1.4.9. Bone turnover markers are poorly characterised in pregnancy, but in small studies, both formation and resorption markers have been shown to be low in the first trimester but to increase steadily thereafter. Markers of bone resorption reach as much as twice normal in the third trimester, and markers of bone formation are low in early pregnancy and reaching normal values by term, such that there is a net resorptive state in late pregnancy (135-142). This is supported by studies of maternal bone mass throughout pregnancy and postpartum, showing that BMD declines by a small amount (less than 5%), particularly at trabecular sites (151).

An interaction between maternal vitamin D status, calcium intake, and markers of bone resorption has been observed (131), and there is some evidence that maternal vitamin D status and calcium intake are negatively associated with markers of bone resorption in pregnancy (113, 130).

C-terminal telopeptide of type I collagen (CTX), a marker of bone resorption, was measured in maternal urine in early and late pregnancy in the MAVIDOS trial. It therefore offered an ideal

opportunity to investigate changes in maternal urinary CTX concentrations in pregnancy, and the effect of gestational vitamin D supplementation on them. As mothers underwent DXA scanning shortly after birth, we were able to analyse associations between CTX and maternal postnatal bone indices.

6.2 Methods

The design of the MAVIDOS trial, a randomised controlled trial of vitamin D supplementation in pregnancy, has been described in chapter 3.

Prior to commencing the study medication at 14 weeks, and again at 34 weeks' gestation, the women attended the research centre for a detailed assessment of diet (including supplement use), lifestyle (smoking, physical activity participation, employment) and health (past medical history, current medication use) using interviewer-led questionnaires. Ethnicity was self-reported by the participant as "White, Black Caribbean, Black African, Black Other, Indian, Pakistani, Bangladeshi, Chinese, Other Asian group or Other (specify)". This was then categorized as White or non-White.

Anthropometric measurements included height, measured to the nearest 0.1 cm using a stadiometer, and weight, assessed to the nearest 0.1 kg using calibrated electronic scales.

To briefly summarise serum maternal 25(OH)D measurement, on the day that the study medication was dispensed (around 14 weeks) and at 34 weeks' gestation, a non-fasted venous blood sample was obtained, and serum stored at -80°C. 25(OH)D was assessed by radioimmunoassay (Liaison RIA automated platform, Diasorin, Minnesota, USA). All samples were analysed in a single batch at the end of the study at MRC Human Nutrition Research, Cambridge, UK.

Maternal second void urine was collected at the same time points (on the day of medication dispensing and at 34 weeks). Urinary CTX was measured using an ELISA measuring both α-CTX and β-CTX (Immunodiagnostic Systems, Boldon, UK) by Fatma Gossiel under the supervision of Professor Richard Eastell at the Academic Unit of Bone Metabolism, University of Sheffield. CTX was expressed as a ratio to urine creatinine (μg/mmol). The assay has an inter-assay coefficient of variation of 6% (521).

Maternal bone indices were assessed by DXA (Hologic Discovery, Hologic Inc., Bedford, MA, USA, or GE-Lunar iDXA, GE-Lunar, Madison, WI, USA) within 2 weeks of delivery. Each instrument underwent daily quality control, with cross-calibration between sites. The total radiation dose was

estimated to be 0·04 mSv, equivalent to about 7 days' exposure to background radiation in the UK. All DXA images were reviewed for movement artefacts and quality by two operators who were blinded to treatment allocation. Those with excessive movement artefact were excluded from the analysis, as described in section 3.1.4.3.2.

6.2.1 Statistical analyses

The analysis was restricted to mothers who had not taken cholecalciferol supplements of greater than 400 IU daily in addition to the study drug. Maternal characteristics in the placebo and vitamin D supplemented groups were compared (using t- tests for normally distributed variables, Wilcoxon-ranksum for non-normally distributed variables; chi-square for categorical variables).

Comparisons between median CTX concentrations in the placebo and cholecalciferol supplemented groups in early pregnancy (14 weeks) and late pregnancy (34 weeks) were made using the Wilcoxon matched-pairs signed-ranks test. Percentage changes in CTX from early to late pregnancy were calculated, statistical differences in median percentage change in the placebo group and cholecalciferol supplemented groups were also tested using the Wilcoxon matched-pairs signed-ranks test. Spearman's rank pairwise correlation was used to test correlations between CTX in early pregnancy and late pregnancy, and between CTX (in early or late pregnancy) and maternal serum 25(OH)D measurements (in early or late pregnancy).

The distribution of CTX was skewed, and was therefore log transformed. Associations between maternal characteristics and CTX were analysed using linear regression analysis and back-transformed, hence the back-transformed estimates are average proportional differences in CTX (hence a coefficient of 1.10 indicates a 10% increase, and 0.90 a 10% decrease), per unit change in the maternal predictor.

When used in regression analysis with DXA-assessed bone outcomes, CTX was standardised to an SD scale for ease of interpretation. Beta coefficients represent change in bone outcome per 1 SD increase in CTX.

All analyses were performed in Stata v14 (Statacorp, College Station, Texas, USA). A p value of < 0.05 was considered statistically significant.

6.3 Results

6.3.1 Characteristics of the participants

Of the 965 women recruited to the MAVIDOS trial who remained in the study until delivery, 630 had a measurement of CTX in either early (n = 493) or late pregnancy (n = 498). 372 women had a CTX measurement at both timepoints (placebo group, n = 188, cholecalciferol supplemented group, n = 184). The characteristics of the mothers with a measurement of urine CTX in early or late pregnancy are summarised in Table 6.1. Their mean age was 30.4 years and 95.0% were of Caucasian ethnicity and 44.5% were nulliparous. In this subgroup analysis, there were no statistically significant differences between the mothers randomised to placebo versus those randomised to cholecalciferol for the majority of early pregnancy characteristics (age, height, weight, pregnancy weight gain, parity, ethnicity, educational level, smoking or level of strenuous physical activity). Early pregnancy BMI was greater in the placebo group [25.5 kg/m^2 (IQR 22.9, 29.5)] than the cholecalciferol supplemented group [24.4 kg/m^2 (IQR 22.3, 28.1); p = 0.02].

Baseline maternal 25(OH)D concentration (measured at recruitment, 14 weeks) was similar in both groups. Mean maternal 25(OH)D concentration at 34 weeks gestation was significantly higher in the women who received cholecalciferol [66.0 nmol/l (SD 20.4)] than in those who received placebo [42.5 nmol/l (SD 20.6); p < 0.0001].

Table 6.1 Characteristics of MAVIDOS mothers with a measurement of CTX available in early or late pregnancy, in the study overall, and in the placebo group and cholecalciferol supplemented group separately, n = total number of participants with available data, N = number with characteristic.

[a] Characteristics at early pregnancy study visit; [b] p-value for t-test; [c] p-value for Wilcoxon-ranksum; [d] p-value for Chi-squared test

	All			*Placebo*			*Cholecalciferol 1000 IU/day*			**P-value**
	n	**Mean**	**SD**	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	
Age [a]	**607**	30.4	5.3	**296**	30.4	5.3	**311**	30.3	5.3	0.86[b]
Height (cm) [a]	602	165.4	6.5	292	165.5	6.7	310	165.2	6.4	0.51[b]
Weight (kg) [a]	607	71.8	15.0	296	72.8	14.4	311	70.8	15.5	0.11[b]
Pregnancy weight gain (kg)	536	9.5	3.6	264	9.3	3.7	272	9.7	3.4	0.20[b]
25(OH)D at 14 weeks (nmol/l)	630	44.6	16.2	312	44.1	16.1	318	45.0	16.4	0.46[b]
25(OH)D at 34 weeks (nmol/l)	561	54.2	23.6	282	42.5	20.6	279	66.0	20.4	**< 0.001[b]**
Adjusted calcium at 14 weeks (mmol/l)	630	2.38	0.08	312	2.38	0.09	318	2.37	0.07	0.15
Adjusted calcium at 34 weeks (mmol/l)	560	2.34	0.11	280	2.34	0.12	280	2.34	0.10	0.91
	n	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**	**Median**	**IQR**	
BMI (kg/m^2) [a]	602	24.9	22.5-28.6	292	25.5	22.9-29.5	310	24.4	22.3-28.1	**0.02[c]**
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	
First Pregnancy	604	269	44.5	294	134	45.6	310	135	43.5	0.62[d]
White (Caucasian) ethnicity	604	574	95.0	296	285	96.3	308	289	93.8	0.17[d]
A Level or higher qualification	602	450	74.8	293	214	73.0	309	236	76.4	0.35[d]
Smoker [a]	605	54	8.9	295	27	9.2	310	27	8.7	0.85[d]
More than one hour strenuous exercise per week [a]	553	79	14.2	270	33	12.2	283	46	16.3	0.18[d]

6.3.2 Descriptive epidemiology of maternal urinary CTX in pregnancy and its relationship with 25(OH)D

6.3.2.1 Maternal urinary CTX in early and late pregnancy

In both the placebo and 1000 IU/day cholecalciferol supplemented mothers, urinary CTX increased from 14 to 34 weeks gestation [median 14 and 34 week CTX: placebo group, 223.8 and 445.3 μg/mmol creatinine; cholecalciferol supplemented group, 217.5 and 420.0 μg/mmol creatinine; (both p-difference < 0.0001)], as shown in Table 6.2. Median late pregnancy CTX was 25.3 μg/mmol greater in the placebo group compared with the cholecalciferol supplemented group, but this difference was of borderline statistical significance (p = 0.06).

In the groups combined, maternal CTX approximately doubled from 14 weeks to 34 weeks gestation (median percentage change in CTX 101.7%). The percentage increase in CTX from early to late pregnancy was statistically significantly greater in the placebo group than the cholecalciferol supplemented group [placebo group, median (IQR) percentage increase 111.1% (47.1%, 210.9%); cholecalciferol group 88.8% (22.8%, 183.1%), p difference = 0.02].

As shown in Figure 6.1, both early and late pregnancy urinary CTX were in a right skewed distribution; a few women had very high CTX measures (> 1500 μg/mmol creatinine). The increase in median CTX between early and late pregnancy within the population is demonstrated in this figure.

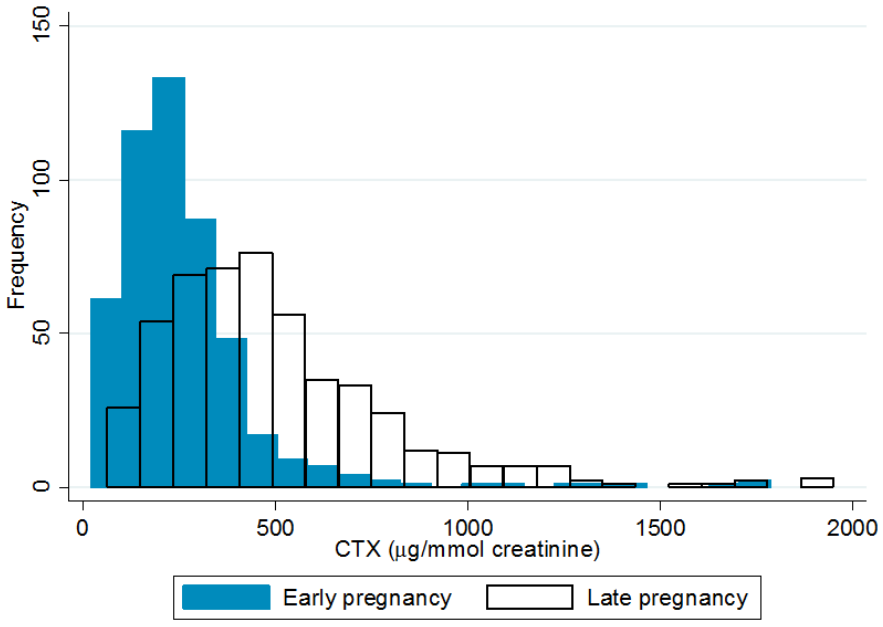


Figure 6.1 Frequency distribution of maternal urinary CTX (μg/mmol creatinine) in early (14 weeks) and late pregnancy (34 weeks) in all mothers with a measurement of CTX.

Table 6.2 Maternal urinary CTX in early (14 weeks) and late pregnancy (34 weeks) and percentage change in CTX from early to late pregnancy, in all mothers in the MAVIDOS trial and in the placebo group and cholecalciferol 1000 IU supplemented groups separately. Maternal DXA characteristics at the lumbar spine and total hip are also presented. Percentage change in CTX is defined as a ratio of late pregnancy to early pregnancy CTX.

P values represent outcomes of tests for differences between the placebo group and vitamin D supplemented group (Chi squared tests for CTX, t tests for DXA bone indices).

	All			*Placebo*			*Cholecalciferol 1000 IU/day*			*p-value*
	n	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**	**Median**	**IQR**	
Early pregnancy (14 week) CTX (μg/mmol creatinine)	493	219.4	154.7, 306.4	242	223.8	155.0, 308.6	251	217.5	154.5, 305.2	0.67
Late pregnancy (34 week) CTX (μg/mmol creatinine)	498	437.4	292.3, 633.0	252	445.3	317.6, 657.3	246	420 .0	252.2, 608.5	0.06
Percentage change in CTX (%)	372	101.7	31.1, 203.4	188	111.1	47.1, 210.9	184	88.8	22.8, 183.1	**0.02**
	n	**Mean**	**SD**	**n**	**Mean**	**SD**	**N**	**Mean**	**SD**	
Lumbar spine										
BMC (g)	343	60.52	9.91	168	60.88	9.51	175	60.17	10.30	0.51
BMD (g/cm²)	343	1.03	0.11	168	1.03	0.11	175	1.03	0.11	0.41
Area (cm²)	343	58.36	5.29	168	58.48	5.27	175	58.25	5.33	0.69
Total hip										
BMC (g)	339	30.78	4.68	166	31.16	4.43	173	30.43	4.89	0.15
BMD (g/cm²)	339	0.94	0.10	166	0.94	0.10	173	0.94	0.11	0.55
Area (cm²)	339	32.68	3.16	166	32.98	3.27	173	32.38	3.04	0.08

6.3.2.2 Correlations between early and late pregnancy urinary CTX and maternal serum 25(OH)D

In both the placebo and cholecalciferol supplemented mothers, early (14 week) and late (34 week) pregnancy CTX were significantly correlated (placebo group r = 0.31, treatment group r = 0.45, both p < 0.0001) as shown in Table 6.3. Early and late pregnancy 25(OH)D were significantly correlated (placebo group, r = 0.36, p < 0.0001; cholecalciferol supplemented group r = 0.22, p = 0.0003); with a greater correlation coefficient in the placebo group. No significant correlations were demonstrated between early or late pregnancy serum 25(OH)D measurements and CTX measures.

Table 6.3 Correlations between maternal urinary CTX in early (EP, 14 weeks) and late (LP, 34 weeks) pregnancy, and between early (14 week) and late (34 week) pregnancy measures of serum 25(OH)D. *Numbers in each correlation, pairwise Spearman's Rank correlation coefficients and p-values are shown.*

	EP CTX	LP CTX	EP Vit D	LP Vit D
EP CTX		**n = 188** **R = 0.31** **p<0.0001**	n = 240 R = 0.09 p = 0.16	N/A
LP CTX	**n = 184** **R = 0.45** **p<0.0001**		n = 251 R = -0.10 p = 0.11	n = 246 R = -0.07 p = 0.25
EP Vit D	n = 248 R = 0.07 p = 0.28	n = 245 R = -0.04 p = 0.57		**n = 280** **R = 0.36** **p<0.0001**
LP Vit D	N/A	n = 243 R = 0.01 p = 0.83	**n = 275** **R = 0.22** **p = 0.0003**	

Placebo

Cholecalciferol 1000 IU/day

6.3.2.3 Maternal predictors of CTX in early and late pregnancy

Maternal characteristics were analysed as predictors of CTX in early pregnancy and late pregnancy using linear regression analyses, as shown in Table 6.4 and Figure 6.2.

Receiving cholecalciferol supplementation (1000 IU/day) from 14 weeks gestation was associated with a 10% reduction in late pregnancy CTX (average proportional difference 0.90), [95% CI 0.81 to 1.00 (p = 0.045)], compared with allocation to placebo.

Greater maternal age was associated with lower early pregnancy CTX (by 2% per year, p = 0.001), and late pregnancy CTX (by 1% per year, p = 0.007). Being a smoker in early pregnancy was

associated with 26% greater (on average) early pregnancy CTX (p = 0.028), though was not associated with late pregnancy CTX, compared with being a non-smoker.

Parity also had a large effect on CTX – having one or more previous children was associated with early pregnancy CTX being 25% lower, and late pregnancy CTX being 18% lower, compared to primagravida mothers (both p < 0.001).

Maternal height was significantly associated with CTX in late pregnancy (1% lower per cm increase in height, p = 0.006), and associations between maternal early pregnancy BMI and late pregnancy CTX were of borderline statistical significance (p = 0.074).

Table 6.4 Maternal determinants of CTX in early and late pregnancy, average proportional differences per unit change in maternal predictor.

[a]Maternal CTX in late pregnancy adjusted for treatment group, except when associations between vitamin D supplemented group and CTX are being tested. Significant p-values are highlighted in bold.

	Average proportional difference in maternal urinary CTX in early pregnancy (14 weeks gestation)			*Average proportional difference in maternal urinary CTX in late pregnancy [a] (34 weeks gestation)*		
Predictor	β	**95% CI**	**p-value**	β	**95% CI**	**p-value**
Maternal age (years)	0.98	(0.97, 0.99)	**0.001**	0.99	(0.98, 1.00)	**0.007**
Strenuous exercise (> 1 hr /week, Y/N)	1.00	(0.94, 1.07)	0.956	1.00	(0.94, 1.06)	0.883
Smoking in early pregnancy (Y/N)	1.26	(1.03, 1.55)	**0.028**	0.88	(0.73, 1.07)	0.199
Parity (One or more, Y/N)	0.75	(0.75, 0.67)	**< 0.001**	0.82	(0.74, 0.92)	**< 0.001**
Caucasian ethnicity (Y/N)	0.91	(0.69, 1.19)	0.483	0.86	(0.66, 1.11)	0.244
Weight (kg)	1.00	(0.99, 1.00)	0.128	1.00	(1.00, 1.01)	0.486
Height (cm)	0.99	(0.98, 1.00)	0.073	0.99	(0.98, 1.00)	**0.006**
BMI (kg/cm^2)	0.99	(0.99, 0.98)	0.330	1.01	(1.00, 1.02)	0.074
14 week 25(OH)D (nmol/l)	1.00	(1.00, 1.00)	0.144	1.00	(0.99, 1.00)	0.147
14 week Calcium (mmol/l)	1.29	(1.29, 0.63)	0.484	1.44	(0.78, 2.64)	0.242
34 wk. 25(OH)D (nmol/l)				1.00	(1.00, 1.00)	0.541
34 wk. calcium (mmol/l)				1.08	(0.69, 1.70)	0.738
Change in 25(OH)D from 14 to 34 wks. (nmol/l)				1.00	(1.00, 1.00)	0.627
Vit D supplemented group (Y/N)				0.90	(0.81, 1.00)	**0.045**
Season of delivery, summer (proxy for season of measurement)	1.04	(0.92, 1.18)	0.52	1.04	(0.94, 1.16)	0.44

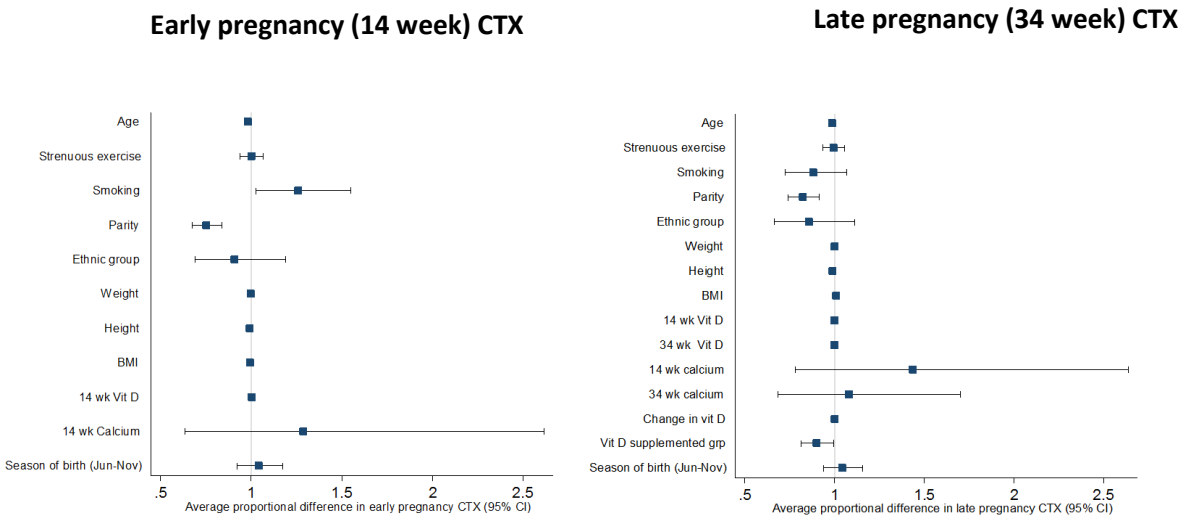


Figure 6.2 Maternal determinants of early (14 weeks) and late (34 weeks) CTX.

Outcomes are the result of separate regression analyses (average proportional differences in late pregnancy CTX, 95% confidence interval). Predictors of late pregnancy CTX (except vitamin D supplemented group) are adjusted for treatment group.

6.3.2.4 Associations between maternal serum 25(OH)D in late pregnancy and late pregnancy urinary CTX

Maternal cholecalciferol supplementation group was associated with maternal urinary CTX in late pregnancy, and therefore regression analyses were used to further explore the relationships between maternal concentrations of 25(OH)D and urinary CTX. In univariate regression analyses, maternal early pregnancy 25(OH)D was not associated with early pregnancy CTX or late pregnancy CTX. Late pregnancy 25(OH)D was also not a predictor of late pregnancy urinary CTX, in the maternal randomisation groups separately, or combined.

In the UK, season has been shown in the MAVIDOS trial and others (377), to be strongly associated with maternal 25(OH)D in pregnancy. After adjustment for season of delivery, negative associations between maternal 25(OH)D in late pregnancy and CTX became statistically significant [in both groups combined, β -0.998 average proportional difference, representing a 0.002% reduction in CTX per nmol/l 25(OH)D (p = 0.054)]. These associations were slightly stronger in the placebo group [β -0.996 (p = 0.036)]. Owing to baseline differences in BMI in early pregnancy between the randomisation groups, BMI was also adjusted for, and the observed associations remained. Maternal age, early pregnancy smoking, parity and height were maternal predictors of late pregnancy CTX at the univariate level: when added to a multivariate model, associations

between increasing maternal serum 25(OH)D concentrations and decreasing maternal urinary CTX in late pregnancy remained.

Further exploration of seasonal trends showed no differences in associations between late pregnancy 25(OH)D and maternal late pregnancy CTX by season when the groups were combined. However, in women giving birth in spring (March to May) in the placebo group only, greater maternal 25(OH)D status in late pregnancy was significantly associated with lower maternal late pregnancy CTX [β -0.993 (p = 0.049)].

Overall, a trend of declining late pregnancy CTX with increasing late pregnancy vitamin D was shown, as in Figure 6.3.

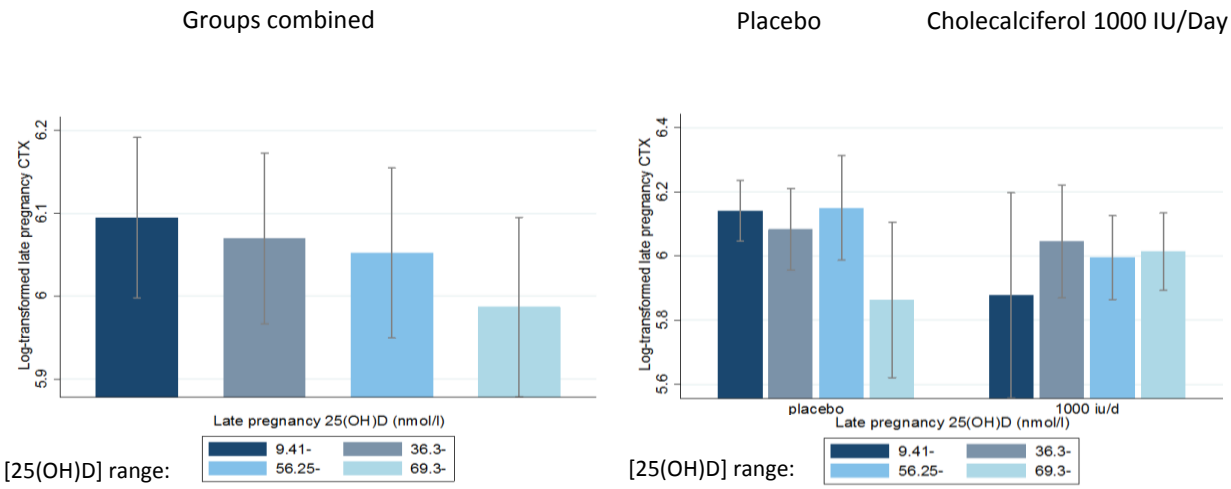


Figure 6.3 Associations between late pregnancy (34 week) 25(OH)D concentrations (nmol/l) expressed in quarters of the distribution and log-transformed late pregnancy CTX (μg/mmol creatinine): all subjects, n = 489 (left) and by randomisation group (right), placebo n = 246, cholecalciferol n = 243.

Table 6.5 Associations between maternal late pregnancy (34 week) serum 25(OH)D (nmol/l) and late pregnancy maternal urinary CTX, expressed as average proportional differences. Outcomes are displayed for the randomised groups combined and separately. *Owing to large seasonal variations in vitamin D levels, outcomes are also stratified by season of delivery.*

	Average proportional difference in late pregnancy maternal urinary CTX											
	Groups combined				*Placebo*				*Cholecalciferol 1000 IU/day*			
Late pregnancy 25(OH)D nmol/l, adjustments:	**n**	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**
Unadjusted	489	0.998	(0.997, 1.000)	0.139	246	0.998	(0.994, 1.001)	0.140	243	1.001	(0.997, 1.005)	0.612
Season	479	0.998	(0.995, 1.000)	**0.054**	244	0.996	(0.992, 1.000)	**0.030**	235	1.000	(0.997, 1.004)	0.762
Season and EP BMI	447	0.998	(0.996, 1.000)	0.056	224	0.996	(0.992, 1.000)	**0.036**	223	1.001	(0.997, 1.005)	0.700
Season, EP BMI, age, smoking, parity, height	443	0.998	(0.996, 1.000)	**0.051**	221	0.996	(0.992, 1.000)	**0.037**	222	1.001	(0.996, 1.005)	0.754
	Average proportional difference in late pregnancy maternal urinary CTX by season of delivery											
	Groups combined				*Placebo*				*Cholecalciferol 1000 IU/day*			
Late pregnancy 25(OH)D nmol/l	**n**	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**
Winter (Dec-Feb)	89	0.998	(0.993, 1.003)	0.423	41	0.997	(0.987, 1.007)	0.528	48	0.997	(0.986, 1.007)	0.502
Spring (Mar-May)	136	0.997	(0.993, 1.001)	0.132	73	0.993	(0.987, 1.000)	**0.049**	63	1.002	(0.993, 1.010)	0.679
Summer (Jun-Aug)	142	0.998	(0.994, 1.003)	0.408	69	0.996	(0.989, 1.004)	0.302	73	1.004	(0.997, 1.011)	0.268
Autumn (Sep-Nov)	112	0.998	(0.992, 1.004)	0.491	61	0.998	(0.989, 1.006)	0.573	51	0.999	(0.999, 1.004)	0.802

6.3.3 Maternal CTX in late pregnancy and maternal postpartum bone health

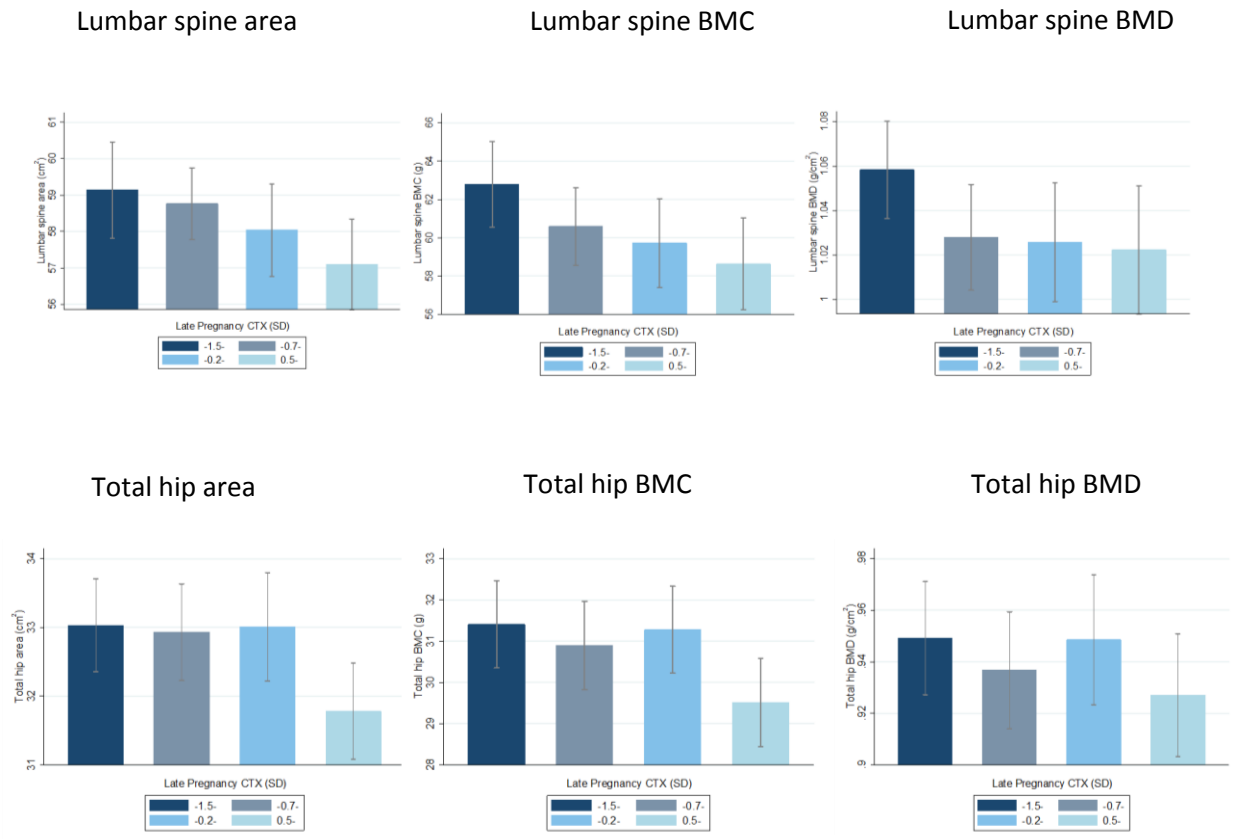
Linear regression analysis was used to explore the associations between late pregnancy CTX and maternal DXA bone outcomes at the lumbar spine and femoral neck, measured within two weeks postpartum (Table 6.6).

In the 301 mothers with a late pregnancy measure of urine CTX and postpartum lumbar spine DXA, greater 34-week maternal urinary CTX was associated with lower maternal postpartum lumbar spine bone area [β -0.82 cm^2/SD (95% CI -1.45, -0.19), p = 0.01], BMC [β -1.63 g/SD (95% CI -2.80, -0.46), p = 0.01] and BMD [β -0.01 g/cm^2/SD (95% CI -0.03, -0.01), p = 0.04], in the treatment and placebo groups combined. In the placebo group alone, these associations persisted for BMC and bone area, but not BMD. Negative associations between late pregnancy CTX and lumbar spine BMD were, however, present in the cholecalciferol supplemented group [β -0.02 g/cm^2/SD (95% CI -0.04, -0.0001), p = 0.05].

In the 297 mothers with available total hip DXA, greater 34-week maternal urinary CTX was significantly associated with lower maternal postpartum total hip BMC [β -0.71 g/SD (95% CI -1.30, -0.15), p = 0.01], and bone area [β -0.53 cm^2/SD (95% CI -0.90, -0.16), p = 0.01], but not BMD, in the treatment and placebo groups combined. In the placebo group alone, there were no statistically significant associations between late pregnancy maternal CTX and bone area, BMC or BMD at the total hip. In the cholecalciferol supplemented group, negative associations between late pregnancy CTX and total hip area and BMC were observed, [β -0.64 cm^2/SD (95% CI -1.14, -0.13), p = 0.02] and [β -0.94 cm^2/SD (95% CI -1.78, -0.10), p = 0.03], respectively. It should be noted that the magnitude and direction of these associations did not differ greatly between placebo and cholecalciferol supplemented groups. Adjustment for serum maternal 25(OH)D concentrations in late pregnancy did not change the magnitude or level of statistical significance of the associations between late pregnancy CTX (in placebo and cholecalciferol supplemented groups combined) and maternal bone indices. Following adjustment for previously identified predictors of maternal CTX, which may act as confounders of the association between maternal CTX and bone indices (maternal age, early pregnancy smoking and parity), statistically significant outcomes at the 5% level remained only for total hip bone area.

Table 6.6 Associations between late pregnancy urine CTX (expressed in standard deviations) in the vitamin D supplemented and placebo groups combined, and maternal lumbar spine and total hip area, bone mineral content (BMC) and bone mineral density (BMD).

	Total population				*Placebo*				*Cholecalciferol 1000 IU/day*			
	n	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**
Lumbar spine												
Bone area (cm^2)	301	-0.82	(-1.45, -0.19)	**0.01**	146	-1.01	(-1.87, -0.14)	**0.02**	155	-0.63	(-1.55, 0.29)	0.18
BMC (g)	301	-1.63	(-2.80 ,-0.46)	**0.01**	146	-1.61	(-3.17, -0.05)	**0.04**	155	-1.69	(-3.46, 0.07)	0.06
BMD (g/cm^2)	301	-0.01	(-0.03, -0.01)	**0.04**	146	-0.01	(-0.03, 0.01)	0.30	155	-0.02	(-0.04,-0.0001)	**0.05**
Total hip												
Bone area (cm^2)	297	-0.53	(-0.90, -0.16)	**0.01**	144	-0.46	(-1.00, 0.08)	0.10	153	-0.64	(-1.14, -0.13)	**0.02**
BMC (g)	297	-0.71	(-1.30, -0.15)	**0.01**	144	-0.52	(-1.25, 0.21)	0.16	153	-0.94	(-1.78, -0.10)	**0.03**
BMD (g/cm^2)	297	-0.01	(-0.02, 0.01)	0.26	144	-0.002	(-0.02, 0.01)	0.78	153	-0.01	(-0.03, 0.01)	0.18



CTX divided into quarters of the distribution: -1.5 SD to -0.7 SD; -0.7 SD to -0.2 SD, -0.2 SD to +0.5 SD, +0.5 SD and above

Figure 6.4 Late pregnancy urinary CTX (shown in quarters of the distribution, SD) and maternal postpartum lumbar spine area, BMC and BMD (n =301) and total hip area, BMC and BMD (n = 297) assessed by DXA.

6.4 Discussion

6.4.1 Summary of findings

In a randomised controlled trial setting, MAVIDOS, maternal gestational cholecalciferol supplementation has been shown to be associated with a reduction in bone resorption markers in late pregnancy. Whilst maternal urinary CTX almost doubled from 14 weeks to 34 weeks gestation in both vitamin D supplemented and placebo groups, the percentage increase in CTX from early to late pregnancy was significantly reduced in the cholecalciferol supplemented group. Absolute measures of late pregnancy CTX, however, did not differ between the groups.

CTX at 14 and 34 weeks were significantly correlated in both the placebo and supplemented groups, and so women with a lower CTX in early pregnancy were likely to have a lower CTX in late pregnancy. Early and late pregnancy maternal serum 25(OH)D concentrations were not correlated with measures of urinary CTX at either timepoint.

However, on univariate regression analysis, randomisation to the cholecalciferol supplemented group was associated with a reduction in mean late pregnancy CTX. On further investigation via a multivariate regression analysis with the placebo and cholecalciferol supplemented groups combined, greater serum 25(OH)D concentrations in late pregnancy were associated with lower urinary CTX measures at the same timepoint, following adjustment for season of delivery. Adjustment for maternal early pregnancy BMI (which differed between the groups at baseline), and other maternal characteristics shown to be associated with increased CTX (smoking in early pregnancy) or decreased CTX (greater maternal age, greater parity, and greater height) did not alter the magnitude or statistical significance of these associations. When the placebo and cholecalciferol supplemented groups were studied separately, associations between increasing maternal 25(OH)D and lower urinary CTX were only observed in the placebo group. Analysis of maternal postpartum DXA findings were consistent with its role as a resorption marker, as, overall, greater 34-week CTX was associated, similarly in either group, with lower maternal total hip bone area and BMC, in addition to lumbar spine BMC.

Our findings suggest that vitamin D supplementation may contribute in part towards a protective effect on maternal bone resorption in pregnancy. We have already demonstrated in the previously published outcomes of the MAVIDOS trial at birth, that maternal cholecalciferol supplementation was associated with improved neonatal bone indices in winter-born infants; it may, therefore provide a beneficial effect for both mother and baby (377).

6.4.2 Descriptive epidemiology of maternal urinary CTX in pregnancy and its relationship with 25(OH)D

6.4.2.1 Maternal urinary CTX increased from early to late pregnancy, cholecalciferol supplementation reduced the magnitude of this increase

Fetal requirements for calcium for mineralisation of the skeleton are highest in the third trimester, at which time around 80% of the fetus's approximately 30 g of calcium are accrued (522). This requirement may be fulfilled by increased maternal dietary intake, higher intestinal calcium absorption, greater mobilization of calcium from the maternal skeleton, and greater placental calcium transport, which maintains a positive calcium gradient between mother and fetus (136, 523). Bone turnover markers offer a non-invasive way of monitoring the possible maternal skeletal contribution to the fetus, and a number of studies are consistent with our finding of markers of bone resorption being around double in late pregnancy in comparison to early pregnancy (135-142).

The role of vitamin D in the modulation of calcium transfer to the fetus is unclear. We know that the concentration of $1,25(OH)_2D$ (calcitriol) increases during pregnancy with (most importantly) the maternal kidney and possibly the placenta, decidua and fetal kidney providing the necessary 1-α-hydroxylase activity (136, 141, 524). Increased $1,25(OH)_2D$ is thought to contribute to increasing maternal intestinal calcium absorption (525), through fetal PTH, and maternal and fetal PTHrp, (with the balance between PTH and PTHrp set by the calcium sensing receptor (CaSR)) (526-528). A host of other factors are known to come into play in regulating the balance between maternal and fetal bone formation and resorption (osteoblast and osteoclast activity), including RANKL and OPG, Sclerostin and FGF-23, the Wnt pathway, oestrogens and prolactin (523).

It is therefore unlikely that the study of one potential mediating factor (maternal serum 25(OH)D concentrations), and one measure of bone resorption (urinary CTX), are likely to provide conclusive information about the system, though it does provide an incremental step towards our understanding of this complex process. Indeed, the European Food Safety Authority have identified the associations between vitamin D, bone turnover markers and bone health as a current knowledge gap, as outlined in their 2016 scientific opinion paper (529).

To my knowledge, MAVIDOS is the first randomised controlled trial of vitamin D in pregnancy to study the effects of vitamin D on bone turnover markers, and is the first to report associations between maternal 25(OH)D supplementation and lower late pregnancy bone resorption markers. The mechanism for this difference could be related to greater calcium availability from maternal intestinal absorption for the developing fetus in mothers with improved 25(OH)D status, reducing the requirement for the release of calcium from maternal bone resorption. Our finding that

increasing maternal serum 25(OH)D concentrations in late pregnancy are associated with lower late pregnancy CTX, after adjustment for season, would support this hypothesis. Additionally, the fact that the strongest negative associations between maternal 25(OH)D in late pregnancy and urinary CTX were observed in mothers who delivered babies in the spring in the placebo group may support the notion that the greatest levels of maternal bone resorption occur in those with the lowest serum 25(OH)D concentrations in late pregnancy. The seasonal nadir of 25(OH)D concentrations are known to occur in late winter or early spring (239, 530), and in the MAVIDOS trial the greatest decreases in 25(OH)D concentrations were observed in the placebo group mothers delivering in winter and spring (377). This does not explain why seasonal effects were not observed in mothers giving birth in winter; the reduction in participant numbers from the trial overall to this subgroup analysis of mothers with a CTX measurement could be one explanation. Also, CTX measurement was taken on average six weeks prior to delivery, so over half of the mothers in this study giving birth in winter (December – February) would have had their serum 25(OH)D and urinary CTX assessed in autumn.

Studies of calcium and vitamin D in pregnant women provide supportive evidence for our hypothesis that maternal 25(OH)D status in late pregnancy is important in regulating maternal bone turnover to ensure calcium availability for the developing fetus. One previous RCT of calcium supplementation in pregnancy showed that daily consumption of an 1100 mg calcium supplement led to a 15% reduction in urinary NTX during the second and third trimesters (144). A prospective cohort showed that higher calcium intake in late pregnancy was associated with reduced bone resorption in late pregnancy, with greater effects in winter, when vitamin D mediated calcium absorption is less (131). Other longitudinal studies of maternal 25(OH)D status in pregnancy demonstrated that vitamin D repletion was associated with lower markers of bone resorption, but that this association is more likely to be apparent in pregnancy when calcium requirements are greatest. Park et al., in a small study of 26 pregnant women and 21 healthy non-pregnant controls, showed that greater 25(OH)D status, and greater levels of the vitamin D metabolites ($1,25(OH)_2D$, $24,25(OH)_2D$) tended to be associated with lower NTX levels in the second and third trimesters and postpartum period; whilst no correlation between 25(OH)D and NTX was seen in the controls (113). In a Turkish study of 30 pregnant women and 30 non-pregnant health controls, again, in pregnant women only, 25(OH)D levels were negatively correlated with serum concentrations of CTX in late pregnancy (but not early pregnancy) (130).

In non-pregnant individuals, a high bone turnover in those who are vitamin D deficient has been observed, with vitamin D supplementation attenuating this effect in some studies (132) but not others (288, 531).

6.4.2.2 Predictors of maternal bone resorption markers include vitamin D supplementation group, maternal age, smoking status, parity and height

In addition to our finding that 25(OH)D supplementation attenuated increases in late pregnancy CTX, we were able to explore other maternal determinants of CTX levels. We showed that early pregnancy CTX was higher in women who smoked. This relationship was not observed with late pregnancy CTX, but this may be due to the fact that lower numbers of women smoked in the later stages of pregnancy. Smoking is widely recognised as a risk factor for osteoporosis, and, interestingly, has been linked to lower 25(OH)D levels (532). Smoking has also been shown to be associated with greater NTX levels in women aged 30 to 45 years (134).

Both early and late pregnancy CTX were lower in women who had one or more children. Previous studies have demonstrated lower bone turnover in women of higher parity and with longer periods of lactation compared with women of the same age (162). It is possible that reduced bone resorption following childbirth is one of the mechanisms by which pregnancy is not detrimental in the long term to bone health or fracture risk in women of good nutritional status (158), despite increases in resorption in late pregnancy and lactation (143).

Associations between maternal height and late pregnancy CTX were observed: taller women had lower urinary CTX in late pregnancy, although height was not significantly associated with early pregnancy CTX. Greater maternal height has been associated with greater fetal femur volume in utero (359), in addition to improved bone indices in neonates (90) and children (360). However, tall maternal height has also been associated with increased hip fracture risk in adulthood (193). The mechanisms behind greater maternal height and lower bone resorption markers are not clear, perhaps in a taller mother reserves of calcium in the skeleton are greater, so, proportional to body size, less bone is resorbed. Alternatively, perhaps taller mothers have lower baseline CTX to ensure that their larger quantity of bone is mineralised.

Maternal weight and BMI were not associated with CTX in late pregnancy, other epidemiological studies have shown that low maternal BMI is associated with increased markers of bone resorption (145).

Serum calcium concentrations at 14 weeks and 34 weeks calcium were not associated with CTX, though as maternal serum calcium remains stable throughout pregnancy this is unsurprising. Future analyses of the association between maternal dietary calcium intake and CTX would be more informative, as would serum 25(OH)D concentrations adjusted for dietary calcium intake as a predictor of pregnancy bone resorption.

6.4.3 Greater maternal CTX is associated with poorer maternal postpartum bone indices

Higher measures of maternal urinary CTX in late pregnancy were associated with lower lumbar spine bone area, BMC and BMD, and lower total hip bone area and BMC (but not BMD) with similar patterns observed in both the cholecalciferol and supplemented groups. CTX, released by osteoclastic resorption of bone, has been shown to be significantly correlated with lower total hip BMD, distal radius BMD and lumbar spine BMD (121). For each SD increase in urinary CTX, fracture risk has been demonstrated to increase by approximately 50% in older women (123, 124). Associations between bone turnover markers and fracture risk in younger women however are poorly characterised, and not all studies have correlated bone resorption markers with fracture risk, though the majority have demonstrated associations between CTX and bone indices assessed by DXA, pQCT or histology (126, 127).

We did not find evidence of differences between the placebo and cholecalciferol supplemented groups in the associations between late pregnancy CTX and bone indices postpartum, and when analysing the groups combined in a multivariate regression model, adjustment for 25(OH)D in late pregnancy did not alter the relationships. As negative associations between late pregnancy CTX and bone indices are observed in healthy individuals, it is not clear whether this is of clinical significance. However, as greater late pregnancy 25(OH)D in mothers was associated with lower CTX, and lower CTX was associated with better bone indices at the spine and hip, it is possible that gestational vitamin D supplementation may have a beneficial effect in preserving maternal bone health.

This suggestion comes with a number of caveats, the first being that maternal $1,25(OH)_2D$ may not be the main factor mediating the increase in maternal intestinal calcium absorption. The biologically active or free concentration of $1,25(OH)_2D$ is only elevated during the third trimester, which occurs months after the first trimester increase in calcium absorption, a pattern which has been observed in both humans and rodents (335, 533). Models of severe vitamin D deficiency and absence of the vitamin D receptor (in VDR knockout mice) do not prevent the increase in calcium absorption observed in pregnancy, as different calcium transporters are upregulated (534). However, in mice, absence of a functional VDR or the 25(OH)D-1α-hydroxylase enzyme (CYP27B1) does create a bone and growth plate phenotype that mimics severe vitamin D deficiency in humans (535). Of course, for ethical reasons, no human studies of the effects of severe vitamin D deficiency or hereditary vitamin D disorders on intestinal calcium absorption during pregnancy have been examined. As far as I am aware, no previous randomised controlled trials of gestational cholecalciferol supplementation have studied the influence of supplementation on bone turnover

markers, or the associations between bone turnover and measures of maternal bone health. Therefore, further evidence is required from other populations to determine whether our findings reflect real and meaningful impacts on maternal bone health, particularly as the existing evidence does not support the notion that pregnancy has a negative impact on maternal health in the long run (158).

6.4.4 Limitations

Limitations regarding the use of CTX as a marker of bone resorption should also be recognised, and are discussed further in section 8.4.5. These include its circadian rhythm and relationship with food intake (though fasting, early morning, second void urine was used to attempt to reduce this variation). We have not accounted for haemodilution and individual changes in maternal GFR during pregnancy (thereby affecting renal clearance of CTX) (138), maternal $1,25(OH)_2D$ status or measures of vitamin D binding protein (DBP), PTH or PTHrP. An estimate of maternal dietary calcium intake would also be important as a potential confounding factor (113, 130, 131), as would be sensitivity analyses on the effect of maternal vitamin D supplementation use external to the study drug (though we restricted these analyses to mothers who reported use of 400 IU cholecalciferol or less). The assay used in this study does not distinguish between the different isoforms of CTX (α–CTX is predominantly released from growing young bone, and may cross the placenta), therefore it is not possible to quantify the fetal contribution to the mother's urinary CTX (145).

In terms of maternal DXA measures, maternal breastfeeding is known to play a large role in determining levels of bone resorption and postpartum BMD, particularly at trabecular sites (153). Lumbar spine BMD has been shown to decline by a mean of 5-10% during exclusive breastfeeding (153). Though mothers underwent DXA within 2 weeks postpartum, number of days spent breastfeeding prior to DXA assessment may be important, this data was not available at the time of analysis. Late pregnancy CTX was measured at 34 weeks, therefore there could be as much as an 8 week lag between the last CTX measurement and maternal DXA. Further limitations to the MAVIDOS trial are discussed in section 8.4.2.

6.4.5 Conclusion

In conclusion, we have shown, for the first time in a randomised controlled trial setting, that urinary CTX rises from early to late pregnancy, appears to be reduced by gestational cholecalciferol supplementation, and is inversely associated with maternal post-partum measures of bone area, BMC, and BMD at the lumbar spine, and bone area and BMC at the hip. These findings suggest that cholecalciferol supplementation could have beneficial effects on maternal

bone health, and inform our understanding of bone resorption in pregnancy and potential relationship with vitamin D metabolism.

Further analysis of the associations between maternal 25(OH)D status and the change in CTX from early and late pregnancy, adjustment for factors previously shown to be associated with late pregnancy bone health such as calcium intake, maternal BMI, parity, season (157) and where available, measures of $1,25(OH)_2D$, DBP, PTH and PTHrP, may be helpful in better understanding this association. Umbilical cord venous plasma CTX measures (in small numbers), in addition to neonatal DXA outcomes are also available, which will be useful in determining associations between maternal and neonatal CTX and bone indices of the babies of the MAVIDOS trial. Long term follow-up of both mothers and offspring, with repeat assessments of bone indices (the mothers are undergoing DXA in the 4-year follow-up), will enable us to better determine the lasting impact of cholecalciferol supplementation in pregnancy.

Chapter 7: Associations between cholecalciferol supplementation and musculoskeletal health at age 4 years: findings from the MAVIDOS trial

7.1 Background and aims

As discussed previously, there is increasing evidence that the in-utero environment can modify fetal bone development, with persisting effects into later childhood. As vitamin D is important for bone mineralisation and the developing fetus is dependent on the placental transfer of 25(OH)D from the maternal circulation, there is growing interest in investigating the role for maternal 25(OH)D in pregnancy in determining offspring bone mass.

A Health Technology Assessment systematic review of observational studies concluded that there was some evidence to support a positive association between maternal 25(OH)D status and offspring bone mass, though it was not possible to deduce a vitamin D threshold at which improved bone mass could result, or indeed whether there was a threshold at all (507). A variety of intervention studies of vitamin D supplementation in pregnancy, the majority of which (other than the MAVIDOS study) were underpowered, have shown mixed evidence of beneficial effects on offspring musculoskeletal health (371, 536, 537). A meta-analysis of intervention trials, which included MAVIDOS, showed no significant effect of vitamin D supplementation in pregnancy on neonatal bone mineral content or infant bone mineral content (538).

The neonatal outcomes of the MAVIDOS trial showed that, although overall there were no differences in whole body or LS BMC, bone area or aBMD between the babies born to the cholecalciferol supplemented or placebo groups (n = 736), a significant interaction was observed between season of birth and maternal randomisation group (p for interaction for BMC 0.04) (377). Whole body BMC and BMD were approximately 9% and 5% higher, respectively, in the children born in winter (December – February) to mothers randomised to cholecalciferol compared to those randomised to placebo, a difference greater than 0.5 SD. Associations between 25(OH)D status in pregnancy and offspring bone mass have been shown to persist into adulthood in the Western Australian Pregnancy Cohort (RAINE) (361). In addition, the effect size observed in MAVIDOS winter births (a 0.5 SD difference in whole body BMC and BMD) is substantially larger than the difference in BMC and BMD observed between children with and without fractures (Clark et al. showed that children with fractures at age 9 years had a 0.1 SD lower mean BMD than those without, n = 2692) (378). Hence, if such differences persist into later childhood, this is likely

to be clinically relevant in terms of fracture prevention. Of note, this finding was consistent with the observation from the Southampton Women's Survey showing that neonates born in the winter when background 25(OH)D levels are low, have lower BMC than do those born in the summer months (539). Longer term follow up is required to assess whether the effect of gestational vitamin D supplementation on increased bone mineralisation in children born in winter does persist beyond the neonatal period.

No RCTs have determined the effect of antenatal vitamin D supplementation on bone or other measures of musculoskeletal health in later childhood (such as muscle mass). Similarly, to my knowledge, muscle strength has never been considered as an outcome in any intervention study of gestational vitamin D supplementation.

The aim of this study was therefore to determine whether 1000 IU/day maternal cholecalciferol supplementation versus placebo was associated with differences in bone mass, lean mass, or grip strength of the offspring at age 4 years.

7.2 Methods

7.2.1 The four year follow-up of the MAVIDOS study

The MAVIDOS study methodology is described in detail in Chapter 3. The follow-up at 4 years of age commenced in March 2013 and was completed in October 2018. The WBLH region of interest was used in this analysis to maximise the number of usable scans, as head movement was common. Scans were reviewed by a clinician blinded to treatment allocation (EMC or RJM). Those with movement artefact were reviewed by two clinicians (EMC, RJM or NCH) and a decision was made to keep or exclude them. Scans with movement artefact affecting the whole body, both legs, or both arms, were removed from the analysis. In scans with movement artefact in one limb, the ROI of the limb with movement artefact was removed and the opposite limb ROI measurements were transposed to that region, as described in Chapter 3.

7.2.2 Statistical analyses

Comparisons were made between randomisation groups and attendees versus non-attendees using t-tests, Mann-Whitney U tests and χ^2 tests for normally distributed, non-normally distributed and categorical variables respectively. DXA outcomes and grip strength were transformed to Z scores (representing standard deviations) for ease of comparison of outcomes,

grip strength was measured three times in each hand; both a maximum value and a mean value of the six attempts was analysed.

Owing to differences in body composition between boys and girls, DXA outcomes were adjusted for sex using linear regression. DXA outcomes were also further adjusted for age. Grip strength was adjusted for height and sex.

Owing to chance differences in the baseline mean BMI of the randomly assigned pregnant women in the MAVIDOS trial [placebo n = 523, mean (SD) 25.7 (23.0-30.0) kg/m^2; cholecalciferol n = 533, 24.7 (22.3-28.6) kg/m^2], maternal BMI was also adjusted for (377). Higher maternal BMI at 14 weeks gestation has been shown to be associated with attainment of a lower late pregnancy 25(OH)D status following cholecalciferol supplementation (341). Higher maternal BMI is also relevant to offspring bone mass (482, 540, 541).

Model building by iterative forward selection was used to determine which covariates should remain in a multivariate regression model examining the association between randomisation group and offspring bone or lean mass outcomes, or grip strength. The following covariates, chosen due to evidence from previous studies suggesting an association with offspring bone mass, 25(OH)D status, or both, were tested: child's sex, child's age, child's height, offspring gestational age at birth, maternal age at delivery, maternal parity, maternal BMI in early pregnancy, maternal smoking in early or late pregnancy, maternal moderate to strenuous physical activity in late pregnancy, maternal educational attainment (A-level or above), offspring season of birth, child's weekly milk intake at age 4 years as a proxy for calcium intake, and child's weekly "screen time" (watching TV or computer screen) as an inverse measure of physical activity (sedentary time assessed by questionnaire has been shown to be negatively associated with physical activity assessed by accelerometry in primary school children (542, 543)).

Multivariate linear regression was subsequently performed including all variables with a $p < 0.2$ from the univariate model, in order to assess their importance after adjustment for other important covariates.

In the primary trial analysis, season of birth was classified according to the UK Meteorological office recommendations, (www.metoffice.gov.uk) as winter (December-February), spring (March-May), summer (June-August), and autumn (September-November). As 25(OH)D concentrations are not linearly associated with season, and for ease of comparison, and to maximise power, this classification has been into two groups, "winter/spring" (the months in which 25(OH)D concentrations tended to be lowest, December – May) and "summer/autumn" (the months in which 25(OH)D concentrations tended to be highest, June – November).

In sensitivity analyses, associations between randomisation group and DXA outcomes and grip strength were examined, stratifying baseline 25(OH)D using a threshold of ≤ 30 nmol/l (a threshold advised by the Institute of Medicine (228) and the Global Consensus Recommendations on the Prevention and Management of Nutritional Rickets (229) to define vitamin D deficiency. The influence of adjustment for maternal late pregnancy moderate to strenuous physical activity was examined in a separate multivariate model (missing data reduced the overall numbers substantially). In further sensitivity analyses, firstly data from children who were born preterm (< 37 weeks) was excluded, then analysis was limited to children of mothers of white ethnicity, and finally analysis was limited to scans without any movement artefact.

7.3 Results

7.3.1 Characteristics of the participants

Within the Southampton-based recruitment to the MAVIDOS study, 723 babies were born at term. 564 attended the 4 year visit (286 in the placebo group, 278 in the cholecalciferol group), 78.0 % of eligible children. 561 children (99.5% of attendees) underwent anthropometric measurement, and 515 (91.3% of attendees) underwent at least one measurement of grip strength. Numbers of DXA scans were lower, either due to participant refusal or technical issues. 508 children (90.1% of attendees) underwent DXA scanning, and 452 children had a useable DXA scan (89.0% of all DXAs). 90 DXAs (19.9% of the useable DXAs) had movement artefact in one leg, one arm, or one leg and one arm and underwent transposition of data from the ROI of the opposite side.

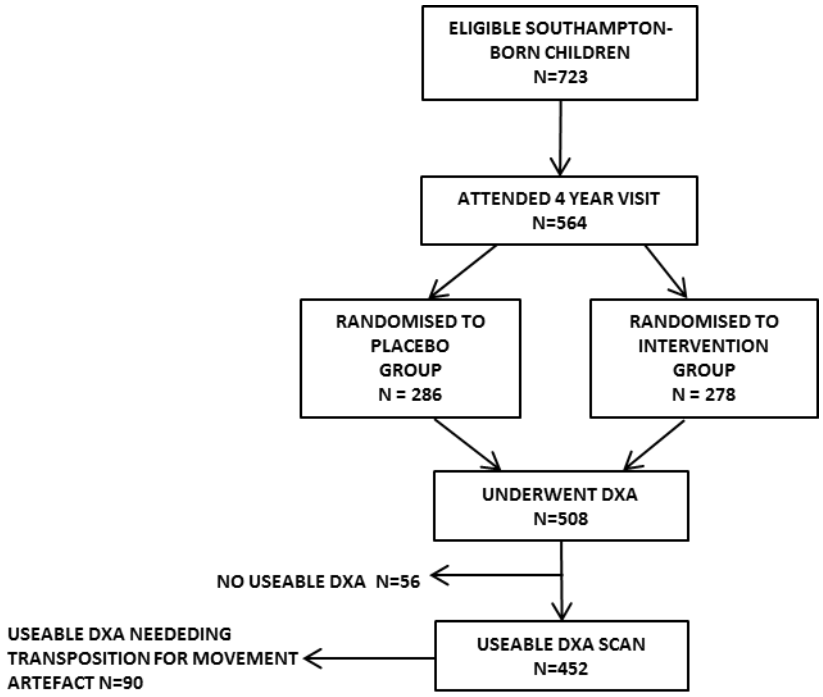


Figure 7.1 MAVIDOS Trial Consort Diagram for the Southampton –based 4 year followup.

Maternal characteristics did not differ between the two randomisation groups for those attending the 4 year visit (all $p > 0.05$), as shown in Table 7.1. No statistically significant differences were observed in early pregnancy plasma 25(OH)D concentrations between the groups. Plasma 25(OH)D concentrations obtained in late pregnancy were significantly higher in the mothers in the cholecalciferol supplemented group ($p < 0.001$). Late pregnancy 25(OH)D was significantly greater in the cholecalciferol supplemented groups regardless of season of delivery. Late pregnancy 25(OH)D was greater in the placebo group in "summer/autumn" births than "winter/spring" births, therefore the increase in 25(OH)D attributable to supplementation was greater in "winter/spring" births ("winter/spring" placebo group late pregnancy mean 25(OH)D 24.55 nmol/l, compared with "summer/autumn" placebo group late pregnancy mean 25(OH)D 52.8 nmol/l). In comparison to the mothers who remained in the study to delivery, mothers attending the 4 year follow-up visit were older at time of delivery, of higher educational attainment and a smaller proportion smoked in early pregnancy (all $p < 0.001$) or late pregnancy ($p = 0.009$), as shown in Table 7.2.

Table 7.1 Characteristics of the mothers of the children attending the MAVIDOS 4 year followup.

"Winter/Spring" births are defined as December to May, "Summer/Autumn" births are defined as June to November. Early pregnancy (EP) is 14 weeks gestation, late pregnancy (LP) is 34 weeks gestation. Shown as mean (SD), n (%) or median (IQR)+*

	n	*Placebo*	*n*	*Cholecalciferol 1000 IU/day*	*p difference*
Maternal age	286	32.1 (4.7)	278	32.0 (4.7)	0.844
*White ethnicity**	269	260 (96.7)	263	248 (94.3)	0.190
*Nulliparous**	267	114 (42.7)	265	115 (43.4)	0.871
*Educated to A level or higher**	266	216 (81.2)	264	221 (83.7)	0.448
Height	265	166.3 (6.4)	266	165.6 (6.3)	0.224
BMI+	265	25.5 (22.8, 29.6)	266	24.9 (22.3, 28.5)	0.149
*Early pregnancy smoking**	268	14 (5.2)	265	11 (4.2)	0.558
*Late pregnancy smoking**	254	13 (5.1)	245	12 (4.9)	0.910
Moderate/strenuous physical activity in LP (hrs/week)	181	0.83 (0.52)	174	0.88 (0.74)	0.427
*Offspring sex (male)**	285	144 (50.5)	278	161 (57.9)	0.079
Maternal vitamin D					
EP 25(OH)D (nmol/l)+	280	45.05 (33.93, 56.35)	273	44.95 (33.85, 57.35)	0.952
LP 25(OH)D (nmol/l)+	257	42.35 (23.25, 56.40)	252	67.40 (56.15, 80.30)	**< 0.001**
"Winter/Spring" births: EP 25(OH)D (nmol/l)+	134	47.05 (35.25, 56.45)	127	47.20 (34.75, 59.9)	0.699
"Winter/Spring" births: LP 25(OH)D (nmol/l)+	122	24.55 (16.9, 42.35)	113	64.10 (54.4, 74.45)	**< 0.001**
"Summer/Autumn" births: EP 25(OH)D (nmol/l)+	145	42.9 (32.65, 55.5)	146	43.0 (30.8, 55.4)	0.788
"Summer/Autumn" births: LP 25(OH)D (nmol/l)+	135	52.8 (38.65, 63.6)	139	69.4 (58.45, 83.35)	**< 0.001**

Table 7.2 Comparison of the mothers attending the four year follow up visit with mothers remaining in the study until delivery. *Shown as mean (SD), n (%)* or median (IQR)+*

	n	*4-year visit attendees*	*n*	*4-year visit non-attendees*	*p difference*
Maternal age at delivery (years)	564	32.03 (4.71)	402	29.86 (5.48)	**< 0.001**
*Ethnicity (white vs non-white)**	532	508 (95.5)	397	373 (94.0)	0.296
*Parity (nulliparous)**	532	229 (43.0)	395	174 (44.1)	0.093
*Educational attainment (A level or higher)**	530	437 (82.5)	396	281 (71.0)	**< 0.001**
Height (cm)	531	165.97 (6.37)	396	165.20 (6.53)	0.070
BMI (kg/m^2)+	531	25.00 (22.62, 29.21)	396	25.06 (22.51, 29.31)	0.746
*Early pregnancy smoking**	533	25 (4.69)	397	47 (11.84)	**< 0.001**
*Late pregnancy smoking**	499	25 (5.01)	341	33 (9.68)	**0.009**
Moderate/strenuous physical activity in LP (hrs/week)	355	0.86 (0.64)	193	0.91 (0.57)	0.355
*Offspring sex (male)**	563	305 (54.2)	402	212 (52.7)	0.659

Table 7.3 Characteristics of the children of the Southampton arm of the MAVIDOS trial attending the four year follow-up visit. *Shown as mean (SD), n (%)* or median (IQR)+*

	n	*Placebo*	*n*	*Cholecalciferol 1000 IU/day*	*p difference*
Age (years)+	284	4.08 (4.03, 4.16)	276	4.07 (4.03, 4.15)	0.61
Gestational age at birth (weeks)+	285	40.29 (39.29, 41.00)	278	40.29 (39.29, 41.00)	0.95
*Male sex**	286	144 (50.3)	278	161 (57.9)	0.08
Weight (kg)	285	17.20 (2.06)	275	17.37 (2.20)	0.34
Height (cm)	281	104.78 (4.43)	274	105.19 (4.38)	0.27
BMI (kg/m^2)	281	15.65 (1.26)	274	15.66 (1.24)	0.91
OFC (cm)	274	50.88 (1.59)	266	50.98 (1.57)	0.49
MUAC (cm)	274	17.42 (1.23)	263	17.44 (1.27)	0.88
Triceps SFT (mm)	251	10.13 (2.26)	234	9.95 (2.35)	0.38
Subscapular SFT (mm)+	240	6.08 (5.37, 7.13)	231	5.97 (5.07, 7.00)	0.09

The median (IQR) age of children attending the four year visit was 4.08 (4.03, 4.16) years in the placebo group and 4.07 (4.03, 4.15) years in the cholecalciferol group. Characteristics and anthropometry of the children attending the four year visit did not differ significantly, numerically there were more boys in the cholecalciferol group, and weight and height were greater. Subscapular SFT was lower in the cholecalciferol group though the difference was of borderline statistical significance (p = 0.09).

7.3.2 Maternal cholecalciferol supplementation and offspring bone indices, lean mass and grip strength at 4 years of age

Table 7.4 Whole body less head DXA, lumbar spine DXA and grip strength assessed at age 4 years in the children born to mothers randomised to placebo or cholecalciferol 1000 IU/day in pregnancy. *Outcomes are unadjusted. Values are mean (95% confidence interval) unless otherwise stated.*

	n	*Placebo*	*n*	*Cholecalciferol 1000 IU/day*	*p difference*
Bone outcomes:					
Whole body (less head):					
BA (cm^2)	246	756.7 (750.2, 763.2)	248	756.0 (749.3, 762.7)	0.878
BMC (g)	246	356.7 (351.2, 362.2)	248	361.2 (355.7, 366.7)	0.252
BMD (g/cm^2)	246	0.470 (0.466, 0.475)	248	0.477 (0.472, 0.481)	**0.048**
Lumbar spine:					
BA (cm^2)	248	24.8 (24.3, 25.3)	248	25.3 (24.8, 25.9)	0.153
BMC (g)	248	14.3 (13.9, 14.6)	248	14.6 (14.2, 15.0)	0.212
BMD (g/cm^2)	248	0.576 (0.570, 0.583)	248	0.576 (0.569, 0.583)	0.930
Body composition:					
Whole body (less head):					
Lean (g)	248	9006.3 (8830.2, 9182.4)	248	9248.3 (9080.0, 9416.5)	**0.051**
Fat (g) (median, IQR)	248	4516.9 (3882.8, 5360.0)	248	4447.0 (3779.8, 5276.2)	0.172
Lean mass percentage	248	64.3 (63.5, 65.0)	248	65.1 (64.4, 65.8)	0.091
Fat mass percentage	248	33.1 (32.4, 33.9)	248	32.4 (31.7, 33.0)	0.124
Grip strength:					
Maximum (kg)	262	5.74 (5.52, 5.97)	253	5.92 (5.69, 6.15)	0.271
Mean (of 6 attempts) (kg)	262	4.53 (4.34, 4.73)	253	4.67 (4.48, 4.86)	0.328

Whole body (less head) bone mineral density was statistically significantly greater in the children of gestational cholecalciferol supplemented mothers (a difference of 0.007 g/cm^2, p = 0.048), as shown in Table 7.4. Whole body (less head) BMC and BA did not differ between the two groups, though BMC was numerically greater in the cholecalciferol supplemented group. Lumbar spine BMC and BA were greater in the children of cholecalciferol supplemented mothers, though these differences did not reach statistical significance. In terms of body composition, whole body (less head) lean mass was 241.98 g greater in the 4 year old offspring of the cholecalciferol supplemented group, of borderline statistical significance (p = 0.051). A trend towards a slightly greater lean mass percentage (0.85% greater in the cholecalciferol vs. placebo group) was observed (p = 0.091). There were no statistically significant differences in fat mass, though it was numerically greater in the children of placebo group mothers.

Grip strength, recorded as maximum grip strength and mean of six attempts (three in each hand) did not differ significantly between the two groups (p = 0.271 and p = 0.328 respectively), though was numerically greater in the children of cholecalciferol supplemented mothers (a difference of 0.18 kg maximum grip strength).

Whole body less head bone outcomes: multivariate regression analysis

On multivariate regression analysis of the association between gestational cholecalciferol supplementation versus placebo and offspring whole body bone outcomes, receiving 1000 IU/day cholecalciferol supplementation from 14 weeks gestation was associated with 0.18 SD (95% CI 0.00, 0.35) greater offspring bone mineral density at age 4 years, following adjustment for sex and age (n = 489, p = 0.047) as shown in Table 7.5 and Figure 7.2. Following additional adjustment for maternal early pregnancy BMI, the associations were slightly attenuated [β = 0.16 SD (-0.02, 0.33), p = 0.083] though 28 women did not have a measurement of BMI available (n = 461). In the fully adjusted model (adjusted for sex, age, gestational age at birth, early pregnancy BMI, and child's weekly "screen time" (time spent watching TV or looking at a computer screen each week) these associations remained similar in magnitude [β = 0.16 SD (-0.01, 0.34), p = 0.072, n = 460]. Maternal treatment group was not statistically significantly associated with offspring bone area [β = 0.02 SD (-0.16, 0.19), p = 0.858] or bone mineral content [β = 0.12 SD (-0.06, 0.30), p = 0.189] at age four years, though associations remained in the positive direction.

When the season of birth of the children was split into two groups, "Winter/Spring" (December to May) and "Summer/Autumn" (June to November), offspring born in "Winter/Spring" saw a greater benefit of maternal cholecalciferol supplementation than those born in "Summer/Autumn" as shown in Figure 7.3. Though not reaching statistical significance, offspring of vitamin D supplemented mothers born in the winter months had greater age and sex-adjusted bone area [β = 0.16 SD (-0.09, 0.41), p = 0.204], bone mineral content [β = 0.23 SD (-0.04, 0.50), p = 0.093] and bone mineral density [β = 0.22 SD (-0.05, 0.48), p = 0.106]. These associations were unchanged after adjustment for maternal BMI and after full adjustment.

Lumbar spine bone outcomes: multivariate regression analysis

In the four year old children, associations between maternal treatment group and measures of lumbar spine bone health were very similar to the whole body (less head) outcomes as shown in Figure 7.4. Maternal cholecalciferol supplementation was associated with greater lumbar spine area, bone mineral content and bone mineral density, though these associations did not reach statistical significance. However, offspring born in "winter/spring", whose mothers however had received the cholecalciferol supplement, had significantly greater lumbar spine bone mineral content [β = 0.27 SD (0.00, 0.53), p = 0.043, n = 230] than those receiving placebo, after

adjustment for sex and age (Figure 7.5). The magnitude of this association remained after adjustment for maternal early pregnancy BMI and after full adjustment, though statistical significance was slightly reduced (p = 0.061 and p = 0.069).

Lean mass and fat mass: multivariate regression analysis

Whole body (minus head) lean mass was measured in 491 children. Maternal supplementation with cholecalciferol in pregnancy was associated with a trend towards greater lean mass in children at age 4 years, [β = 0.15 SD (-0.02, 0.31), p = 0.081 after adjustment for sex and age]. Following adjustment for sex, age, gestational age at birth, maternal BMI in early pregnancy, and child's "screen time", a surrogate marker for sedentary behaviour, associations were slightly strengthened [β = 0.17 SD (0.00, 0.34), p = 0.048]. As for the bone outcomes, there was a trend towards greater effects of cholecalciferol supplementation on lean mass in children born in the "winter/spring" months [β = 0.21 SD (-0.04, 0.45), p = 0.097], versus the "summer/autumn" months [β = 0.09 SD (-0.13, 0.32), p = 0.410, adjusted for sex and age]. Full adjustment attenuated these seasonal differences markedly.

No associations between lean mass percentage or fat mass percentage and randomisation group were observed. However, when analysed by season of birth, statistically significant associations between maternal treatment group and offspring lean mass percentage in "summer/autumn" births [β = 0.24 SD (0.04, 0.45), p = 0.021] were observed, following adjustment for age and sex, though these associations were of borderline significance after full adjustment (p = 0.079). As would be expected, in the same group there was a reciprocal effect of similar magnitude, with maternal cholecalciferol supplementation being associated with lower fat mass in the "summer/autumn" born children [β = -0.26 SD (-0.46, -0.05), p = 0.016] after adjustment for sex and age.

Grip strength: multivariate regression analysis

Grip strength was measured in 512 four year old children. Although both maximum and mean grip strength were numerically higher in the cholecalciferol supplemented group, these differences were very slight and not statistically significant, following adjustment for sex and height. Seasonal differences were observed, in the same direction as in bone outcomes and lean mass, with a trend towards greater effects of maternal cholecalciferol supplementation in "winter/spring" born children though these associations were non statistically significant. For example, for maximum grip strength, "winter/spring" born children had 0.15 SD greater maximum grip strength if their mothers were supplemented with cholecalciferol [(-0.08, 0.40), p = 0.097],

whereas the effect was smaller in the “summer/autumn” born children [β = 0.09 SD (-0.13, 0.32), p = 0.410]. Adjustment for sex, height, gestational age at birth, parity, maternal smoking in late pregnancy and child’s screen time did not alter the magnitude or direction of these relationships, but they remained not statistically significant.

Table 7.5 Associations between maternal treatment group (cholecalciferol 1000 IU/ day versus placebo) and whole body less head DXA outcomes in their children assessed at age 4 years. *Regression coefficients are expressed in standard deviations (z scores). "Winter/Spring" births: children born December to May. "Summer/Autumn" births: children born June to November.*

	Cholecalciferol vs placebo Adjusted for sex and age				*Cholecalciferol vs placebo Adjusted for sex, age and maternal BMI*				*Cholecalciferol vs placebo Adjusted for sex, age, gestational age at birth, EPBMI, child's screen time*			
WBLH DXA outcomes	**N**	**β (SD)**	**95% CI**	**P value**	**n**	**β (SD)**	**95% CI**	**P value**	**n**	**β (SD)**	**95% CI**	**P value**
Bone area	489	0.02	(-0.16, 0.19)	0.858	461	0.01	(-0.17, 0.19)	0.934	460	0.02	(-0.16, 0.20)	0.864
"Winter/Spring" births	228	0.16	(-0.09, 0.41)	0.204	224	0.15	(-0.10, 0.41)	0.243	224	0.14	(-0.12, 0.39)	0.293
"Summer/Autumn" births	261	-0.12	(-0.36, 0.13)	0.356	237	-0.10	(-0.36, 0.15)	0.423	236	-0.07	(-0.32, 0.19)	0.601
Bone mineral content	489	0.12	(-0.06, 0.30)	0.189	461	0.10	(-0.08, 0.28)	0.279	460	0.11	(-0.07, 0.29)	0.241
"Winter/Spring" births	228	0.23	(-0.04, 0.50)	0.093	224	0.20	(-0.07, 0.48)	0.142	224	0.19	(-0.09, 0.46)	0.178
"Summer/Autumn" births	261	0.02	(-0.22, 0.25)	0.886	237	0.02	(-0.22, 0.27)	0.850	236	0.05	(-0.20, 0.31)	0.679
Areal bone mineral density	489	0.18	(0.00, 0.35)	**0.047**	461	0.16	(-0.02, 0.33)	0.083	460	0.16	(-0.01, 0.34)	0.072
"Winter/Spring" births	228	0.22	(-0.05, 0.48)	0.106	224	0.19	(-0.08, 0.45)	0.169	224	0.17	(-0.09, 0.44)	0.204
"Summer/Autumn" births	261	0.14	(-0.10, 0.37)	0.243	237	0.14	(-0.10, 0.38)	0.254	236	0.16	(-0.09, 0.40)	0.205

Table 7.6 Associations between maternal treatment group (cholecalciferol 1000 IU/ day versus placebo) and lumbar spine DXA outcomes in their children assessed at age 4 years. *Regression coefficients are expressed in standard deviations (z scores). "Winter/Spring" births: children born December to May. "Summer/Autumn" births: children born June to November.*

	Cholecalciferol vs placebo Adjusted for sex and age				*Cholecalciferol vs placebo Adjusted for sex, age and maternal BMI*				*Cholecalciferol vs placebo Adjusted for sex, age, gestational age at birth, LP BMI, child's screen time*			
Lumbar spine DXA outcomes	**N**	**β (SD)**	**95% CI**	**P value**	**n**	**β (SD)**	**95% CI**	**P value**	**n**	**β (SD)**	**95% CI**	**P value**
Lumbar spine Bone area	481	0.12	(-0.03,0.30)	0.182	463	0.13	(-0.05, 0.31)	0.164	451	0.03	(-0.15, 0.22)	0.718
"Winter/Spring" births	222	0.20	(-0.06, 0.45)	0.131	226	0.18	(-0.08, 0.44)	0.169	218	0.12	(-0.16, 0.40)	0.385
"Summer/Autumn" births	259	0.06	(-0.19, 0.31)	0.640	237	0.09	(-0.17, 0.34)	0.509	233	-0.06	(-0.31, 0.20)	0.653
Lumbar spine Bone mineral content	491	0.12	(-0.06, 0.29)	0.204	463	0.13	(-0.05, 0.31)	0.168	462	0.13	(-0.06, 0.31)	0.177
"Winter/Spring" births	230	0.27	(0.00, 0.53)	**0.043**	226	0.25	(-0.01, 0.52)	0.061	226	0.24	(-0.02, 0.51)	0.069
"Summer/Autumn" births	261	-0.01	(-0.26, 0.23)	0.914	237	0.02	(-0.24, 0.28)	0.881	236	0.02	(-0.24 0.27)	0.903
Lumbar spine Areal bone mineral density	491	0.01	(-0.16, 0.19)	0.886	463	0.03	(-0.16, 0.21)	0.764	462	0.03	(-0.15, 0.22)	0.725
"Winter/Spring" births	230	0.18	(-0.09, 0.45)	0.198	226	0.17	(-0.10, 0.45)	0.217	226	0.17	(-0.11, 0.45)	0.233
"Summer/Autumn" births	261	-0.13	(-0.37, 0.10)	0.269	237	-0.10	(-0.35, 0.14)	0.407	236	-0.08	(-0.33, 0.18)	0.557

Table 7.7 Associations between maternal treatment group (cholecalciferol 1000 IU/ day versus placebo) and whole body (less head) lean mass assessed by DXA, lean mass percentage, fat mass and fat mass percentage. *Regression coefficients are expressed in standard deviations (z scores). "Winter/Spring" births: children born December to May. "Summer/Autumn" births: children born June to November.*

	Cholecalciferol vs placebo Adjusted for sex and age				*Cholecalciferol vs placebo Adjusted for sex, age and maternal EP BMI*				*Cholecalciferol vs placebo Adjusted for sex, age, gestational age at birth, EP BMI, child's screen time*			
Body composition DXA outcomes	**n**	**β (SD)**	**95% CI**	**P value**	**n**	**β (SD)**	**95% CI**	**P value**	**n**	**β (SD)**	**95% CI**	**P value**
Lean mass	491	0.15	(-0.02, 0.31)	0.081	463	0.16	(-0.01, 0.33)	0.060	462	0.17	(0.00, 0.34)	**0.048**
"Winter/Spring" births	229	0.21	(-0.04, 0.45)	0.097	225	0.19	(-0.06, 0.44)	0.138	225	0.17	(-0.07, 0.42)	0.166
"Summer/Autumn" births	262	0.09	(-0.13, 0.32)	0.410	238	0.15	(-0.08, 0.38)	0.197	237	0.18	(-0.05, 0.42)	0.125
Lean mass percentage	491	0.11	(-0.05, 0.27)	0.175	463	0.09	(-0.07, 0.25)	0.250	462	0.08	(-0.08, 0.24)	0.305
"Winter/Spring" births	229	-0.04	(-0.28, 0.20)	0.748	225	-0.03	(-0.27, 0.22)	0.833	225	-0.01	(-0.26, 0.23)	0.907
"Summer/Autumn" births	262	0.24	(0.04, 0.45)	**0.021**	238	0.20	(-0.01, 0.41)	0.057	237	0.19	(-0.02, 0.40)	0.079
Fat mass	491	0.01	(-0.16, 0.18)	0.896	463	0.04	(-0.13, 0.21)	0.655	462	0.05	(-0.12, 0.23)	0.537
"Winter/Spring" births	228	0.25	(-0.01, 0.51)	0.062	224	0.23	(-0.04, 0.49)	0.095	224	0.20	(-0.06, 0.47)	0.129
"Summer/Autumn" births	263	-0.20	(-0.43, 0.02)	0.077	239	-0.12	(-0.35, 0.10)	0.273	238	-0.09	(-0.32, 0.13)	0.427
Fat mass percentage	491	-0.09	(-0.24, 0.07)	0.281	463	-0.07	(-0.22, 0.09)	0.412	462	-0.06	(-0.21, 0.10)	0.496
"Winter/Spring" births	228	0.10	(-0.13, 0.34)	0.394	224	0.09	(-0.14, 0.33)	0.450	224	0.08	(-0.16, 0.31)	0.518
"Summer/Autumn" births	263	-0.26	(-0.46, -0.05)	**0.016**	239	-0.21	(-0.42, 0.00)	0.053	238	-0.20	(-0.41, 0.02)	0.076

Table 7.8 Associations between maternal treatments group (cholecalciferol 1000 IU/ day versus placebo) and grip strength assessed by Jamar dynamometer.

*Outcomes are displayed as maximum grip and mean (of six attempts, three in the left hand and three in the right). Mean Regression coefficients are expressed in standard deviations (z scores). "Winter/*Spring*" births: children born December to May. "Summer/Autumn" births: children born June to November.*

	Cholecalciferol vs placebo Adjusted for sex and height				*Cholecalciferol vs placebo Adjusted for sex, height and maternal EP BMI*				*Cholecalciferol vs placebo Adjusted for sex, height, gestational age at birth, parity, LP smoking, child's screen time*			
Grip strength outcomes	**n**	**β (SD)**	**95% CI**	**P value**	**n**	**β (SD)**	**95% CI**	**P value**	**n**	**β (SD)**	**95% CI**	**P value**
Maximum grip	512	0.04	(-0.12,0.20)	0.613	480	0.06	(-0.11, 0.23)	0.516	428	0.02	(-0.16, 0.20)	0.797
"Winter/Spring" births	242	0.15	(-0.08, 0.40)	0.202	238	0.15	(-0.09, 0.39)	0.226	208	0.19	(-0.08, 0.46)	0.160
"Summer/Autumn" births	270	-0.06	(-0.28, 0.16)	0.593	242	-0.03	(-0.27, 0.21)	0.777	220	-0.17	(-0.42, 0.06)	0.149
Mean grip	512	0.03	(-0.13,0.19)	0.746	480	0.04	(-0.13, 0.20)	0.667	428	-0.02	(-0.20,0.15)	0.784
"Winter/Spring" births	242	0.12	(-0.12, 0.36)	0.324	238	0.12	(-0.12, 0.37)	0.321	208	0.12	(-0.14, 0.38)	0.361
"Summer/Autumn" births	270	-0.06	(-0.27, 0.16)	0.616	242	-0.05	(-0.28, 0.19)	0.698	220	-0.20	(-0.44, 0.03)	0.093

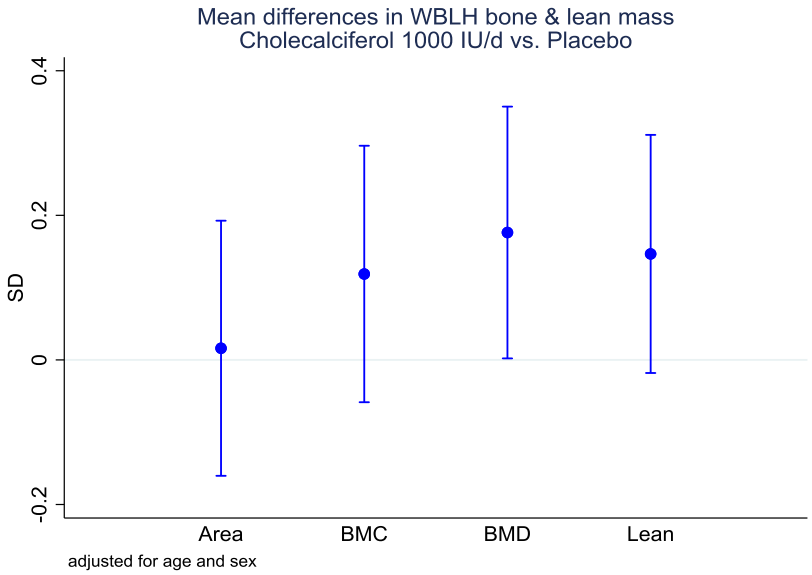


Figure 7.2 The effect of gestational cholecalciferol supplementation (1000 IU/day from 14 weeks gestation) compared with placebo on offspring whole body (less head) bone area, bone mineral content, bone mineral density and lean mass assessed by DXA at age 4 years. *Each bar is the outcome of a separate linear regression adjusted for age and sex, outcomes are expressed as z scores (SDs, 95% CI).*

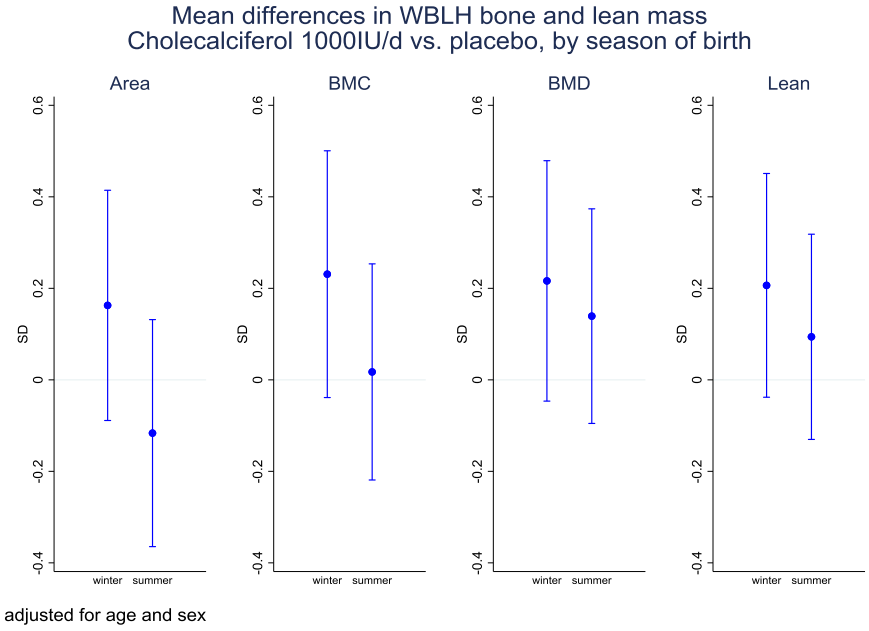


Figure 7.3 The effect of gestational cholecalciferol supplementation compared with placebo on offspring whole body (less head) bone area, bone mineral content, bone mineral density and lean mass assessed by DXA at age 4 years, stratified by season of birth ("winter/spring", labelled winter, December to May; "summer/autumn", labelled summer, June to November). *Each bar is the outcome of a separate linear regression adjusted for age and sex, outcomes are expressed as z scores (SDs, 95% CI).*

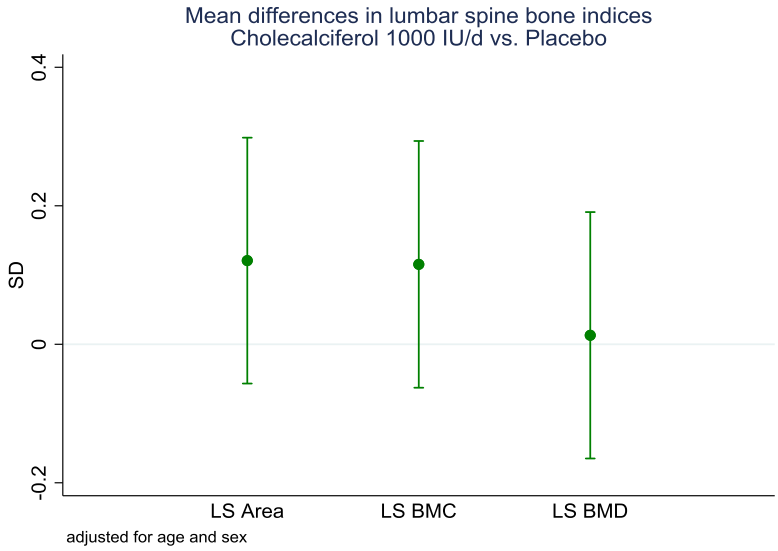


Figure 7.4 The effect of gestational cholecalciferol supplementation compared with placebo on offspring lumbar spine bone area, bone mineral content and bone mineral density assessed by DXA at age 4 years. *Each bar is the outcome of a separate linear regression adjusted for age and sex, outcomes are expressed as z scores (SDs, 95% CI).*

Figure 7.5 The effect of gestational cholecalciferol supplementation compared with placebo on offspring lumbar spine bone area, bone mineral content and bone mineral density at age 4 years stratified by season of birth ("winter/spring", labelled winter, December to May; "summer/autumn", labelled summer, June to November). *Each bar is the outcome of a separate linear regression adjusted for age and sex, outcomes are expressed as z scores (SDs, 95% CI).*

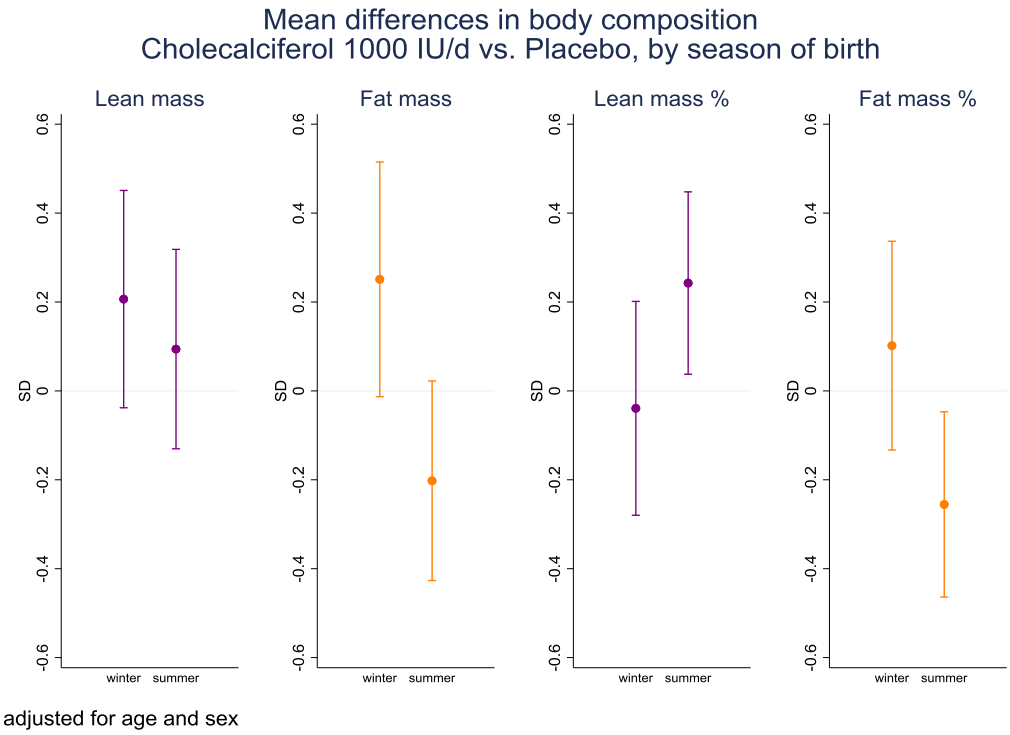


Figure 7.6 The effect of gestational cholecalciferol supplementation compared with placebo on offspring whole body (less head) lean mass, fat mass, lean mass percentage and fat mass percentage at age 4 years stratified by season of birth ("winter/spring", labelled winter, December to May; "summer/autumn", labelled summer, June to November). *Each bar is the outcome of a separate linear regression adjusted for age and sex, outcomes are expressed as SDs, (95% CI).*

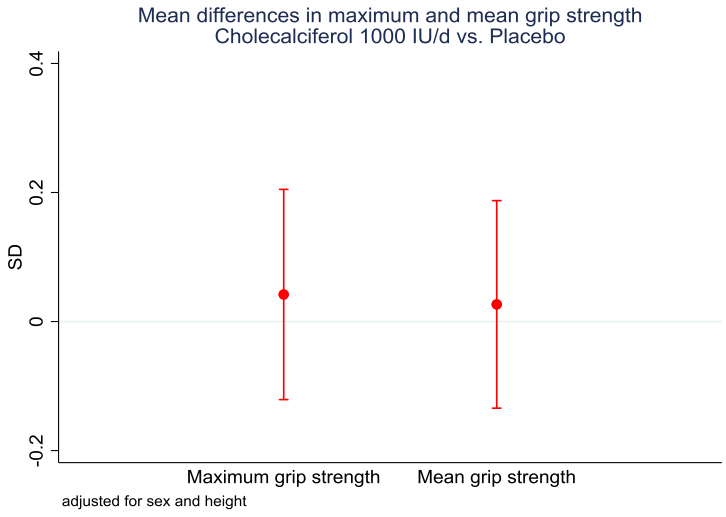


Figure 7.7 The effect of gestational cholecalciferol supplementation compared with placebo on offspring maximum grip strength and mean grip strength at age 4 years. *Each bar is the outcome of a separate linear regression adjusted for height and sex, outcomes are expressed as z scores (SDs, 95% CI).*

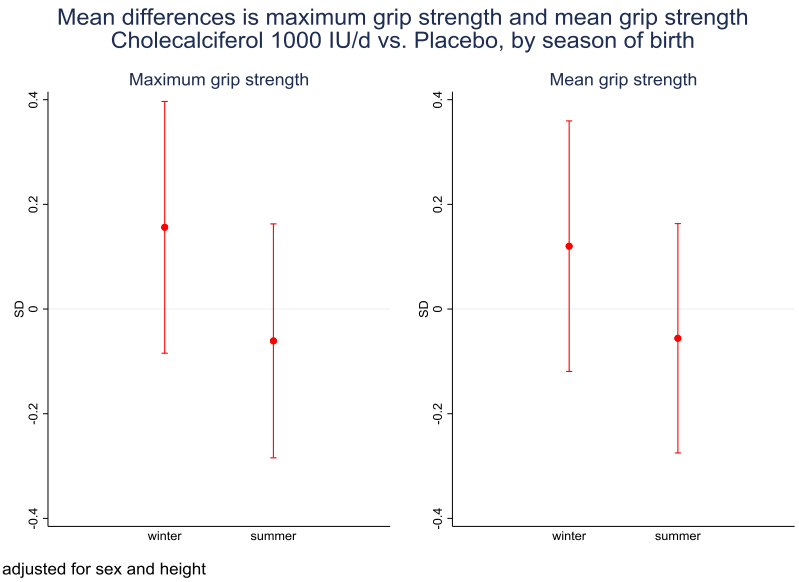


Figure 7.8 The effect of gestational cholecalciferol supplementation compared with placebo on offspring maximum grip strength and mean grip strength at age 4 years, stratified by season ("winter/spring", labelled winter, December to May; "summer/autumn", labelled summer, June to November). *Each bar is the outcome of a separate linear regression adjusted for height and sex, outcomes are expressed as z scores (SDs, 95% CI).*

7.3.3 Sensitivity analyses: exclusion of preterm babies, exclusion of participants of non – white ethnicity, exclusion of DXA data for children with limb movement and cross-imputation of limb data.

Sensitivity analysis restricted to babies born from 37 weeks gestation

Exclusion of preterm babies born prior to 37 weeks gestation reduced the sample size by 18 (n = 471). Maternal cholecalciferol supplementation was still associated with greater offspring bone mineral density (whole body less head) at age 4 years, though reduction in sample size meant that this association was no longer statistically significant [β = 0.16 SD (-0.02, 0.33), p = 0.086 following adjustment for sex and age]. Associations with offspring lean mass were attenuated further [β = 0.12 SD (-0.05, 0.29), p = 0.151, following adjustment for sex and age].

Sensitivity analysis restricted to white Caucasian offspring

This also led to a reduction of sample size by 18 (n = 471), and loss of statistical significance of associations between maternal cholecalciferol and BMD at age 4 years though associations were similar in magnitude [β = 0.17 SD (-0.01, 0.35), p = 0.064 following adjustment for sex and age].

Associations with offspring lean mass were, again, further attenuated [β = 0.13 SD (-0.04, 0.30), p = 0.124, following adjustment for sex and age].

Sensitivity analysis restricted to scans without cross-imputation of data to account for limb movement

156 scans with limb movement were removed from the analysis, which led to a loss of associations between maternal cholecalciferol supplementation and offspring BMD and lean mass [n = 356, β = 0.09 SD (-0.12, 0.29), p = 0.413] following adjustment for sex and age). Similarly, the observed associations with lean mass were lost [n = 357, β = 0.04 SD (-0.16, 0.23), p = 0.709 following adjustment for sex and age].

7.4 Discussion

7.4.1 Summary of findings

In a population of women with baseline 25(OH)D concentrations ranging from 25-100 nmol/l, 1000 IU/day cholecalciferol supplementation from 14 weeks gestation until delivery was associated with greater offspring WBLH bone mineral density at age 4 years (a difference of 0.007 g/cm^2, p = 0.048, n = 494). On multivariate regression analysis, following adjustment for sex and age, this was equivalent to 0.18 SD (95% CI 0.00, 0.35) greater bone mineral density in the children. Following full adjustment (for sex, age, gestational age, late pregnancy maternal BMI, child's screen time) these associations were slightly attenuated [β = 0.16 SD (-0.01, 0.34), p = 0.072, n = 460].

WBLH bone mineral content, and lumbar spine bone area and bone mineral content were greater in the offspring of gestational cholecalciferol supplemented mothers, but the differences between the groups did not reach statistical significance.

Maternal cholecalciferol supplementation was also associated with differences in offspring body composition. WBLH lean mass at age 4 years was 242.0 g greater in the offspring of mothers receiving gestational cholecalciferol supplementation compared with placebo (p = 0.051). Following multivariate regression analysis with full adjustment, lean mass was 0.17 SD (0.00, 0.34) greater in the offspring of cholecalciferol supplemented mothers, compared with placebo (p = 0.048). No statistically significant differences in fat mass were observed, though it was numerically lower in the children of cholecalciferol supplemented mothers.

Maximum and mean grip strength, though numerically higher in the offspring of cholecalciferol supplemented mothers, were not statistically significantly different.

When stratified by season of birth, there was a trend towards greater effects of maternal cholecalciferol supplementation on bone outcomes (WBLH and lumbar spine) and lean mass in children born in the "winter/spring" months. For example, in four year old children born between December and May, WBLH bone mineral content was 0.23 SD [(-0.04, 0.50) p = 0.093] greater if their mothers received gestational cholecalciferol supplementation, and lean mass was 0.21 SD [(-0.04, 0.45) p = 0.097] greater, after adjustment for age and sex. Seasonal differences in bone indices were more prominent at the lumbar spine, and reached statistical significance. In "winter/spring" born offspring of cholecalciferol supplemented mothers, cholecalciferol supplementation was associated with 0.27 SD [(0.00, 0.53) p = 0.043] greater lumbar spine bone mineral content at age 4 compared to placebo (adjusted for age and sex).

When body composition was analysed as percentages of the total body mass, the greatest magnitude of effect was observed in the "summer/autumn" born offspring of cholecalciferol supplemented mothers. In "summer/autumn" births, lean mass percentage was 0.24 SD [(0.04, 0.45) p = 0.021] greater than the placebo group; correspondingly, fat mass percentage was -0.26 SD [(-046, -0.05) p = 0.016] lower than the placebo group.

7.4.2 Vitamin D supplementation in pregnancy and offspring bone health in childhood

These findings, demonstrating that maternal cholecalciferol supplementation is associated with greater whole body (less head) BMD in children (age 4) are novel as, to my knowledge, MAVIDOS is the first trial to test these associations. As detailed previously, in the MAVIDOS trial at birth, there were no significant differences between the randomisation groups in neonatal WBLH bone area, BMC or BMD. However, a significant interaction was observed between season of birth and maternal randomisation group (p for interaction between season and BMC = 0.04). Whole body BMC and BMD were approximately 9% and 5% higher (> 0.5 SD greater), respectively, in the children born in winter (December – February) to mothers randomised to cholecalciferol compared to those randomised to placebo (377). In this study, at 4 years of age, greater WBLH BMD (0.18 SD, p = 0.047) was seen in the offspring of the cholecalciferol supplemented mothers, regardless of birth season, after age and sex adjustment; a similar but non-significant difference in WBLH BMC was observed between the groups (0.12 SD, p = 0.189).

In keeping with the findings at birth, a greater effect of cholecalciferol supplementation on bone outcomes was observed in children born in the winter. Outcomes were stratified by dividing the

year into two halves, “winter/spring”, December to May, and “summer/autumn”, June to November (to maintain power as participant numbers were lower in the 4-year follow-up n = 496 with DXA, versus n = 665 at birth). A trend towards greater WBLH bone area, BMC, and BMD in the “winter/spring” born 4 year old children of cholecalciferol supplemented mothers than placebo was observed. At the lumbar spine, between-group differences stratified by season were more marked. “Winter/spring” born offspring of cholecalciferol supplemented mothers had 0.27 SD (p = 0.043, adjusted for sex and age) greater lumbar spine BMC than the offspring of placebo group mothers. Even after adjustment for a range of confounding factors (sex, age, gestational age at birth, maternal BMI in late pregnancy and child’s screen time), the effect size was almost unchanged (0.24 SD, p = 0.069). In the “summer/autumn” born offspring however, no significant differences in lumbar spine BMC between the cholecalciferol and placebo groups were observed. The reason behind more marked seasonal differences at the lumbar spine could be attributed to the fact that the lumbar spine is a site of greater trabecular bone mass, and it is possible that lasting effects of gestational cholecalciferol supplementation on childhood bone mass could differentially affect trabecular, as opposed to cortical bone sites. Though this hypothesis has not been tested in animal models of gestational vitamin D deficiency, discordant effects on trabecular and cortical bone have been observed in vitamin D deficient growing rodents, with greater relative loss of trabecular bone (544). In addition, due to greater scan numbers at the lumbar spine, there was greater power to detect a possible seasonal difference (the lumbar spine DXA scan is shorter in duration so fewer had to be excluded due to movement artefact).

Attrition of participant numbers led to reduced power to detect a difference in outcomes between treatment groups- 78% of eligible children in the Southampton arm of the study attended the four year follow-up. Other limitations to the MAVIDOS study, including the use of DXA and grip measurement are described in section 8.4.2. It should be recognised that any positive finding in this study is part of a post hoc secondary analysis of a randomised trial, and therefore should be interpreted with caution prior to replication in other populations.

Aside from the MAVIDOS trial, a few small intervention studies have investigated the effects of antenatal vitamin D supplementation on offspring bone mineralisation. In a non-randomised study 64 women of Asian ethnicity living in the UK, 1000 IU vitamin D and calcium supplementation during the third trimester was not associated with significant differences were observed in the forearm BMC of the offspring at birth assessed using single photon absorptiometry (536). In an Iranian study, 153 women were randomised to placebo or 2000 IU/day cholecalciferol from 26-28 weeks until delivery, but only 25 neonates (16% of the original cohort) had DXA assessment. No significant differences in whole body BMC, BMD or bone area

were found between the two groups, but, again, power to detect a difference in these outcomes was lacking (371). In a study based in India, 300 women were divided into three groups, one group receiving 400 IU/day cholecalciferol daily (the "placebo" group), a second group receiving 60 000 IU cholecalciferol every 4 weeks and a third group receiving 60 000 IU cholecalciferol every 8 weeks from around 14 weeks until delivery. Only half of the women were followed up until delivery, and 52 children (17% of the original cohort) underwent DXA at 12-16 months of age. Contrary to expectations, the children in the placebo group had significantly higher measurements of whole body BMC and BMD than the vitamin D supplemented group, though these relationships were confounded by differences in age at DXA scan. Following multivariate analysis, randomisation group was not a significant predictor of BMC or BMD (297). A meta-analysis of these trials, including the MAVIDOS trial, showed no significant effect of vitamin D supplementation in pregnancy on neonatal bone mineral or infant bone mineral content, though it did not stratify the neonates by season of birth (538).

The only other intervention study supporting a possibly beneficial effect of gestational vitamin D supplementation on offspring bone health assessed fetal skeletal development using 2D ultrasound in a population with a high prevalence of vitamin D deficiency, Iran. 140 women were randomised to 1000 IU per day cholecalciferol versus placebo from 6-8 weeks gestation. In the cholecalciferol supplemented group, fetal femur length and humerus length were significantly greater, when assessed in both the second and third trimester. They also demonstrated increased growth of fetal bones in the cholecalciferol supplemented group, as longitudinal measurements at the femoral and humeral proximal metaphysis, midshaft and distal metaphysis were significantly greater, though exact gestation was not reported and was not corrected for in the analyses and accurate diameter measurements are limited in 2D ultrasound (373). As described in Chapter 1.7.9, Mahon et al. used 3D ultrasound to measure fetal femur length and distal metaphyseal cross sectional area, together with the ratio of metaphyseal cross-sectional area to femur length (femoral splaying index) in pregnant mothers at 19 and 34 weeks gestation in the Southampton Women's Survey. They demonstrated an increase in the femoral splaying index (as is seen in childhood rickets) in mothers with lower 25(OH)D concentration but no relationships with femur length were observed (358). Unfortunately, femoral splaying index could not be calculated in the Vafaei et al. study; and anthropometry or DXA characteristics of the children at birth have not been reported.

Observational studies, described in detail in 1.7.9, provide conflicting evidence of associations between maternal vitamin D status and offspring bone development. The majority of published studies support the hypothesis that lower maternal serum 25(OH)D concentrations are associated

with poorer offspring bone indices. Some used season of birth as a proxy for maternal 25(OHD) status; in the SWS, there was marked seasonal variation in 25(OH)D status in both early and late pregnancy (340), this pattern was observed in other population studies from similar latitudes (325, 382). Whilst several observational studies suggested that neonates born in the summer months or to mothers with higher UVB exposure had higher BMC, in line with higher maternal 25(OH)D concentrations (in the SWS, summer/autumn births had 2.0 g greater neonatal BMC than winter/spring births, p = 0.03) (351, 362, 545), others did not (352).

Similarly, conflicting results have come from studies measuring 25(OH)D status in maternal blood samples or offspring cord blood. A Canadian study showed that higher infant plasma 25(OH)D measurements in cord blood were associated with higher whole body BMC and femoral BMC in the neonates (adjusted for body weight) (546) , though BMD was not reported. Concordant results were produced by a Finnish study, using maternal 25(OH)D measurements in early pregnancy and two days postpartum as predictors, and neonatal tibial pQCT measures as outcomes. Whilst positive associations between maternal 25(OH)D and tibial BMC and CSA were observed, no differences in BMD were present (354). At 14 month follow-up, these associations were attenuated, such that only tibial CSA was significantly higher (355). In a multi-ethnic cohort in the USA, maternal 25(OH)D deficiency, (measured at multiple time-points from 13 weeks gestation onwards and averaged across the pregnancy) was associated with lower neonatal BMD assessed by DXA in both sexes, and lower lean mass in males – though only in the exact gestation, and the magnitude of the effect was small (356). Finally, in a subset of the SWS, in which mother, father and neonate had DXA scanning and assessment of various characteristics, maternal serum 25(OH)D concentrations in late pregnancy were positively associated with neonatal bone size. The mean whole body BA of the female offspring of mothers with 25(OH)D levels less than 33 nmol/l was 110 *vs*. 119 cm^2 in the offspring of mothers more than 33 nmol/l (p = 0.04). The results for whole body BMC were 58 *vs*. 63 g (p = 0.04), respectively. These associations were independent of any paternal relationships with offspring bone indices (482).

No association between maternal 25(OH)D status and offspring bone indices have been reported by others– though in a Gambian study, no mothers in the study group had low 25(OH)D (357), and in the ALSPAC study (363), there was by chance a strong correlation between maternal UVB exposure and the age of offspring DXA assessment which may have confounded any association. In a subset of the Southampton Women's Survey, maternal 25(OH)D concentration measured at 34 weeks was associated with fetal femur size on 3D ultrasound, but was not significantly associated with offspring bone indices, assessed by DXA shortly after birth (359). Unfortunately, comparing the outcomes and effect sizes of these observational studies is difficult due to

differences in timing of maternal 25(OH)D measurement or estimation, and differences in methods and timing of skeletal assessment in the offspring.

No studies have reported, either in an observational or interventional setting, associations between maternal 25(OH)D status and skeletal indices in their offspring at age 4 years, limiting opportunities for comparison with our findings. Data from two Southampton – based cohorts, the Princess Anne Cohort (324) and the Southampton Women's Survey, have demonstrated associations between maternal 25(OH)D in later pregnancy and offspring WBLH and lumbar spine DXA outcomes at 8 years (n = 198) and 6-7 years (n = 1030) respectively (360). In the Princess Anne Cohort, reduced concentration of 25(OH)D in mothers at 34 weeks gestation was associated with lower whole body and lumbar spine BMC and bone area, but not BMD, at age 9 years. In addition, it was noted that mothers who reported vitamin D-containing supplement use (only 15% of the sample, n = 30), had children with significantly greater WBLH BMC (0.42 SD, p = 0.027) and bone area (0.45 SD, p = 0.024) than non-users, but not areal BMD; at the lumbar spine, similar effects on BMC (0.38 SD, p = 0.055), bone area (0.23 SD, p = 0.24) and areal BMD (0.41, p = 0.040) were observed. In the SWS, bone mineralisation at age 6 years in children born to mothers with 25(OH)D < 25 nmol/l in late pregnancy was compared to those with a 25(OH)D above this threshold, and were shown to have lower WBLH bone area [-26.0 cm^2 (-45.5, -6.5)], bone mineral content [-30.8 g (-53.8, -7.7)] and areal bone mineral density [-0.016 g/cm^2 (-0.031, -0.001)]; plus lower lumbar spine BMC [-1.0 g (-1.8, -0.2)]. The magnitude of the difference between the bone indices in the 4 year old offspring of mothers receiving cholecalciferol supplementation in the MAVIDOS study was not as great (a difference in WBLH BMD 0.007 g/cm^2, 0.18 SD after adjustment for sex and age). This attenuation of effect may be explained by the fact that mothers with early pregnancy 25(OH)D < 25 nmol/l were excluded from the trial.

A variety of mechanisms could be responsible for an association between maternal 25(OH)D status and offspring bone health. As previously discussed, the main action of $1,25(OH)_2D$ is to increase uptake of dietary calcium through the intestinal enterocytes, but it also enables PTH induced mobilisation of calcium and phosphate from bone mineral. Alterations in calcium and phosphate metabolism occur in pregnancy to allow the accretion of the fetal skeleton particularly in the third trimester (136). Maternal calcitropic hormones, including $1,25(OH)_2D$, are likely have an important role in these adaptations, as total $1,25(OH)_2D$ increases during the second and third trimesters. Concurrently vitamin D binding protein levels increase, meaning that the available pool of free (unbound) 25(OH)D may reduce (though studies are conflicting) (141, 547). With supplementation, the concentration of free 25(OH)D has been shown to increase (341), hence more will be available for conversion to $1,25(OH)_2D$ to enable intestinal calcium absorption,

meaning maternal to fetal calcium transfer may be greater. Our findings (discussed in Chapter 6) that maternal markers of bone resorption (CTX) decrease in response to vitamin D supplementation would support this.

Vitamin D (specifically $1,25(OH)_2D$) is known to play a role in the regulation of placental calcium transport (79, 82, 324) and amino acid transport, implying that the fetus in a more "vitamin D rich" mother may benefit from the increased availability of bone mineral and collagen components (438). Several amino acid transporters have vitamin D response elements (VDRE) in their promoter regions and therefore could be regulated at a transcriptional level by vitamin D (specifically, by $1,25(OH)_2D$ binding the VDR and interacting with VDRE) (548, 549). As VDREs are found in the promoter regions of various genes, vitamin D bound to the VDR can have pleiotropic effects on transcription on a variety of cell types. As 25(OH)D is readily able to cross the placenta, with studies showing that maternal and umbilical cord venous 25(OH)D are moderately well correlated, downstream gene transcription effects may occur in both the mother and offspring (550).

Furthermore, vitamin D has been shown to have various epigenomic effects. Genes in the vitamin D signalling system (such as the VDR gene, CYP2R1 (25 hydroxylase) gene and CYP27B1 (1α–hydroxylase genes) have large CpG islands in their promoter regions meaning they are responsive to DNA methylation. The VDR can interact with co-activator and co-repressor proteins which are in contact with chromatin modifiers such as histone acetylases and deacetylases, and chromatin modifiers. Chromatin modifier genes themselves are among those with a VDR target site (444). As discussed previously, we have shown in MAVIDOS that maternal vitamin D supplementation is associated with reduced levels of DNA promoter methylation at the *RXRA*, important in the downstream effects of the VDR, one of its heterodimers (514). DNA methylation at the CDKN2A locus, important in cell cycle regulation and senescence have also been associated with both childhood bone mass and fat mass. Hence, vitamin D is important in a variety of cellular, placental and epigenetic processes relevant to bone metabolism.

7.4.3 Seasonal differences in the effect of cholecalciferol supplementation on childhood bone health

Seasonal differences in the magnitude of effect of maternal gestational cholecalciferol supplementation on offspring bone indices at age 4 in the MAVIDOS trial, showing consistently greater effects of supplementation versus placebo on WBLH bone indices and lumbar spine bone indices in offspring born in winter, are consistent with findings at birth.

The seasonal variation of 25(OH)D is well documented; with a half-life of around 3 weeks, the nadir occurs in late winter or early spring (239, 530). Taking the influence of season into account, an individual's 25(OH)D within the population distribution is known to track from early to late pregnancy, as demonstrated by data from the Southampton Women's Survey in which 25(OH)D concentrations were measured at 11 and 34 weeks ($r = 0.53$, $p < 0.0001$) (340). Maternal gestational vitamin D supplementation was linked to increases in late pregnancy 25(OH)D corrected for season, as was greater physical activity. In both the SWS and MAVIDOS, greater pregnancy weight-gain was also associated with a lower late pregnancy 25(OH)D (see Figure 1.18 (341)), with maternal genotype providing further variation in response to supplementation (551).

In the MAVIDOS trial, seasonal fluctuation in maternal 25(OH)D status in late pregnancy was observed in both groups, however, there was a reduction in amplitude of the seasonal variation in late pregnancy 25(OH)D in the cholecalciferol supplemented group as shown in Figure 7.9.

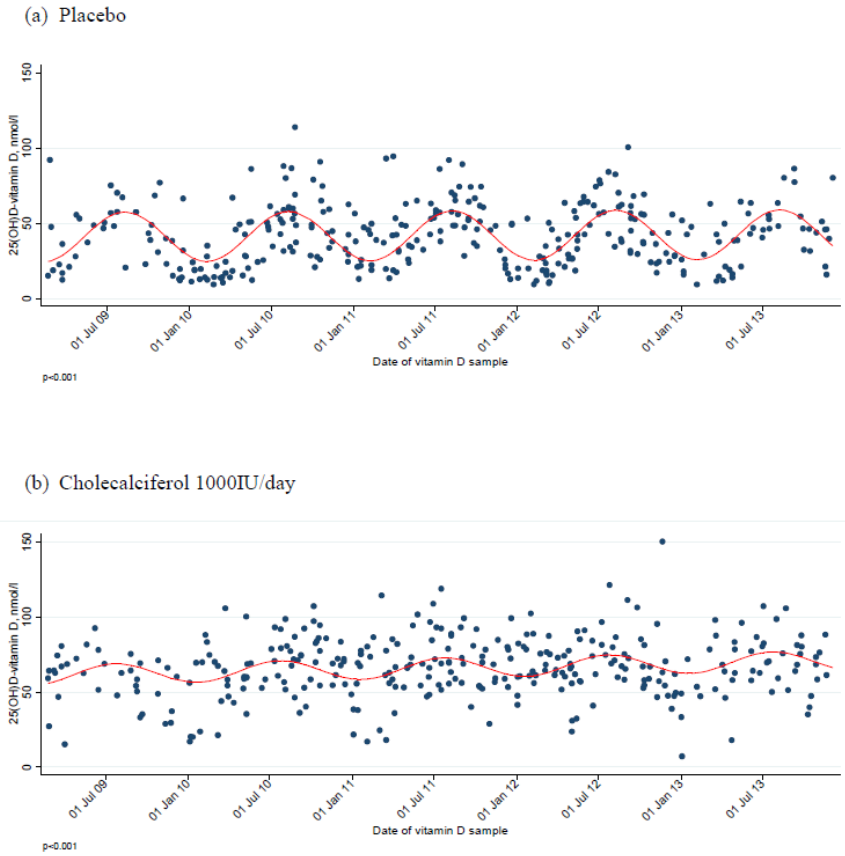


Figure 7.9 Plasma 25(OH)D concentrations at 34 weeks gestation by date of blood collection, among mothers randomised to (a) placebo and (b) vitamin D supplementation in the MAVIDOS trial. Statistically significant sinusoidal distributions were observed ($p < 0.01$) using a Fourier transformation in both treatment and placebo groups, but the supplemented mothers had higher average values in late pregnancy. There was a

marked reduction in seasonal amplitude resulting from the correction in trough values in mothers giving birth to babies in the winter months.

As shown in Figure 1.21, in MAVIDOS mothers giving birth in winter or spring, a distinct fall in 25(OH)D concentration was observed from 14 weeks to 34 weeks, but a rise was observed in the treatment group. As most calcium is accrued during the third trimester of pregnancy (76), in theory, reversal of this drop in 25(OH)D concentration would could permit greater maternal intestinal calcium absorption and transfer across the placenta to the developing fetus. However, in the MAVIDOS birth outcomes, a statistically significant effect was seen in winter and not spring births, which would have been expected from the timing of the 25(OH)D nadir. At 4 years, the only statistically significant seasonal difference was seen in BMC at the lumbar spine in "winter/spring" [0.27 SD ($p = 0.043$)] greater lumbar spine BMC in the cholecalciferol supplemented group than the placebo group; only a trend towards a seasonal effect was seen in other bone outcomes.

However, the fact that there is consistency across BMC and BMD in particular at both WBLH and lumbar spine does support the hypothesis that vitamin D supplementation has the greatest benefit in the offspring of participants with an absolute decline in 25(OH)D above a notional threshold, or those in whom 25(OH)D remains below a certain threshold. Whether this benefit occurs through effects on calcium transfer, epigenetic effects or through another mechanism will require further laboratory based studies. Clearly, replication in other populations is required, preferably including those with low 25(OH)D concentrations. As no significant differences in offspring height, weight, BMI or bone area were observed between the groups, these differences are not driven purely by chance differences in offspring size.

The reasons for associations between maternal gestational cholecalciferol supplementation and offspring bone mass being present only in winter-born neonates but in the children at 4 years of age, regardless of season of birth, are yet to be elucidated. Several longitudinal studies attest to the tracking of bone mass throughout childhood and adolescence, and bone mineral gain is substantial in early childhood. Children may be set on a track towards greater bone mass due to physiological differences as a consequence of epigenetic modifications, greater placental calcium or amino acid transport (438) (or some other mechanism) triggered by exposure to greater *in utero* 25(OH)D levels. If a child on a higher bone mineral accrual trajectory is compared several years later to another child on a lower trajectory, skeletal characteristics may have diverged further, and therefore be more detectable, than at birth. An epigenetic mechanism would be a

credible explanation as epigenetic modifications can occur as a consequence of a transient exposure, and lead to persistent effects on an individual's health. For example, those exposed in utero to the Dutch Hunger Winter in 1944-5 showed persistent epigenetic differences in the insulin-like growth factor 2 gene (*IGF-2*) six decades later compared to their unexposed siblings (380). Vitamin D has been shown to have effects on the epigenome at multiple levels, and, in the MAVIDOS trial we have demonstrated lower levels of *RXRA* methylation in the offspring of cholecalciferol supplemented mothers (514).

Other reasons for associations between cholecalciferol supplementation and offspring bone indices being present at 4 years but not at birth may be methodological. Owing to limitations in the use of DXA in neonates – they have lower absolute bone mass, are difficult to position, and have a tendency to move- sensitivity to detect differences in bone mass is therefore reduced in this age group in comparison to older children. Selection bias is another possibility, as we have shown that the characteristics of the mothers attending the four year follow-up visit were different from those who did not – they were older, of higher educational attainment, and were less likely to smoke. However, although the distribution of measures may be different in this population, there is no reason to suppose that the effect of vitamin D supplementation, particularly in the context of a randomised controlled trial, would differ. Of course, chance differences always remain a possibility-given that both the neonatal and childhood results are secondary findings, it will be essential to establish reproducibility of these results in other studies, for example, in the SPRING Trial (described in section 8.3).

Finally, the observed associations between maternal cholecalciferol supplementation and offspring bone mass at 4 years may be an example of a type 1 error (p-values for the differences between BMD and lean mass were both 0.05), and although WBLH bone mineral content and lumbar spine BMC and BMD were greater in the offspring at age 4 in the maternal cholecalciferol supplemented group, the differences between groups were not statistically significant.

The relevance of this finding to fracture prevention on a population level will be determined by the persistence of the effects of maternal cholecalciferol supplementation on offspring bone health into adulthood. In the Western Australian Pregnancy Cohort (RAINE) (361), associations between 25(OH)D status in pregnancy and offspring bone mass have been shown to persist to age 20 years. Hence, if such differences persist into later childhood, affecting the trajectory to peak bone mass, this is likely to be clinically relevant in terms of fracture prevention. The difference in WBLH BMD ranged from 0.43% to 2.3% between the supplemented and non-supplemented groups at age 4 years. In older adults aged 65, a 5% difference in BMD is associated with a 20%

difference in the risk of osteoporotic fracture and a 50% difference in the risk of hip fracture, so the magnitude of these differences could be clinically important should they persist (552).

7.4.4 Gestational Vitamin D supplementation and offspring lean mass and muscle strength in childhood

In the 4 year follow-up of the MAVIDOS trial, we have identified associations between maternal gestational cholecalciferol supplementation and a trend towards greater offspring muscle development, a trend which strengthened following adjustment for potential covariates such as age, sex, gestational age at birth, maternal BMI in early pregnancy, and child's "screen time", a surrogate marker for sedentary behaviour. Consistent with seasonal differences in effects of supplementation on bone indices, there was a trend towards greater effects of cholecalciferol supplementation on lean mass in children born in the "winter/spring" months, versus the "summer/autumn" months. To further characterise body composition, we calculated % lean mass and % fat mass as more adipose children are known to have higher absolute lean mass (384). We observed associations between gestational cholecalciferol supplementation and greater offspring % lean mass (and consequently lower % fat mass) though these associations did not reach statistical significance, except in "summer/autumn" born children.

Our findings are in support of an association between maternal 25(OH)D status and offspring muscle size, but not strength; although both maximum and mean grip strength were numerically higher in the cholecalciferol supplemented group, these differences were very slight and non-statistically significant.

Various lines of evidence support a role for vitamin D in muscle health – clinically, individuals with vitamin D deficiency may suffer from muscle pain and weakness (306, 307), and the VDR is expressed in skeletal muscle, with its activation promoting de novo protein synthesis (308-310). A range of observational and interventional studies in children, adolescents and adults have suggested a positive association between 25(OH)D status or supplementation and lean mass (313, 314, 318) or muscle strength and function (315-317); though others have not (296, 311, 320). In general, supplementation in adults with vitamin D deficiency, rather than those who were vitamin D replete, is associated with beneficial effects in muscle power, though not muscle mass, as demonstrated in a recent meta-analysis (312).

This is the first mother- offspring randomised placebo controlled trial to report associations between maternal cholecalciferol supplementation and childhood lean mass. Mothers and children were well characterised, though, as previously mentioned, the characteristics of the

mothers attending the 4 year follow-up visit differed from the overall trial recruits at baseline - they tended to be older, more educated and were less likely to smoke, providing potential sources of selection bias. Other limitations of the trial are described in section 8.4.2. Physical activity monitoring was not undertaken in the MAVIDOS trial 4 year follow-up, for this reason a child's "screen time" was used as a proxy. However, when testing for potential confounders of the association between maternal cholecalciferol supplementation and offspring lean and bone mass, this was a significant negative mediator of the association with WBLH lean mass and BMD (both $p < 0.05$) suggesting it is likely to be a useful measure of physical activity. Modes of infant feeding were not included in our adjusted models due to lack of available data – duration of breastfeeding and the timing of the introduction of solid foods, have been shown to be associated with bone indices in childhood and adolescence (553).

Intervention studies of gestational cholecalciferol supplementation reporting on offspring muscle mass and strength are lacking, the available evidence comes from observational studies. In the SWS, maternal 25(OH)D status was measured at 34 weeks of gestation, and offspring lean mass (measured by DXA) and grip strength were assessed at age 4 years. Contrary to our findings, in an analysis of 678 mother-child pairs, maternal 25(OH)D concentrations were significantly positively associated with offspring height-adjusted hand grip strength, and persisted after adjustment for maternal confounding factors, duration of breastfeeding and child's physical activity at age 4 years (assessed in a subset using 7-day accelerometry). In partial agreement with our findings, maternal 25(OH)D was also associated with offspring percent lean mass (but not total lean mass) – though this relationship did not persist after adjustment for confounding factors (383).

The Generation R study, again in a mainly white population, produced supportive evidence of a link between maternal 25(OH)D deficiency and lower offspring lean mass percentage and greater fat mass percentage measured by DXA (with a large sample size of 4903 mother offspring pairs, 1142 with 25(OH)D concentrations below 25 nmol/l). In mothers with 25(OH)D concentrations < 25 nmol/l, $\beta = -0.13$ % lean mass/nmol/l, demonstrating a continuous relationship in this subgroup. Significant associations were not observed between maternal 25(OH)D concentrations > 25 nmol/l and offspring lean mass, nor were associations with umbilical cord blood 25(OH)D demonstrated, albeit in a smaller sample; muscle strength was not assessed (385).

Other studies reporting associations with lean mass or grip strength use less robust assessments of either the predictor or outcome. Evidence from the Mysore Parthenon Study (relating maternal serum 25(OH)D concentrations at 28-32 weeks gestation with offspring grip strength and anthropometric approximations of muscle area at 5 and 9.5 years, like MAVIDOS, did not show an

significant association with grip strength but did demonstrate that the offspring of vitamin D deficient mothers had smaller muscle area in comparison with children born to mothers without deficiency. Maternal vitamin D deficiency was also associated with higher percentage body fat in the offspring (particularly in boys at age 5 years), though the lack of pubertal assessment limits the interpretability of the outcomes at 9.5 years (57). The ALSPAC study, using estimated UVB exposure as an approximate measure of maternal vitamin D status in the third trimester, also demonstrated positive associations with offspring lean mass assessed by DXA at 9.9 years, though due to chance collinearity between estimated UVB exposure and age of childhood DXA this finding should be interpreted with caution (382). As mentioned previously, comparing the outcomes and effect sizes of these studies with the MAVIDOS trial is difficult due to differences in timing of maternal 25(OH)D measurement or estimation, and variation in the methods and timing, of offspring muscle mass and strength assessment, plus differences in analysis methods and covariates adjusted for.

It should also be noted that observational data in relation to maternal vitamin D and offspring muscle health are at risk of risk confounding and reverse causality, as, for example, mothers who undertake more physical activity outdoors are known to have a higher 25(OH)D, and have more active children with greater muscle strength and mass. Vitamin D correlates between parents and pre-school children in developed countries, therefore challenges exist in disentangling whether maternal vitamin D is causally related to an outcome such as offspring lean mass, or is simply collinear with other related characteristics (554). Vitamin D is also negatively associated with markers of inflammation – in individuals who do not have chronic illness, 25(OH)D concentrations are higher (555). Mothers without chronic inflammatory conditions are likely to have healthier children – not just in developmental terms, but in their ability to spend time outdoors, have a healthier diet, and enjoy wealthier socioeconomic status (556). Relationships between maternal 25(OH)D status and offspring muscle health may differ in developing countries, in which exposure to outdoor manual work is more common in women of lower socioeconomic status, hence the observed associations could differ between population groups.

The mechanisms by which maternal 25(OH)D concentrations during pregnancy may affect offspring muscle size or strength are poorly understood. Animal studies support a role for 25(OH)D in fetal muscle development, with evidence for storage of 25(OH)D in fetal muscle (386) and has been shown to have stimulatory and proliferative effects on myogenesis (387, 388) as described in section 1.7.10.2. Myoblasts of newborn rat offspring born to vitamin D deficient mothers have been shown to be smaller, with evidence of differences in muscle gene expression

and the time spent in the proliferative phase of the cell cycle pointing towards possible effects on the offspring epigenome; similar findings have been published in pigs and sheep (389-391).

Vitamin D, in addition to its role in placental calcium transport (79, 82, 324), has also been implicated in transport of amino acids across the placenta. Research using placental samples from the SWS has demonstrated that placental amino acid transport is partly regulated by maternal vitamin D status and vitamin D binding protein levels. Total lean mass and bone mineral density in the offspring at age 4 years were positively associated with the expression of different amino acid transporters, though associations with muscle mass have not been reported (438). Several amino acid transporters have vitamin D response elements (VDRE) in their promoter regions and therefore could be regulated at a transcriptional level by vitamin D (specifically, by $1,25(OH)_2D$ binding the VDR and interacting with VDRE) (548, 549).

A role for maternal vitamin D status on fat mass accumulation, thereby modifying the body composition balance of fat and lean, is plausible. Adult BMI has been shown to differ with season of birth, and is highest in winter and spring. Studies in India (57), the Netherlands (385) and the UK (239) have demonstrated an association between lower maternal vitamin D status and greater fat mass – in neonates and in older children. However in an earlier UK study in a smaller cohort of children, body composition was not related to vitamin D status (557). Adipose tissue and muscle share a common mesenchymal origin, and adipose tissue also expresses VDRs and has the ability to synthesise $1,25(OH)_2D$. *In vitro* studies have suggested that $1,25(OH)_2D$ could inhibit adipogenesis and lipid accumulation (558, 559), through the inhibition of expression of PPAR-gamma (560). Recently, *in vivo* studies of higher vitamin D diets (compared to low vitamin D diets) in mice were shown to be associated with lower levels of lipid deposition in skeletal muscle (both inside and outside muscle fibres), and lower PPAR-gamma expression (561).

RXRA may also play a role – it is known to interact with several different nuclear hormone receptors – including the VDR, thyroid hormone receptor and PPAR-gamma. As we have already demonstrated that offspring *RXRA* methylation is altered by maternal cholecalciferol supplementation (514), and is associated with measures of both bone mass and adiposity in childhood (441, 443). Therefore, levels of RXRA protein production in the developing fetus could influence PPAR-gamma dependent gene expression, having downstream effects on adipocyte maturation and hence body composition. This complex set of inter-relationships between vitamin D, RXRA, PPAR-gamma and other nuclear hormone receptors could explain the differences in seasonal effects observed between in the MAVIDOS follow-up at 4 years – with cholecalciferol supplementation having greater beneficial effects on bone outcomes in “winter/spring”-born

children and on fat and lean mass percentages in “summer/autumn” born children. These questions, also acknowledging the fact that vitamin D may interact with the epigenome on multiple levels, (439, 444, 509, 515, 516), must remain the focus of future research.

In conclusion, we have observed that maternal cholecalciferol supplementation is associated with greater lean mass in the offspring at age 4 years, after adjustment for covariates. We also observed greater lean mass percentage, and lower fat mass percentage in the offspring of cholecalciferol supplemented mothers, born in the “summer/autumn” months. An improvement in muscle function in childhood could have long term health benefits. Low grip strength, as a marker of poorer skeletal muscle function in adulthood is associated with increased cardiovascular and all-cause mortality in older age (59), and a greater risk of hospital admission over the following decade (60). As muscle strength peaks in young adulthood before declining, interventions to improve muscle mass and strength early in life could have lasting benefits to the individual, in addition to addressing the increasing burden of sarcopenia.

Studies have demonstrated evidence of tracking of muscle function and mass through childhood and adolescence (562-564), and one study has demonstrated tracking of muscle strength into early adulthood. Indeed, studies of fetal and postnatal growth from the SWS suggest that most children have settled onto a sustained growth trajectory by the age of 4 years (198). Accordingly, the greater muscle mass observed in the offspring of mothers receiving gestational vitamin D supplementation in the MAVIDOS study would be expected to track into adulthood, and these individuals may be at lower risk of sarcopenia. Muscle strength was identified in the 1980s as being associated with the risk of hip fracture (565). In a recent Australian study, of older adults followed up over a 10 year period, low hand grip strength (in the lowest 20% of the population) was associated with an increased risk of incident fracture [RR 1.55 (95% CI 1.09, 2.20)] and low (bottom 20%) appendicular lean mass relative to BMI was associated with an increased mortality risk [RR 1.54 (95% CI 1.14, 2.08)] (566). Therefore, our observed effect size of lean mass increases of 0.17 SD (95%CI 0.00, 0.34) in the 4 year old offspring of cholecalciferol supplemented mothers compared to placebo could have clinically important effects in reducing falls, fractures and perhaps mortality. Further testing of these associations as the children grow up, to establish whether such benefits persist into later childhood and adulthood, and replication in other populations will be needed before any clinical recommendations are developed.

Chapter 8: Summary Discussion

8.1 Main findings

In this thesis I have investigated a variety of mechanistic links potentially underlying musculoskeletal development in early life, using both observational and intervention studies. In an observational mother-offspring cohort, the SWS, DNA methylation at a locus linked to ageing and cell cycle regulation *(CDKN2A)* has been shown to be associated with childhood bone health. Then, building on previous observations in this cohort, methylation at another locus, linked to vitamin D signalling *(RXRA),* was reduced by maternal vitamin D supplementation, and associated with neonatal bone mass, in a randomised controlled trial setting, MAVIDOS. In the MAVIDOS trial, maternal vitamin D supplementation was associated with a reduction in a marker of bone resorption in pregnant women, and was also associated with greater measures of bone and muscle mass in their offspring at the age of 4 years. These novel results are summarised below:

8.1.1 Associations between methylation of *CDKN2A* and offspring bone health at 4 and 6 years: findings from the Southampton Women's Survey

- Higher levels of methylation in umbilical cord of specific CpG dinucleotides within the *CDKN2A* gene locus were associated with lower total whole body minus head bone area, BMC and areal BMD at 4 and 6 years of age in the SWS.

8.1.2 The influence of Vitamin D supplementation on RXRA methylation and associations with bone outcomes at birth: findings from the MAVIDOS trial

- In a randomised controlled trial setting (MAVIDOS), supplementation with 1000 IU/day cholecalciferol from 14 weeks gestation to delivery of the baby is associated with reduced methylation levels at specific regions near to the *RXRA* promoter in fetal DNA derived from the umbilical cord of the offspring. These results are consistent with our previous observational findings in the Southampton Women's Survey, in which a negative association was found between an estimate of maternal free 25(OH)-vitamin D and methylation in this region.
- The lower levels of methylation in the cholecalciferol supplemented group were most prominent in "summer/autumn"-born babies.
- In the population as a whole, there were modest positive associations between *RXRA* methylation in umbilical cord tissue and neonatal BA, BMC and aBMD. On stratification

according to treatment allocation, associations were noted in the placebo but not in the cholecalciferol supplemented groups.

- *In silico* functional analysis demonstrated that the *RXRA* DMR is a region of significant functional activity across a range of cell types in terms of transcription factor binding, enhancer activity and DNAse1 hypersensitivity.

8.1.3 Urine β-C-terminal telopeptide of type I collagen (CTX) in pregnancy: descriptive characteristics, influence of cholecalciferol supplementation and associations with maternal bone health: findings from the MAVIDOS trial

- In a randomised controlled trial of cholecalciferol supplementation in pregnancy, maternal gestational cholecalciferol supplementation was associated with a reduction in the increase in a maternal bone resorption marker (CTX) normally observed between early and late pregnancy.
- Predictors of lower levels of CTX in late pregnancy included older maternal age, greater parity, taller height, and being randomised to a 1000 IU/day cholecalciferol supplement.
- Lower concentrations of CTX were associated with greater maternal bone mass measures at the lumbar spine and total hip in the early postpartum period.

8.1.4 Associations between cholecalciferol supplementation and musculoskeletal health at age 4 years: findings from the MAVIDOS trial

- Antenatal supplementation with 1000 IU/day cholecalciferol was associated with greater whole body (less head) BMD in the offspring at age 4, regardless of season of birth. This is in contrast to the findings in the offspring of the MAVIDOS trial in the neonatal period, in whom greater offspring bone indices with maternal cholecalciferol were observed in winter-born infants only.
- Upon stratification by season of birth, a greater effect of cholecalciferol supplementation on bone outcomes in 4 year old offspring was observed in those born in the winter months, at the whole body (less head) and lumbar spine sites.
- Maternal cholecalciferol supplementation was also associated with greater lean mass in the offspring at age 4 years, after adjustment for covariates. It was not, however, associated with greater offspring grip strength.

- A greater lean mass percentage, and lower fat mass percentage was observed in the offspring of cholecalciferol supplemented mothers, born in the “summer/autumn” months.

8.2 Synthesis of main findings: contribution to our understanding of the developmental origins of osteoporosis

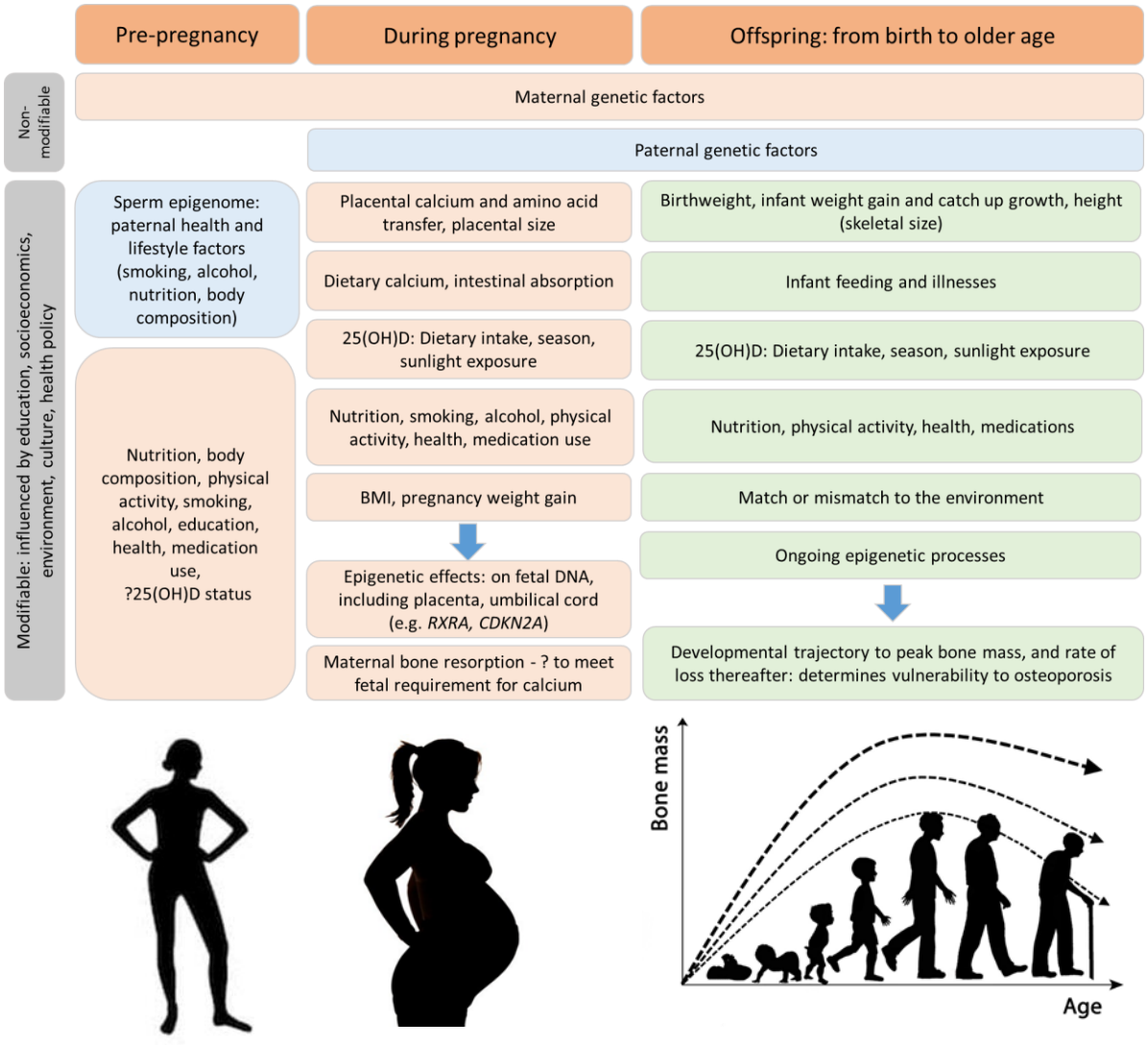


Figure 8.1 A conceptual framework of the developmental origins of osteoporosis.

Maternal factors (orange), paternal factors (blue) and postnatal factors (green boxes) are listed.

The risk of osteoporotic fracture ultimately depends on two factors – the mechanical strength of bone, and the forces applied to it. Bone mass (considered as a composite measure including contributions from bone size and from its volumetric mineral density), is an established determinant of bone strength. The bone mass of individual in later life (when most osteoporotic fractures occur) depends upon the peak attained during skeletal growth, and the subsequent rate of loss. As described in this thesis and in figure 8.1, a variety of factors contribute to an individual's peak bone mass, and come together in a complex interplay of processes – some non-modifiable (e.g. genotype, age), and others modifiable.

Recently, a genome-wide association study (GWAS) for bone mineral density was performed, using genetic and heel quantitative ultrasound data (eBMD) from 426,824 individuals from UK Biobank. Bone mineral density, the most clinically relevant risk factor for fracture, was shown to be a highly polygenic trait; 518 genome-wide significant loci were identified, explaining 20% of its variance. 13 loci were shown to be associated with fractures, in a cohort combining UK Biobank and 23andMe (1.2 million individuals) with linkage to Hospital Episode Statistics (179). Therefore, as yet unidentified genetic factors (potentially including many sites with BMD associations below genome-wide statistical significance), epigenetic modifications to DNA, or environmental factors may potentially contribute to the remaining 80% of the population variance in BMD.

There is evidence that various environmental influences during childhood and puberty benefit bone mineral accrual, and can contribute to peak bone mass. However, the relatively rapid rate of bone mineral gain during intrauterine and early postnatal life, coupled with the plasticity of development in utero, means that there are many possibilities for interactions between the maternal environment and the fetal genome at this lifecourse stage.

In this thesis, two examples of epigenetic developmental plasticity have been described. In addition, further characterisation of the possible effects of cholecalciferol supplementation in pregnancy on the mother's skeleton, and its lasting impact on her offspring's skeletal characteristics at age 4 years have been demonstrated.

Earlier investigations led by researchers at the MRCLEU resulted in the identification of the epigenetic loci of interest: an epigenetic array covering 24,134 genes (the NimbleGen Systems HG_17_min_promoter array), was performed in samples from children aged 9 years in a mother-offspring cohort, the Princess Anne Hospital Study. Methylation in the region near the promoter of *CDKN2A* and *RXRA* was associated with DXA measurements of fat mass. Methylation near the promoter regions of both loci was also associated with offspring childhood fat mass in the SWS (502). Given the common mesenchymal origins of fat and bone, further analysis of methylation in

these regions with respect to bone development was warranted. DNA methylation at various genes, including cyclin dependent kinase inhibitors such as *CDKN2A*, has been shown to play a role in skeletal development, homeostasis and bone cell activity. Thus, methylation has been implicated in mechanisms of osteoblastic differentiation (455-457) and osteoclastogenesis (459), together with the transition from osteoblast to osteocyte (460-463).

In analyses presented in this thesis, perinatal *CDKN2A* methylation was negatively associated with bone mass (bone area, bone mineral content and bone mineral density) in 669 children aged 4 years in the SWS, with similar relationships observed at age 6 years. Later functional work (performed by Dr Murray and Professor Lillycrop) demonstrated that these CpG sites may play a role in the level of expression of the long non-coding RNA ANRIL, which regulates cell survival. SNPs in the *CDKN2A* gene did not appear to be associated with adult eBMD in the UK Biobank GWAS. However, other genes encoding proteins similar to, or known to interact with, products of the CDKN2A gene were shown to be important, (*CKD2AP1*, which regulates $p14^{ARF}$ levels (500), and *CDK-5*, a member of the cyclin family important in cell cycle regulation (501)) providing support for a role for the *CDKN2A* region in bone health. It is not clear whether fetal *CKDN2A* methylation occurs as a result of a maternal environmental or dietary characteristics, or is genetically driven. Further investigation in other cohorts with different exposures, and replication of these findings, may extend our understanding of this association.

Prior to commencement of this PhD project, lower levels of umbilical cord *RXRA* methylation had been shown to be associated with greater measures of offspring bone size and density in 230 children aged 4 years from the SWS. A negative association between a measure of maternal free 25(OH)D and *RXRA* methylation was documented (443). Hence, a randomised controlled trial of cholecalciferol supplementation in pregnancy provided the ideal opportunity to attempt to replicate this finding. Thus in MAVIDOS, gestational cholecalciferol supplementation was associated with significantly lower levels of umbilical cord *RXRA* methylation, with more marked effects noted in “summer/autumn”-born babies. The point in gestation at which DNA methylation of the *RXRA* promoter occurs is not known. If it occurs in later pregnancy, then babies born in the “summer/autumn” months with the addition of cholecalciferol supplementation would be exposed to the greatest concentrations of 25(OH)D (or its metabolites), suggesting a possible threshold effect.

Relationships between perinatal *RXRA* methylation and offspring bone indices are complex; in the MAVIDOS population as a whole, there were modest positive associations between *RXRA* methylation in umbilical cord tissue and neonatal BA, BMC and aBMD; this was in keeping with

neonatal findings in the SWS. On stratification according to treatment allocation in MAVIDOS, associations were noted in the placebo but not the cholecalciferol supplemented groups. By the age of 4 years in the SWS, however, relationships between *RXRA* methylation and offspring bone indices were reversed, suggesting that non-linear relationships may exist between methylation at this locus, bone indices and maternal / fetal vitamin D status. Further investigation of these relationships at 4 years of age in the MAVIDOS trial is warranted.

In addition to its heterodimerisation with the VDR (with 1,25$(OH)_2$D bound), RXRA interacts with several different nuclear hormone receptors, including thyroid hormone receptor and PPAR-gamma, activation of either of which tends to have detrimental effects on bone. It is likely that complex molecular relationships exist between maternal vitamin D, fetal RXRA and these other nuclear hormone receptors, and downstream effects on the growing skeleton. In the UK Biobank GWAS, SNPs in the *RXRA gene*, the VDR (*GC*) gene, or other vitamin D metabolism pathway genes (*DHCR7, CYP2R1* and *CYP24A1*) were not shown to be associated with eBMD in adulthood. However, SNPs in the thyroid hormone receptor and *PPAR-gamma* genes were associated with adult eBMD, providing an insight into the potential importance of RXRA interaction with these proteins (179). A GWAS and Mendelian randomisation study of the genetic and clinical determinants of fracture risk in adulthood also did not demonstrate an association between SNPs in vitamin D pathway genes and fracture risk, or between genetically determined variation in 25(OH)D status and fracture risk (567).

However, genetic risk scores for 25(OH)D concentrations only explain about 2% of its variation (395), and Mendelian randomisation assumes a linear relation between the risk factor and the outcome(567). There is evidence to suggest that vitamin D and bone health associations in an individual, and between a mother and her offspring, are likely to be non-linear, threshold effects. Mendelian randomisation studies testing associations between maternal 25(OH)D concentrations and offspring bone outcomes have not yet been performed, and will require large genetic consortia to achieve the required sample size. Furthermore, this approach carries a number of sources of bias, statistical assumptions and limitations (396, 568). Genotyping and methylation array analyses of mothers and children in the SWS and MAVIDOS are being performed as part of the ALPHABET project, with the intention of combining these data with other mother-offspring cohorts such as ALSPAC, Generation R and the Polish mother and Child Cohort (Repro_PL). Within this collaboration, it may be possible to test whether maternal 25(OH)D status, and offspring DNA methylation are on the causal pathway in determining future musculoskeletal health.

Candidate-focussed studies, of which our analyses of *CDKN2A* and *RXRA* methylation are examples, are at odds with current array analyses using the Illumina Human Methylation 450 BeadChip (an array covering methylation marks at over 450 000 CpG sites) (446). Amongst 819 mother–offspring pairs in the Norwegian Mother and Child Cohort and 597 mother–offspring pairs in ALSPAC, there were no convincing associations between maternal mid-pregnancy 25(OH)D status and methylation profile in cord blood DNA. Additionally, use of the same array technology in blood DNA from 5515 adults of European descent identified only one CpG site associated with femoral neck BMD (569). However, these studies did not specifically address links between perinatal epigenetic marks and offspring bone development, hence there are a number of important considerations. These include (i) the tissue specificity of epigenetic marks and the use of blood DNA compared with perinatal tissues, such as umbilical cord, which contain mesenchymal stem cells that have potential to develop into target tissues such as bone, muscle and fat; (ii) despite coverage of > 450 000 CpG sites, the array targets a tiny percentage of the number of potentially methylated CpG sites across the genome; and (iii) although the variance explained by molecular phenotype is generally greater than that explained by fixed genetic variation, the sample sizes of these consortium-based studies have been relatively small compared with what has recently been possible with genetic analysis (570). Large numbers of SNPs were found to be associated with eBMD (179, 571) and with fracture risk (567) in the UK Biobank and 23andMe cohorts, more than two orders of magnitude greater in sample size. This may mean that future epigenetic arrays covering greater numbers of CpG sites (such as the Illumina Infinium MethylationEPIC BeadChip), and in larger numbers of individuals, may provide different new epigenetic regions of interest.

With these new tools, candidate studies such as the analyses of *CDKN2A* and *RXRA* are likely to become less common. However, this does not detract from the findings, particularly as they have been replicated in different cohorts and, in the case of *RXRA*, derived in a randomised controlled trial setting.

The MAVIDOS trial provided a unique opportunity to study other mechanisms by which maternal gestational cholecalciferol supplementation could influence offspring musculoskeletal development, and contribute to maternal bone health. The measurement of biochemical markers of bone resorption gave some insight into the impact of pregnancy on the maternal skeleton, and whether vitamin D supplementation has an influence on this. Maternal cholecalciferol supplementation was associated with lower levels of a bone resorption marker (CTX) in late pregnancy. Lower maternal CTX was associated with greater maternal bone indices assessed by DXA in the early postpartum period – however differential effects between the cholecalciferol and

placebo groups were not observed. This provides supportive evidence for the hypothesis that maternal 25(OH)D status in late pregnancy is important in regulating maternal bone turnover to ensure calcium availability for the developing fetus. However, this comes with some caveats, as negative associations between CTX and bone indices are observed in healthy individuals. Also, studies in animals have shown that intestinal calcium absorption in animal models is not a vitamin D dependent process, and can occur in severe 25(OH)D deficiency and in VDR knockout animals. We were not able to take into account the impact of breastfeeding on bone health postpartum, maternal dietary calcium intake, or measures of other important vitamin D and bone mediators (DBP, $1,25(OH)_2D$, PTH, PTHrP). Further evidence from other populations and replication in other RCTs of gestational cholecalciferol supplementation will determine whether our findings reflect meaningful impacts on maternal bone health.

Follow-up of the children of the MAVIDOS trial at the age of 4 years demonstrated potential lasting benefits of maternal vitamin D supplementation in pregnancy. Maternal allocation to cholecalciferol was associated with greater offspring whole body minus head bone mineral density (by 0.18 SD, 95% CI 0.00, 0.35) and lean mass (by 0.17 SD, 95% CI 0.00, 0.34) at the age of 4 years, regardless of season of birth of the child; a similar but non-significant trend was observed in offspring BMC. Consistent effects were observed at the lumbar spine, though only statistically significantly in winter-born children. In the outcomes of the MAVIDOS trial at birth, increases in neonatal bone indices were detected only in the offspring of supplemented mothers born in winter months. The fact that these observations were present regardless of season, could suggest that greater maternal vitamin D status (or prevention of maternal vitamin D deficiency), might set the growing child on a trajectory towards improved bone health in the future (see figure 8.1).

Pulling the results together, these findings support the hypothesis that developmental factors contribute to an individual's bone and muscle health. DNA methylation in umbilical cord tissue at two loci, *CDKN2A* and *RXRA,* previously demonstrated to be of importance in aging and cellular senescence, and in vitamin D nuclear signalling respectively, was associated with childhood bone indices. *RXRA* methylation was additionally shown to be influenced by maternal cholecalciferol supplementation in pregnancy in a randomised controlled trial. Furthermore, such supplementation in pregnancy had effects on both maternal and offspring bone health, being associated with reduction of a marker of maternal bone resorption, and, importantly, being associated with greater offspring BMD and lean mass at the age of 4 years. The longer term impact of these factors remains to be demonstrated, but they do provide evidence to support early interventions to improve offspring musculoskeletal health.

8.3 Proposed future work

Two major aspects to future work in this area may be considered. First, a greater understanding of the underlying mechanisms needs to be gained. Second, replication of these findings is required, in larger intervention-based studies and in different populations, to determine whether they are of public health relevance.

There are many possible mechanistic studies which could usefully contribute, incorporating in vitro, animal and human studies, and studies of epigenetic change at multiple levels, from methylation, to histone modifications, and small non-coding RNAs.

The following describes DNA methylation studies which are possible in the near future or already underway, and directly relevant to this work:

- **A study of the associations between genome-wide DNA methylation in umbilical cord blood and musculoskeletal health in the SWS: contribution to an international consortium**

The *ALPHABET* project, funded through the European Research Area Healthy Diet for a Healthy Life (ERA-HDHL) Biomarkers call, aims to expand the knowledge base regarding interactions between diet, epigenetics and offspring health, characterising biomarkers that may inform future health strategies. As previously mentioned in Section 8.2, methylation array analyses (a mixture of 450k and Illumina Infinium "MethylationEPIC" BeadChip, 850k arrays) are being carried out on umbilical cord blood samples from a variety of mother offspring cohort studies, some of which incorporate careful musculoskeletal characterisation (including ALSPAC, SWS, The Generation R Study, the Eden mother-child study, Repro_PL). Combination of these data will improve the statistical power to detect associations between DNA methylation and offspring musculoskeletal characteristics.

- **Genome-wide DNA methylation in umbilical cord tissue (as opposed to blood) and musculoskeletal health in the SWS**

Array analyses of cord blood versus umbilical cord tissue are likely to yield different findings; both contain a variety of cell types and DNA methylation is tissue specific. For this reason, separate methylationEPIC array analysis of SWS cord tissue samples in relation to musculoskeletal phenotypes are being performed. Cord tissue contains mesenchymal stem cells, sharing a common lineage with bone, muscle and fat cells (which clearly cannot be sampled in healthy

children). Therefore studying cord tissue is likely to provide a closer insight into the relevance of DNA methylation at a tissue specific level.

- **Genome wide DNA methylation in venous blood at age 10-13 years and musculoskeletal health in the SWS**

Ethical approval has been granted to collect venous blood samples from the SWS children (now aged 10-13 years), this follow-up visit is currently underway. MethylationEPIC array analyses using childhood blood samples can be compared to the aforementioned methylation arrays on umbilical venous blood. Changes in DNA methylation over a child's first decade of life can be analysed with respect to musculoskeletal development over this period, collected in the SWS at multiple time points. Important information on the child's health, diet and physical activity are being assessed, including pubertal stage, which will be essential to include as confounding factors.

- **A study of the influence of cholecalciferol supplementation in pregnancy (the MAVIDOS trial) on genome-wide DNA methylation, and associations with musculoskeletal health**

The MethylationEPIC array is currently being performed on umbilical cord tissue samples from the MAVIDOS trial. This will enable assessment of the differences in offspring DNA methylation genome-wide between the 1000 IU/day cholecalciferol supplemented and the placebo groups, and analyse associations between maternal 25(OH)D status and season of birth. Furthermore, assessment of associations between methylation in umbilical cord tissue and bone indices assessed by DXA at birth and at 4 years of age will be possible, incorporating adjustment for factors known to influence DNA methylation, e.g. gestational age, birthweight, offspring sex, maternal smoking, maternal BMI, maternal age, maternal genotype (particularly vitamin D related variants). Replication through pyrosequencing, and in vitro studies of the effect of $1,25(OH)_2D$ on the methylome of mesenchymal stem cells may be possible.

- **Methylation arrays on venous blood samples collected at age 4 and 7 years in the MAVIDOS trial**

With ethical approval, Dr Moon and I have collected venous blood samples from the children of the MAVIDOS trial at the 4 year and 6-8 year follow-up. These samples (currently frozen at -80^{o}C) will be used for further epigenetic analyses, to establish whether cholecalciferol supplementation has persistent effects at the epigenome-wide level, and whether any associations exist between DNA methylation and childhood musculoskeletal health.

- **Associations between *RXRA* and *CDKN2A* methylation and later childhood bone health**

The aforementioned measures of *RXRA* and *CDKN2A* methylation may be re-analysed in relation to bone health in later childhood – with complete data now available at age 4 years in MAVIDOS (with 6-8 year follow-up underway) and 8 years in the SWS (with 10-13 year follow-up underway). This will provide insights into the contribution of methylation at these loci in relation to the musculoskeletal developmental trajectory of the children of the SWS and MAVIDOS studies.

- **Relationships between maternal cholecalciferol supplementation, maternal bone resorption markers in pregnancy, future maternal bone health and offspring bone indices**

As characterisation of both mothers and offspring has continued in the MAVIDOS trial (mothers underwent DXA scanning and pQCT scanning of the tibia as part of the 4 year follow-up), this will enable further study of the influence of cholecalciferol supplementation on both maternal and offspring musculoskeletal indices, and bone resorption markers. This will permit a greater understanding of whether cholecalciferol supplementation has a protective effect on the maternal skeleton, as suggested by the reduction in late pregnancy bone resorption markers in supplemented mothers.

- **Associations between cholecalciferol supplementation and pQCT measures of bone and muscle at age 4 years, and HRpQCT at age 6-8 years in the MAVIDOS trial**

Analysis of tibial pQCT (available at age 4) and HRpQCT data (currently being collected at age 6-8 years) will provide an opportunity for assessment of detailed bone geometry, volumetric BMD, bone microarchitecture and lower leg soft tissue parameters in the MAVIDOS children. Such studies will allow characterisation of measures of the mechanical strength of bone, trabecular and cortical microarchitecture, and to understand in greater detail associations between maternal cholecalciferol supplementation and measures of muscle and fat mass in the lower limb.

Another intervention study of cholecalciferol supplementation in pregnancy is also underway:

- **The SPRING randomised controlled trial: 1000 IU/day cholecalciferol supplementation, and the impact of behaviour change interventions (Healthy Conversation Skills) on maternal and offspring health**

The aim of the SPRING trial is to test, in a 2 by 2 factorial design, the efficacy of two interventions: behaviour change (Healthy Conversation Skills) and vitamin D supplementation, and to explore the efficacy of an intervention that combines both, in improving the diet quality and nutritional status of pregnant women (572). The cholecalciferol dose is the same as that used in MAVIDOS,

and mothers and offspring are undergoing DXA within 14 days of delivery, facilitating replication. This process is essential before any recommendations can be made to change public health policy regarding maternal cholecalciferol supplementation in pregnancy.

8.4 Strengths and limitations of this work

8.4.1 The SWS Cohort

The SWS cohort and the MAVIDOS trial were rigorously conducted studies in which both mothers and offspring were carefully phenotyped.

The uniqueness of the SWS cohort is that, in this prospective mother-offspring cohort, women were recruited and assessed before they became pregnant. This is helpful in epidemiological studies as mothers can be characterised, particularly regarding lifestyle, diet, changes in BMI and adiposity, before changes occurred as a result of pregnancy – therefore when these measures are adjusted for as potential confounders, they represent with greater accuracy long-standing maternal characteristics, avoiding recall bias.

In our analyses of *CDKN2A* methylation and childhood bone indices, the study cohort was a subset of the SWS, but mothers whose children underwent DXA scanning and those whose children did not were broadly similar: the former were on average slightly older and smoked slightly less. A limitation of this approach is that this sub-population may not be representative of the SWS cohort as a whole, a source of selection bias. There is no reason to suppose, however, that relationships between *CDKN2A* promoter methylation in umbilical cord and childhood bone mineral accrual would differ between these two groups.

The SWS is an observational study, therefore causality cannot be observed from observational relationships between *CDKN2A* methylation and bone outcomes at 4 and 6 years of age due to the potential for confounding. As the SWS is so well characterised, a range of maternal and child co-variates could be included in the models, but residual confounding will always remain a possibility in any such analyses.

As the SWS cohort is located in Southampton, UK, a mainly Caucasian population, our findings may only be applicable to the population studied. Ideally, these results would be replicated in a geographically separate, and possibly ethnically diverse cohort. In addition, as associations between maternal 25(OH)D concentrations and childhood bone, muscle and fat outcomes were

observed in the SWS, the MAVIDOS trial offered a unique opportunity to translate observation to intervention.

8.4.2 The MAVIDOS Trial

The MAVIDOS trial is currently the second-largest completed RCT of vitamin D supplementation in pregnancy, the largest (Roth et al.) was published in 2018 (374). It is however, the largest trial to assess neonatal bone and muscle indices and body composition, and the only trial to offer follow-up into childhood. Due to its double-blind, placebo controlled design, high quality evidence can be generated with a low risk of bias, offering greater statistical power than the previous smaller studies of gestational cholecalciferol supplementation. As a five year timeframe was required to recruit adequate numbers of women to the trial (across three centres, Southampton, Sheffield and Oxford), there is necessarily a lag between initial births and results being available for analysis. The follow-up of the children at 6-8 years is ongoing, to be completed in 2022.

The MAVIDOS trial has some limitations which must be recognised. The main limitation is that only women with a relatively narrow range of early pregnancy 25(OH)D concentrations could be included due to the ethics approval process – this meant that participants with baseline 25(OH)D concentrations < 25 nmol/l were excluded. Also, the supplement dose of 1000 IU/day was modest in comparison with a variety of other trials of cholecalciferol supplementation in pregnancy, limiting the potential for biological effects. The Department of Health issued guidance that women should take up to 400 IU/day cholecalciferol in pregnancy, and therefore participants were, for ethical reasons, not discouraged from taking supplements containing cholecalciferol up to this dose (573). This may have reduced the numbers of women with low 25(OH)D concentrations in the placebo group and, since the effect on 25(OH)D is likely to have been more marked at lower 25(OH)D concentrations, and in the absence of the comparator, 1000 IU/day , the likelihood of a null finding was increased. Supplement use was recorded at interview and did not differ between the treatment groups. However, as many observational studies have suggested that negative effects of low 25(OH)D concentrations on musculoskeletal indices are only observed at very low levels, the offspring of the group of women with low 25(OH)D (defined as either < 25 nmol/l or below < 50 nmol/l in a variety of studies) are potentially the most likely to benefit from supplementation (356, 360, 361, 385). Indeed, in the MAVIDOS trial at birth, differences in offspring bone indices between randomisation groups were only seen in the group with the lowest levels of maternal 25(OH)D, winter-born babies (377).

Additionally, the MAVIDOS study population, drawn from antenatal ultrasound clinics in Southampton, Oxford and Sheffield, did not include many women who were of non-Caucasian ethnicity, reducing the generalisability of the findings.

DXA, used to ascertain bone and body composition outcomes, has some limitations which are discussed further in section 1.2.1 and 8.4.7. Children are particularly prone to movement and have lower absolute BMC than adults – however, appropriate paediatric software was used on the DXA instrument and DXA indices were cross – calibrated. The validity of DXA in small animals has been documented in the assessment of both bone mass and body composition (505).

Some participants may have been taking vitamin D in addition to the study drug, which could have led to a trend towards a null effect – however, supplement use was recorded at interview and did not differ between treatment groups.

Finally, it is possible that findings in the populations studied in the analyses presented in this thesis could be attributed to selection bias. The mothers bringing their child to the four-year follow-up were older, had higher educational attainment and lower rates of smoking than the mothers who did not bring their child to the four year visit, meaning the population studied was slightly different from the group of mothers who did not attend. However, although it is relevant in terms of generalisability, there is no reason to suppose that this would have affected associations between cholecalciferol supplementation in pregnancy and offspring DNA methylation or bone indices.

8.4.3 Questionnaire data

Both the SWS and MAVIDOS use interviewer-led questionnaires to collect data on dietary, demographic and health characteristics. However, self-reported data relies on the participants to provide accurate information. Women may be biased towards under-reporting of negative health behaviours, such as drinking alcohol, smoking, and poor dietary choices. This could plausibly lead to biases in the cohort data collected from the SWS. The randomisation in MAVIDOS ought to minimise the effects of such reporting inaccuracies, given the large sample size.

8.4.4 Assays of 25(OH)D in MAVIDOS

Both the 14 week (baseline) and 34 week gestation serum samples were analysed by chemiluminescence array (Diasorin Liaison), by a Cambridge University laboratory which is a member of the DEQAS quality assurance scheme (http://www.deqas.org). The screening 25(OH)D

analysis at 11 weeks gestation, however, was carried out on the local hospital platform. This was the measurement used to determine whether women were eligible for inclusion (25(OH)D concentrations between 25 and 100 nmol/l). Owing to differences between the hospital laboratory assay and measurements by the Diasorin Liaison method, a few women had a 25(OH)D < 25 nmol/l at randomisation. Serum samples were stored at -70^{o}C, and all samples from early and late pregnancy were analysed in a single batch. It has previously been shown that storage of serum at -80^{o}C does not affect the stability of 25(OH)D.

8.4.5 Interpretation of CTX measurement

A variety of factors have been identified as sources of variation in bone turnover markers, these can be compartmentalised into pre-analytical and analytical sources (574). Pre-analytical sources of variability which could be relevant to our maternal population in MAVIDOS include age, ethnicity, stage in pregnancy, fractures and immobility, and diseases - such as diabetes or inflammatory conditions. In addition, an individual's circadian rhythm, season of sampling, physical activity, diet, medication (particularly glucocorticoids, anticonvulsants) could all affect CTX levels. Early morning second-void urine was used to attempt to reduce the circadian and dietary sources of variation, tests for associations between CTX and physical activity (collected by questionnaire) were performed. Individual pregnancy-related factors such as haemodilution and individual changes in maternal GFR (thereby affecting renal clearance of CTX) (138), maternal 1,25$(OH)_2$D status or measures of vitamin D binding protein (DBP), PTH or PTHrP were not available, hence could not be accounted for. An estimate of maternal dietary calcium intake would also be important as a potential confounding factor between 25(OH)D status in pregnancy and bone turnover. This could be assessed in future analyses as maternal dietary data are available (113, 130, 131). Another important factor in pregnancy is the potential for fetal CTX (an 8 amino acid sequence, in the α–CTX isoform), to cross the placenta. The assay used does not distinguish between the different isoforms of CTX, therefore it is not possible to quantify the fetal contribution to the mother's urinary CTX (145).

Technical sources of variability include transport of samples (due to light-sensitivity) and storage conditions in the lab. Our samples were processed at Professor Eastell's laboratory at the University of Sheffield, hence transport conditions between Southampton, Oxford and Sheffield could have been important. In terms of analytical variability, within laboratory variations in the ELISA technique may also have occurred. An automated immunoassay was used (Immunodiagnostic Systems, Boldon, UK); which has an inter-assay coefficient of variation of around 6% (521). It is possible that these variations could have contributed to our finding, though

there is no reason why this would happen in an unbalanced way between maternal cholecalciferol-supplemented and placebo groups, the likely effect, if any, is towards reduced precision.

8.4.6 Anthropometric measurements

Anthropometric measurements in both the SWS and MAVIDOS studies were performed by a trained research nurse or doctor, and to improve the accuracy of the measurements, followed detailed protocols. Research staff underwent regular training sessions with calculations of coefficients of variation.

Children tend to move when being measured, therefore all measurements (except weight) were repeated three times, and an average was taken to improve precision. Maternal measurements of skinfold thickness and circumferences were also repeated three times.

8.4.7 Dual energy X-ray absorptiometry

DXA is the gold standard for the assessment of bone indices and body composition. However, some children (particularly in the 4 year follow-up of the MAVIDOS study) refused DXA, and many struggled to remain still for the duration of the scan. Scans with excessive movement artefact were excluded from the analysis. When movement in one limb was present, this ROI was reflected from the opposite limb without movement artefact. Significant side to side differences in BMC measured by DXA have been shown not to be present in adults (575). However, in the MAVIDOS trial at age 4 years, small but statistically significant differences between limbs were present in the children, possibly due to more subtle movement than is visible on the scan image, or from the placing of the ROIs (576). For this reason, sensitivity analysis was performed, excluding data from the children in whom cross-limb imputation had been carried out: this did not alter the findings overall.

Measurement of bone indices in children is also hampered by their low absolute BMC, and inaccuracies in soft tissue measurements can be difficult due to difficulties with edge detection. This is likely to have been less of a source of error in the children at age 4 years compared to neonates. Specific paediatric software was used, and studies of DXA indices compared to ashed mineral content in piglets (of similar size to small children) have confirmed the accuracy of the technique (505). As discussed in section 1.2.1, the use of DXA does not allow measurement of true volumetric bone density, thus making it difficult to be certain about differential determinants of skeletal size and volumetric density.

8.4.8 Hand dynamometry to assess muscle strength

Grip strength was chosen in the MAVIDOS 4 year follow-up as an easily obtainable measurement of muscle strength in large epidemiological settings, though it presents some difficulties in pre-school children. The children need to be able to understand and follow instructions, and the reproducibility and validity of grip strength measurements has been demonstrated previously in children aged 4-11 years (63, 577). Both maximum and average grip strength were used as outcomes due to the variable behaviour of children when undergoing grip strength measurement – some became aware of what they needed to do after the first attempt and subsequently put in more effort to obtain a higher reading, whereas others lost interest after multiple measurements. Average and maximum grip strength in the MAVIDOS 4-year olds were highly correlated (551).

The Jamar hydraulic dynamometer used in the MAVIDOS study at 4 years of age can be read to the nearest 0.5 kg. It is therefore of relatively low sensitivity, given the (mean) maximum grip strength in both cholecalciferol and placebo groups was around 6 kg. Consequently, it is possible that the sensitivity of the Jamar dynamometer may have been insufficient to detect a small effect of maternal cholecalciferol supplementation on offspring muscle strength.

In the MAVIDOS 6-8 year follow-up (which commenced in December 2016), an electronic Jamar instrument is being used, which digitally reports grip strength to the nearest 0.1 kg. However, poor inter-instrument reliability meant that the newer electronic device could not be used when it became available halfway through the MAVIDOS 4-year follow-up (578).

Other methods of muscle strength, including jumping mechanography and isokinetic dynamometry are available, and can provide more detailed assessment of muscle strength than isometric grip strength measurement. The equipment are more costly and more time consuming to use, and it was felt that the complexity of instructions for participants would preclude their use in preschool children. However, jumping mechanography is planned in a subset of MAVIDOS 6-8 year olds.

8.4.9 Assessment of physical activity

Robust assessments of physical activity were not undertaken in the MAVIDOS trial 4 year follow-up, nor were they collected in the mothers of the SWS (walking speed was collected by self-assessment questionnaire) or MAVIDOS trial (time spent undertaking strenuous physical activity per week was collected). In the MAVIDOS 4 year follow-up, child's "screen time" (hours spent watching television/computer games per day) was assessed by maternal questionnaire. This was

used as an inverse proxy for the children's physical activity, and was adjusted for in the analysis. Objectively evaluated sedentary time has been shown to be negatively associated with physical activity assessed by accelerometry in primary school children (542, 543).

In a subset of the SWS children, objective assessment of the children's physical activity was made at 6 years of age using the Actiheart device (Cambridge Neurotechnology Ltd., UK), though a number of children had reactions to the stickers used to attach the electrodes to the skin, which limited the availability of the data. In the MAVIDOS 6-8 year followup, Geneactiv tri-axial accelerometers (Activinsights, UK), worn on the non-dominant wrist are being used to collect a 7 day snapshot of children's physical activity (474-478). This validated tool will provide more detailed assessment of a child's physical activity which will permit its use as a covariate in future analyses.

8.4.10 Linking DNA methylation to exposure and outcome

There are various limitations to the assumption that DNA methylation, measured at a particular locus, is dependent on an exposure. Genetic *trans* effects are possible, whereby a distant SNP could influence DNA methylation at a particular locus and also influence the child's phenotype, therefore being independent of any external exposure such as a dietary factor. The presence of SNPs at CpG sites of interest at the *CDKN2A* and *RXRA* loci were excluded by sequencing (449) , however, it is not possible to exclude an external genetic effect.

8.4.11 Tissue specificity of DNA methylation

In both the SWS *CDKN2A* study and MAVIDOS *RXRA* study, methylation was measured in cells from whole umbilical cord. Umbilical cord comprises a variety of cell types, so it is therefore possible that the differential methylation we observed arose from variation in the proportions of different component cells (for example fibroblasts, epithelial cells) in individual samples. However, our results in the *CDKN2A* methylation analyses were consistent across both our Discovery and Replication set and results from unpublished work by Professor Lillycrop's group show similar methylation in different umbilical cord cell types. Furthermore, any unaccounted cell-heterogeneity that is being observed as epigenetic change may represent proportional differences that are related to the child's skeletal phenotype, and so could potentially be on the causal pathway between DNA methylation and childhood skeletal indices (518, 519).

Clearly, assessment of *CDKN2A* and *RXRA* DNA methylation in bone itself was not possible due to the ethical considerations of obtaining such samples from children and have therefore used DNA

methylation in the umbilical cord as a proxy. There are a growing number of studies that have found that the methylation status of CpGs in peripheral tissues such as blood correlates with that of internal tissues (579, 580). It is important to note that both bone and cord tissue are derived from the mesoderm and share mesenchymal cell origins; mesenchymal stem cells differentiate into osteoblasts, playing a role in bone formation both in the embryo (503) and in the adult, in fracture and repair mechanisms (504). Therefore, it is possible that methylation patterns in umbilical cord cells may correlate with those in bone tissue.

8.4.12 DNA methylation versus gene expression

In our analyses of *CDKN2A* methylation and *RXRA* methylation, we could not examine whether the changes in methylation at these loci were associated with corresponding differences in gene expression. However; altered gene expression patterns would most likely be cell type dependent and reliant upon cell-specific transcription factor expression whose function is then modulated by altered access to the underlying DNA as a result of altered methylation patterns. Therefore, even if gene expression could be measured in the umbilical cord tissue, it is possible that this would not correspond to similar levels of gene expression at the *CDKN2A* and *RXRA* loci in the bones of the child.

8.5 Implications for clinical practice: a lifecourse approach

> *"At the WHO European Ministerial Conference on the Life-course Approach in the Context of Health 2020, held at Minsk in 2015, Member States signed a declaration in which they agreed that using a life-course approach was an essential step towards the implementation of Health 2020 and the 2030 Agenda on Sustainable Development. Member States also agreed that a life-course approach should incorporate actions that are taken early, are appropriate to transitions in life and confer benefits to the whole population across the lifespan and to future generations. Implementing a life-course approach calls for a focus on a healthy start to life and targeting people during critical periods in their life-course, such as investing in childhood. The aim is to prevent disease and its risk factors at the earliest possible time, and to promote timely investments that would have a high rate of return for Member States."*
>
> *World Health Organisation Europe, Health Evidence Network Synthesis Report 63, 2019*

At a World Health Organisation policy level, a lifecourse approach to health has been recognised as a priority (581). A recent WHO report acknowledges the challenges in implementing a life-course approach in public health policy at a national level. Promoting a "healthy start in life" for the prevention of osteoporotic fractures and sarcopenia in older age is an example of one such challenge. Changing the public mind-set from one which believes that fragile bones, weak muscles and poor mobility are an inevitable part of ageing, to one that understands the importance of nutrition, physical activity, body composition, lifestyle and disease management from preconception to old age, and acts upon this knowledge, is not a simple task.

A lifecourse perspective on musculoskeletal health emphasises the importance of prenatal and early development – Hanson et al. have suggested that, as a health strategy, this can be in conflict with other important values in society. They suggest that, whilst focusing on early life can create more support for parents facing difficult conditions, it can also lead to "the policing of women's bodies during and even before pregnancy, and a tendency to place blame on mothers". They point out that the use of the phrase "developmental programming" holds the danger of supporting determinist perspectives on biology, that view an individual's health throughout life as being determined principally by early life experiences. Individuals who might be "programmed" by their conditions in early life to express unhealthy phenotypes in later life, and go on to develop disease, could be discriminated against - particularly if these differences are framed by characteristics such as gender, ethnicity or socioeconomic status. Therefore, a careful balance

between the social and the biological must be struck when translating research to health policy (582).

These perspectives should be considered in the future, when reduced costs might make widespread early testing for epigenetic markers of maternal exposures and potential adverse consequences viable. In 2012, the 450k array was shown to be able to identify differential DNA methylation in blood samples in relation to smoking in pregnancy (583); a recent study demonstrated that these epigenetic changes persist into middle age, independently of active smoking (584). It is possible that methylation at *CKDN2A* and *RXRA*, associated with neonatal bone health, could be part of an epigenetic screening panel as biomarkers of future risk of osteoporosis, should these associations persist into adulthood.

The outcomes of the MAVIDOS trial are more likely to impact upon public health policy in the shorter term. The UK Department of Health and Social Care currently recommends that women should take a vitamin D supplement containing 400 IU/day throughout pregnancy, independent of ethnicity and other risk factors for VDD (344). Indeed findings from MAVIDOS informed the 2016 vitamin D recommendations from the UK Scientific Advisory Committee on Nutrition, which in turn led to guidance from Public Health England. Evidence from the MAVIDOS trial suggests that even 1000 IU/day is not a large enough dose for all women to achieve 25(OH)D concentrations of > 50 nmol/l (often considered as indicating repletion), particularly for those delivering in the winter months (340, 341). It was, however, shown to be safe; no excess adverse events occurred on doses of 1000 IU/day in the MAVIDOS trial, or indeed in another pregnancy study using doses up to 4000 IU/day (585). Avoidance of vitamin D toxicity by over-supplementation, which could cause harm, is also important – the Institute of Medicine suggests that toxic effects can occur above the threshold of 125 nmol/l, and recommends avoidance of intakes above 4000 IU/day (586).

The neonatal findings of the MAVIDOS trial, demonstrating greater offspring WBLH BMC, BMD and bone area in babies born in the winter months do lend themselves to population advice. However, a public health message needs to be interpretable and easily implemented by all women. Therefore, a message that only women with expected delivery dates in the winter should take vitamin D supplements in pregnancy would be difficult to implement. Also, as the seasons were defined in arbitrary (although as recommended by UK Meteorological Office) three month divisions, women delivering in late autumn or early spring might benefit similarly from vitamin D supplementation, but not receive it. Thus supplementation in pregnancy, regardless of season, is likely to be a simpler and more readily implemented strategy.

Indeed, the findings presented in this thesis from the MAVIDOS trial offspring at age 4 years, demonstrating greater BMD and lean mass in the children of women receiving cholecalciferol supplementation, regardless of season of birth, would support this approach. The 4 year findings provide a clearer message of year-round benefit– though the greatest effects were still seen in children born in the winter. The findings of one study alone, even a rigorously conducted RCT, are not enough to recommend a policy change. The findings are only relevant to the population studied, and the effect sizes were of modest statistical significance. Replication in other RCTs, such as the aforementioned SPRING trial (taking place in a similar population to MAVIDOS) (572), and populations with different ethnicity characteristics, are necessary.

In a collaboration between the MRCLEU and the American University of Beirut-Lebanon, a trial of either 600 IU or 3000IU vitamin D daily or matched placebo from 15 weeks gestation until delivery is being conducted. The primary outcome is the proportion of women achieving 25(OH)D concentrations ≥ 50 nmol/l at delivery, and secondary outcomes include measurement of offspring bone mineral content at the whole body site measured by DXA soon after birth. Childhood follow-up at 4 years with further bone assessment is planned, which, if showing a similar effect to the MAVIDOS trial, would thus provide evidence for generalisability across diverse populations (587).

Another question which will guide public health policy is persistence, and magnitude, of effect. In a study of children in ALSPAC aged 9.9 years with and without a history of fracture, the difference in mean WBLH BMD was 0.005 g/cm^2 (mean difference in MAVIDOS 4 year olds 0.007 g/cm^2, 0.18 SD, 95% CI 0.00, 0.35). From this 0.005 g/cm^2 difference, an inverse relationship between WBLH BMD and fracture risk in childhood was detected (OR per SD decrease = 1.12, 95% CI, 1.02-1.25) (296). If such differences persist into adulthood (in the RAINE cohort study, associations between 25(OH)D status in pregnancy and offspring bone mass were detected in their twenties) this could be of greater relevance to fracture prevention (361). Between the offspring of cholecalciferol supplemented and non-supplemented mothers, the difference in WBLH BMD at age 4 years ranged from 0.43% to 2.3%. In 65 year old adults, a 5% difference in BMD was associated with a 20% difference in the risk of osteoporotic fracture and a 50% difference in the risk of hip fracture (552).

The finding that, at age 4, WBLH lean mass was 0.17 SD greater in the offspring of cholecalciferol supplemented mothers, is less clear in terms of possible future clinical importance, given that differences in grip strength were not detected. Although appendicular lean mass is included in the

EWGSOP definition of sarcopenia (58), it is variably related to falls and mortality, and may not contribute to the prediction of fracture outcomes independently of BMD (588).

It important to recognise that prevention of osteoporotic fractures by improving the trajectory to peak bone mass of the population, could not only have great benefits to the individual, but also to the economy. In Europe in 2010, 22 million women and 5.5 million men were estimated to have osteoporosis; and 3.5 million new fragility fractures were sustained, including 610,000 hip fractures. The economic burden of incident and prior fragility fractures was estimated at € 37 billion (21). A reduction in osteoporotic fracture rates even by a small percentage, could have large economic benefits. If the effect of antenatal cholecalciferol supplementation on offspring BMD is replicated in other studies, ideally its cost effectiveness would also be evaluated (589).

8.6 Conclusions

In conclusion, demonstration that levels of DNA methylation at the *CDKN2A* and *RXRA* loci are associated with a child's bone development adds to the accumulating evidence that bone health throughout the lifecourse may be influenced by *in utero* factors. Findings from the MAVIDOS trial, that cholecalciferol supplementation from 14 weeks gestation to delivery is associated with reduced DNA methylation at *RXRA*, informs our understanding of the early life mechanisms linking maternal vitamin D and offspring bone development. Evidence that maternal bone resorption markers in late pregnancy are reduced in mothers receiving cholecalciferol supplementation suggests that this may be of benefit to maternal, as well as offspring bone health. Differences in BMD and lean mass in the four year old offspring of mothers receiving vitamin D or placebo (regardless of season of birth) implies that these children may be on different trajectories to peak bone and muscle mass. These findings could, in the future, assist us in the assessment of an individual's risk of later osteoporosis through an understanding of epigenetic biomarkers, and may inform public health strategies aimed at reducing the burden of fractures and falls for generations to come.

Figure 8.2 The last child to be seen in the MAVIDOS 4 year follow-up holds her certificate of achievement.

Photo credit: Author's own. Parental permission was granted for publication.

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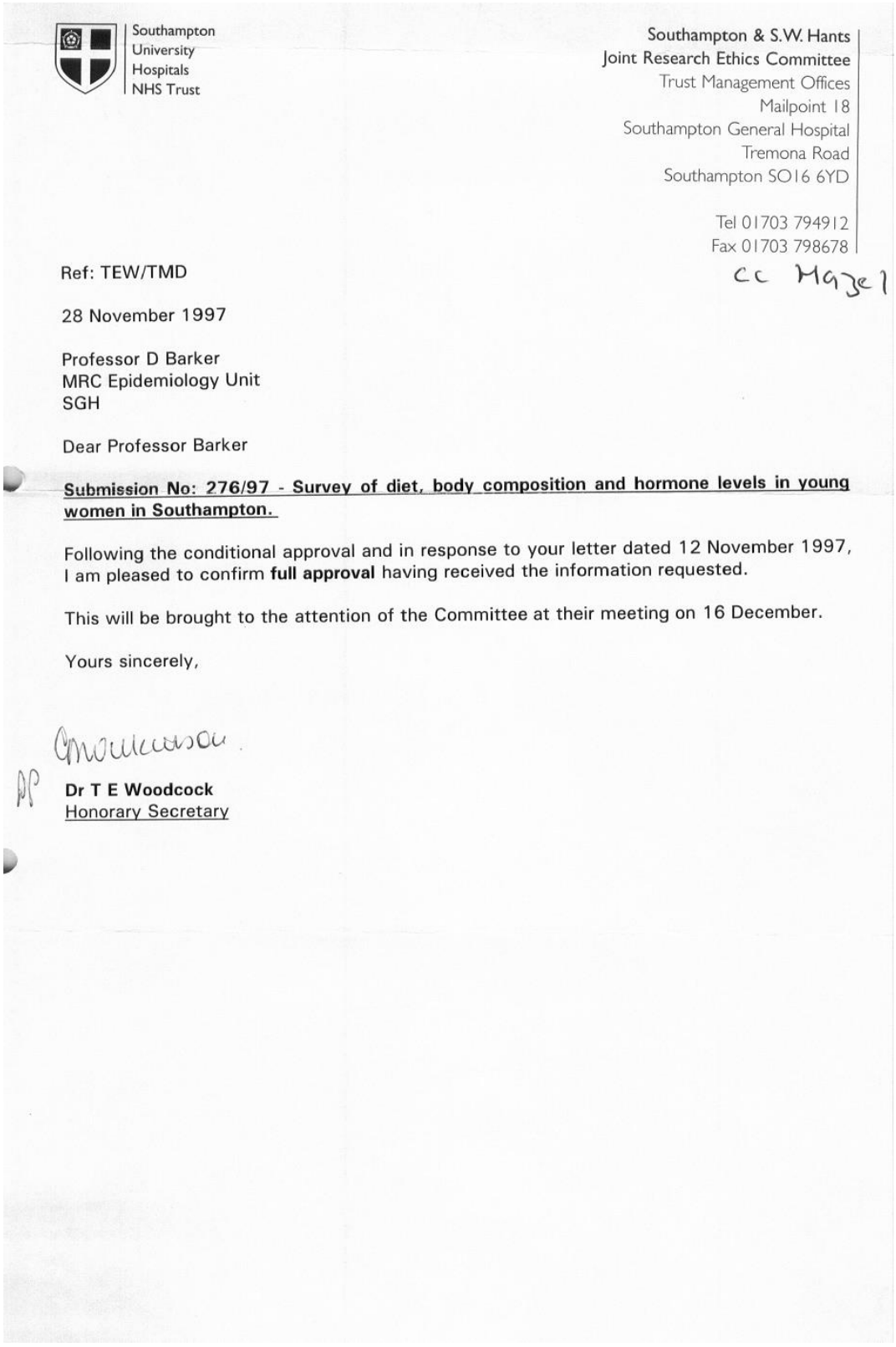
Appendices

Appendix A: Ethical approvals for the Southampton Women's Survey



Southampton University Hospitals NHS Trust

Southampton & S.W. Hants
Joint Research Ethics Committee
Trust Management Offices
Mailpoint 18
Southampton General Hospital
Tremona Road
Southampton SO16 6YD

Tel 01703 794912
Fax 01703 798678

cc Maggi

Ref: TEW/TMD

28 November 1997

Professor D Barker
MRC Epidemiology Unit
SGH

Dear Professor Barker

Submission No: 276/97 - Survey of diet, body composition and hormone levels in young women in Southampton.

Following the conditional approval and in response to your letter dated 12 November 1997, I am pleased to confirm **full approval** having received the information requested.

This will be brought to the attention of the Committee at their meeting on 16 December.

Yours sincerely,

pp

Dr T E Woodcock
Honorary Secretary

Southampton University Hospitals NHS Trust

Southampton & S.W. Hants
Joint Research Ethics Committee
Trust Management Offices
Mailpoint 18
Southampton General Hospital
Tremona Road
Southampton SO16 6YD

Tel 01703 794912
Fax 01703 798678

cc Hazel

Ref: TEW/CPW

28 November 1997

Professor D Barker
MRC Epidemiology Unit
University of Southampton
SGH

Dear Professor Barker

RE: 307/97 - The effects of maternal nutrition, body composition and cardiovascular status on fetal development & metabolic programming.

The Joint Ethics Committee considered your application for the above study at its recent meeting and I am pleased to inform you that **approval was given**. May I draw your attention to the enclosed conditions of approval which must be complied with.

This committee is fully compliant with the International Committee on Harmonisation/Good Clinical Practice (ICH) Guidelines for the Conduct of Trials involving the participation of human subjects as they relate to the responsibilities, composition, function, operations and records of an Independent Ethics Committee/Independent Review Board. To this end it undertakes to adhere as far as is consistent with its Constitution, to the relevant clauses of the ICH Harmonised Tripartite Guideline for Good Clinical Practice, adopted by the Commission of the European Union on 17 January 1997.

The composition of the committee is enclosed for your files and confirms which members were present at the meeting. Most pharmaceutical companies request this information and we would be grateful if you could forward this to them if appropriate.

Should any unforeseen problem of either an ethical or procedural nature arise during the course of this research and you feel the Joint Ethics Committee may be of assistance, please do not hesitate to contact us.

Yours sincerely,

Dr T E Woodcock
Honorary Secretary
Joint Ethics Committee

Southampton University Hospitals NHS Trust

→ Hazel

Southampton & S.W. Hants
Joint Research Ethics Committee
Trust Management Offices
Mailpoint 18
Southampton General Hospital
Tremona Road
Southampton SO16 6YD

Tel 01703 794912
Fax 01703 798678

Ref: CPW/DBL

30
~~26~~th April 1999

Professor D J P Barker - Director
MRC Environmental Epidemiology Unit
(University of Southampton)
Southampton General Hospital

Dear Professor Barker

Submission No:089/99 -Follow-up of infants born to women in the Southampton Women's Survey.

Following the conditional approval and in response to your letter dated 13th April 1999, I am pleased to confirm **full approval** having received the amended consent forms.

This approval was granted on Chairmans action and was brought to the attention of the Committee at their meeting on 28th April 1999.

This committee is fully compliant with the International Committee on Harmonisation/Good Clinical Practice (ICH) Guidelines for the Conduct of Trials involving the participation of human subjects as they relate to the responsibilities, composition, function, operations and records of an independent EthicsCommittee/Independent Review Board. To this end it undertakes to adhere as far as is consistent with its Constution, to the relevant clauses of the ICH Harmonised Tripartite Guideline for Good Clinical Practice, adopted by the Commission of the European Union on 17 January 1997.

Yours sincerely,

C Wilkinson

Clair Wilkinson (Ms)
Research Ethics Administrator

Hampshire and Isle of Wight NHS
Strategic Health Authority

Ref: CPW/HPH

SOUTHAMPTON & SOUTH WEST HAMPSHIRE
LOCAL RESEARCH ETHICS COMMITTEES
1ST Floor, Regents Park Surgery
Park Street, Shirley
Southampton
SO16 4 RJ

Tel: 023 8036 2466
023 8036 3462
Fax: 023 8036 4110

General Enquiries: sharon.atwill@gp-j82203.nhs.uk
clair.wright@gp-j82203.nhs.uk

21 March 2003

Dr M K Jarvaid
ARC Clinical Research Fellow to Professor C Cooper
MRC Environmental Epidemiology Unit
MP 95
SGH

Dear Dr Jarvaid,

Submission No: 005/03/t – Parental determinants of skeletal growth: a longitudinal study.

Following conditional approval and in response to your letter dated 4th March 2003, I am please to confirm **full approval** having responded satisfactorily to the committees concerns.

The following document(s) were re-considered:

- **Letter dated 4th March 2003**
- **Information Sheet**
- **Consent Form**

Please note that all paperwork i.e. Information Sheet etc. should be on departmental headed paper and must carry identification version number and date.

This approval was granted under Chairman's action by Vice Chairman Mr Mervyn Griffiths, and will be recorded by the Committee at their meeting in April.

This committee is fully compliant with the International Committee on Harmonisation/Good Clinical Practice (ICH) Guidelines for the Conduct of Trials involving the participation of human subjects as they relate to the responsibilities, composition, function, operations and records of an independent Ethics Committee/Independent Review Board. To this end it undertakes to adhere as far as is consistent with its Constitution, to the relevant clauses of the ICH Harmonised Tripartite Guideline for Good Clinical Practice, adopted by the Commission of the European Union on 17 January 1997.

Yours sincerely

Mrs Clair Wright
Research Ethics Manager

Chairmen: Dr Audrey Kermode/ Dr David Briggs
Manager: Mrs Clair Wright

Appendix B: Ethical Approvals for the MAVIDOS Trial and Four Year Followup

NHS

National Research Ethics Service

VY/STA/hph

SOUTHAMPTON & SOUTH WEST HAMPSHIRE
RESEARCH ETHICS COMMITTEE (A)
1ST Floor, Regents Park Surgery
Park Street, Shirley
Southampton
Hampshire
SO16 4RJ

Tel: 023 8036 2466
023 8036 3462
Fax: 023 8036 4110

Email: scsha.SWHRECA@nhs.net

03 December 2007

Professor Cyrus Cooper
Professor of Rheumatology, Director of MRC ERC
MRC Epidemiology Resource Centre
MRC ERC
Southampton General Hospital
Southampton
SO16 6YD

Dear Professor Cooper,

Full title of study:	**A double blind randomised placebo controlled trial of vitamin D supplements for pregnant women with low levels of vitamin D in early pregnancy**
REC reference number:	**07/H0502/113**
Protocol number:	**1.5**
EudraCT number:	**2007-001716-23**

Thank you for your letter of 20 November 2007, 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 Vice-Chair.

Confirmation of ethical opinion

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.

In the second page of your response, the Committee have picked up that you have made a few minor changes to the pregnancy questionnaire and it is possible that further minor changes may need to be made during the pilot phase, so The Committee requests to see the final versions at the end of pilot.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		26 July 2007
Investigator CV		
Protocol	1.5	10 November 2007
Covering Letter		25 July 2007
Compensation Arrangements		08 June 2007
Questionnaire: Child Follow Up	1.0	25 July 2007
Questionnaire: 34 Week	1.4	19 November 2007
Questionnaire: 14 Week	1.7	19 November 2007
Letter of invitation to participant	1.1	11 October 2007
GP/Consultant Information Sheets	1.0	07 June 2007
Participant Information Sheet	1.15	19 November 2007
Participant Consent Form: 4 Year	1.1	19 November 2007
Participant Consent Form: Neonatal	1.2	19 November 2007
Participant Consent Form: Initial	1.7	19 November 2007
Response to Request for Further Information		20 November 2007
Obstetric Information Letter	1.0	07 June 2007
Midwife Information Letter	1.0	07 June 2007
Request for Authorisation form MHRA		31 July 2007

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

07/H0502/113	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Mrs Vikkie Yule
Vice-Chair

Email: scsha.SWHRECA@nhs.net

Enclosures: Standard approval conditions

Site approval form

Copy to: Ms Christine McGrath
Southampton University Hospital NHS Trust

Clinical Trials Unit, MHRA

Southampton & South West Hampshire REC (A)

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	07/H0502/113	**Issue number:**	0	**Date of issue:**	03 December 2007
Chief Investigator:	Professor Cyrus Cooper				
Full title of study:	A double blind randomised placebo controlled trial of vitamin D supplements for pregnant women with low levels of vitamin D in early pregnancy				

This study was given a favourable ethical opinion by Southampton & South West Hampshire REC (A) on 30 November 2007. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* (1)
Professor Cyrus Cooper	**Professor of Rheumatology, Director of MRC ERC**	**Southampton University Hospitals NHS Trusts Tremona Road Southampton**	**Southampton & South West Hampshire REC (A)**	**03/12/2007**	

Approved by the Chair on behalf of the REC:

..... (Signature of Co-ordinator)

Mrs. Sharon Atwill

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

NHS
Health Research Authority

NRES Committee South Central - Hampshire A
Bristol Research Ethics Committee Centre
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Tel: 0117 342 1381

26 November 2013

Ms Christine McGrath
University Hospital Southampton NHS Foundation Trust
R & D Department,SUHT
Ground Floor, Duthie Building,MP138
Southampton General Hospital
SO16 6YD

Dear Ms McGrath

Study title:	**A double blind randomised placebo controlled trial of vitamin D supplements for pregnant women with low levels of vitamin D in early pregnancy**
REC reference:	**07/H0502/113**
Protocol number:	**1.3**
EudraCT number:	**2007-001716-23**
Amendment number:	**2.2**
Amendment date:	**03 October 2013**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	2.2	20 September 2013
Participant Information Sheet: Bone density scans in mothers and children	1.0	06 September 2013
Participant Consent Form: 4yr - additional maternal investigations	1.0	09 September 2013
Questionnaire: Child Follow up 4 years old		15 February 2013
Participant Consent Form: 4yr - additional assessments	1.0	09 September 2013

Letter from radiation expert		25 September 2013
Covering Letter		10 October 2013
Letter from Pat Taylor		
Participant Information Sheet: Blood tests in children at 4 years of age	1.0	06 September 2013
European Commission Notification of Substantial Amendment Form	2.2	03 October 2013
Participant Information Sheet: Physical activity monitoring	1.0	06 September 2013

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

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.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

07/H0502/113:	**Please quote this number on all correspondence**

Yours sincerely

Dr Iain MacIntosh
Chair

E-mail: nrescommittee.southcentral-hampshirea@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Prof Cyrus Cooper, MRC Lifecourse Epidemiology Unit*

A Research Ethics Committee established by the Health Research Authority

NRES Committee South Central - Hampshire A

Attendance at Sub-Committee of the REC meeting on 12 November 2013

Name	*Profession*	*Capacity*
Dr Ronja Bahadori	Clinical Trial Coordinator	Expert
Dr Mary Lanyon	Retired Veterinarian	Lay Plus
Dr Iain MacIntosh	Consultant Paediatric Intensive Care	Expert

Also in attendance:

Name	*Position (or reason for attending)*
Mrs Vicky Canfield-Duthie	REC Manager

What are the possible benefits of taking part?

We will perform the NHS ultrasound scan at 19 weeks in our dedicated suite. You will be given a picture of your baby to take home. Your child will have a measurement of their bone mass and you will find out if your vitamin D levels are very low, so that you can receive supplements if needed.

Will my taking part in this study be kept confidential?

All information collected about you will be kept strictly confidential. Your GP and obstetric team will be informed of your participation and we will inform your GP if your vitamin D levels are very low and require treatment.

What will happen to the results of the research?

We will see how taking vitamin D supplements in pregnancy affects bone mass in the baby. These findings will be published in medical journals, and possibly in the local and national press. You will not be identified in these reports/publications in any way. Ultimately, we hope that this study will inform government policy makers.

What if there is a problem?

If you have a concern about any aspect of this study, please ask to speak to the researchers who will do their best to answer your questions (023 8079 4186).

If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure (via Southampton General Hospital). In the unlikely event that you are harmed due to someone's negligence, you may have grounds for compensation but you may have to pay your own legal costs.

Who has reviewed the study?

This study has been reviewed and approved by Southampton and South West Hampshire Research Ethics Committee. Ethics No.: 07/H0502/113

Who is organizing and funding the research?

This study is funded by the MRC and Arthritis Research Campaign, and organized by the MRC Lifecourse Epidemiology Unit and University of Southampton.

Contact for further information

For further information please contact Professor Cyrus Cooper (Chief Investigator) or Dr Nick Harvey (Principal Investigator) at the MRC Lifecourse Epidemiology Unit at Southampton on 023 8077 7624

MRC Lifecourse Epidemiology Unit

You are invited to take part in

Mavid o s

Maternal Vitamin D Osteoporosis Study

A Vitamin D trial in pregnant women:

A double-blind randomised placebo-controlled trial of vitamin D supplements for pregnant women with low levels of vitamin D in early pregnancy.

If you are interested in taking part, please telephone us at the earliest opportunity on:

Telephone: 023 8120 4186

Email: mavidos@mrc.soton.ac.uk

UNIVERSITY OF Southampton

You are being invited to take part in a trial of Vitamin D supplementation in pregnancy. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with anyone you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. A nurse may approach you when you attend for your ultrasound scan.

What is the purpose of this study?

To find out whether giving mothers vitamin D supplements in pregnancy might improve the strength of their child's bones. We also aim to find out if vitamin D affects the immune defence system in the child.

Why might I be approached?

You are currently in early pregnancy and are due to attend hospital for an ultrasound scan.

Do I have to take part?

You decide whether or not to take part. If you do take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

At 12 weeks: After your NHS ultrasound scan, a blood sample will be taken to measure the level of vitamin D in your blood. If this is below the normal level you will not be eligible to take part in the trial and we will arrange for you to receive a vitamin D supplement through your GP. If the level is sufficiently high you will also not be eligible to take part in the trial.

At 14 weeks: If the vitamin D level is intermediate, we will arrange an appointment either at your home or at Princess Anne Hospital (PAH) in the 14th week of your pregnancy. At this visit a nurse will ask you questions about your diet and lifestyle, and take some body measurements, including your partner's height, if they are present. The nurse will take a blood sample (which will include a sample for genetic studies: this will not identify any genetic conditions), and ask for a urine sample. You will be given a supply of study medication for the duration of the trial. You will be randomly allocated to receive either vitamin D tablets 1000 units daily or placebo (dummy) tablets; neither you nor the nurse will know which. You may also be given a light sensitive badge to wear.

At 19 weeks: At PAH, NHS routine ultrasound scan, research ultrasound scan.

At 34 weeks: At PAH, the measurements taken at 14 weeks will be repeated.

At birth: When you have given birth, the midwife will collect samples from the afterbirth (tissue from the placenta and tissue and blood from the cord). This will have no effect on the baby whatsoever. We will also record some information from your hospital notes.

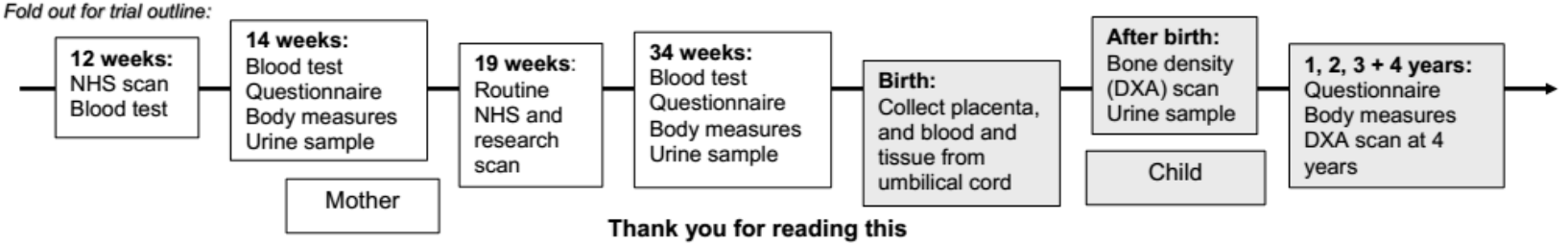
After birth: We will collect a urine sample from your baby and perform a bone density (DXA) scan, and body measurements.

In childhood: When your child is 1, 2, 3 and 4 years old, we will assess your child's height, weight, body build, diet and health. At 4 years we will perform another DXA scan of your child.

What are the possible disadvantages and risks in taking part?

The bone density scan involves exposing your child to a very low dose of x-rays, similar to the x-rays experienced by spending a week outdoors. The dose of vitamin D has been chosen to keep levels in the **normal** range, as there is speculation that high vitamin D levels in pregnancy may make the child slightly more prone to eczema and asthma.

Fold out for trial outline:



Thank you for reading this

Appendix D: MAVIDOS trial 4 year follow-up participant information sheets

MRC Lifecourse Epidemiology Unit
Southampton General Hospital
Southampton S016 6YD

Telephone: 023 8079 4186

Maternal Vitamin D Osteoporosis Study

Bone density scans in mothers and children

Introduction

You are currently participating in the MAVIDOS study. As part of this study your child will have had his/her bone density measured soon after they are born using a DXA bone density scanner. Some mothers also had a DXA scan at the same time.

Your child is invited to have a repeat DXA at 4 years of age. At this visit, we would also like to invite you to have a DXA scan, and for both you and your child to both have another type of bone scan (pQCT).

What if I would prefer not to have these additional scans?

You can decide whether or not you would like you and/or your child to have these additional scans. If you decide not to have it then this will not affect your participation in the MAVIDOS trial in any way, or the medical care you receive.

What do the scans involve?

DXA scan - mother

The DXA scan is similar to the scan your child had just after he or she was born, and is the same scan as your child will have at the 4 year visit. A small scanning arm passes over you about two feet in the air; it does not touch you. The scans are quick: The main scan of the whole body will take around 5 minutes and then scans of your lower spine and both hips will take around 15 seconds each.

pQCT scan – mother and child

The pQCT scan involves sitting on a chair and putting your leg into an open metal tube; it does not touch you. This scan takes around 5 minutes.

Neither of the scans will hurt and the dose of X-rays used in these scans is less than one week's background radiation. You would not be eligible to participate in these studies if you are pregnant. The two scanners are located in the same room and you and your child will both be able to stay in the room whilst the scans are being performed.

It is important that you are wearing light clothing (eg T shirt and tracksuit bottoms) with no metal fixings as these will show on the scan.

If you have any questions about this, please call 023 8079 4186

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MRC Lifecourse Epidemiology Unit
Southampton General Hospital
Southampton S016 6YD

Telephone: 023 8079 4186

Blood tests in children at 4 years of age

Introduction

You and your child are currently participating in the MAVIDOS study. As part of this study you and your child will be invited to attend Southampton General Hospital for a repeat bone scan (similar to the scan you child had shortly after birth) when your child is 4 years of age.

We would also like to invite your child to donate a blood sample to determine your child's vitamin D level and how this affects your child's bone development. We may also use this sample to investigate other markers of your child's health and to look at genes that might be important to bone development, but we will not be testing for individual genetic disorders.

What will happen to my child if I agree to blood sampling?

Some local anaesthetic cream ("magic cream") will be put on your small areas of your child's hands and/or arms. This numbs the skin so that any discomfort is minimised. The blood sample will be taken by a doctor or nurse, who has been specifically trained in taking blood samples from children. Distraction techniques will also be used, which often means children of this age do not realise they have had blood taken. Approximately 10ml (equivalent to two teaspoons) of blood will be taken.

What will happen with the blood sample?

The samples will be stored securely in freezers at the MRC Lifecourse Epidemiology Unit. We will not be testing for any specific disorders and we will not be able to inform you about any blood results for your child. Some samples may be stored for future research.

Does my child have to take part in this additional study?

It is up to you to decide if you would like your child to participate in these studies. If you do not wish to take part in the blood sampling, this will not affect you and your child's participation in the MAVIDOS study or your health care.

If you have any questions about this, please call 023 8079 4186

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Appendix E: MAVIDOS 4 year questionnaire

Study No: ☐☐☐☐

Child Follow-up Questionnaire **4 Years Old** ☐

Date of Birth _____

Date Questionnaire Completed _____

Nurse Number ☐☐

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1. **Food Frequency** Now I am going to ask you about **a few** of the **foods** your child has eaten in the **past 3 months**. I will ask you how often he/she has eaten certain foods and also the amount of food eaten. For some foods, I will show you drawings and models to help you estimate the amount of food. Your child may sometimes have eaten food away from home. If you know the type of food and approximate amount eaten at these times please include them.
Explain the use of spoons, cups, bowl and diagrams.

	Food	never	less than once per month	1-3 times per month	number of times per week 1	2	3	4	5	6	7	more than once per day	no. of times per day	Average amount per serving
bread and crackers														
1.1	white bread, rolls, toast	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	no. of slices ☐•☐ *(1 roll/bagel/croissant = 2 slices bread) (if all crusts gone=0.7 slice)*
1.2	brown bread, rolls, toast	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	no. of slices ☐•☐
1.3	cakes, scones biscuits	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	no. of portions *(1 portion = 2 biscuits, 1 scone, 1 slice of cake)* ☐•☐
1.4	breakfast cereals	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	no. of tbsp (1 weetabix = 4 tbsp 1 minibix = 1 tbsp) ☐•☐
1.5	What are the main types of breakfast cereal used?	Type					Brand							☐☐☐☐☐
		Type					Brand							☐☐☐☐☐
		Type					Brand							☐☐☐☐☐

	Food	never	less than once per month	1-3 times per month	number of times per week 1	2	3	4	5	6	7	more than once per day	no. of times per day	Average amount per serving
1.6	oily fish	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	no. of portions ☐☐.☐ *1 sm can tuna = 2, med= 4;* *1 tbsp= 0.25 portions* *salmon in sandwich = 1*
1.7	eggs	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	no. of eggs ☐☐.☐ *yoke=0.4, white= 0.6*
1.8	cheese	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	*tbsp grated = 0.5* ☐☐.☐ *small triangle = 1 cheese per slice = 1 see drawing*
1.9	What are the main types of cheese used?	Type			Brand									☐☐☐☐☐
		Type			Brand									☐☐☐☐☐
		Type			Brand									☐☐☐☐☐
1.10	baked beans	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	no. of small tins ☐☐.☐ *. small tin = 200g*
1.11	yoghurt & fromage frais	0	0.3	0.5	1	2	3	4	5	6	7	8	☐	no. of grams ☐☐☐ *tiny pot 50g,* *bigger 100g*

	Food	never	less than once per month	1-3 times per month	number of times per week 1	2	3	4	5	6	7	more than once per day	no. of times per day	Average amount per serving
1.12	What are the main types of yoghurt and fromage frais used?	Type			Brand									
		Type			Brand									
		Type			Brand									
1.13	ice-cream	0	0.3	0.5	1	2	3	4	5	6	7	8		no. of tablespoons *1scoop = 4 1 choc ice/ Fab/ Mars i/c etc = 4*
1.14	custard and sweet white sauce	0	0.3	0.5	1	2	3	4	5	6	7	8		no. of tablespoons
1.15	butter & margarine	0	0.3	0.5	1	2	3	4	5	6	7	8		no. of teaspoons *1 sl bread = 1.5 tsp 1 pat =2 tsp*
1.16	What are the main types of spread?	1/			2/				3/					1 2 3
1.17	milky drinks	0	0.3	0.5	1	2	3	4	5	6	7	8		No. standard beakers 7 oz/ 200 ml
1.18	What are the main types of milky drink?	1/			2/				3/					1 2 3

1.19 Now I would like to ask in more detail about your child's milk intake over the past 3 months.

Which types of milk has your child used regularly in drinks and added to breakfast cereals?
(list up to 3 below)

0. None
1. Whole pasteurised
2. Semi-skimmed pasteurised
3. Skimmed pasteurised
4. Whole UHT
5. Semi-skimmed UHT
6. Skimmed UHT
7. Breast milk
8. Formula milk, toddler milks, 'growing up' milk etc
9. Other

Milk 1 ☐ If "Other" *specify* _____

Milk 2 ☐ If "Other" *specify* _____

Milk 3 ☐ If "Other" *specify* _____

1.20 On average over the last 3 months how much of each milk has he/she consumed per day?
(1 average beaker = 0.35 pints; 1 pint = 20oz / 568 mls)

Milk 1 ☐ . ☐☐ Pints

Milk 2 ☐ . ☐☐ Pints

Milk 3 ☐ . ☐☐ pints

1.21 During the past 3 months have you given him/her any vitamins or minerals, including vitamin D, iron and fluoride drops or tablets?

0. No go to Q2 ☐
1. Yes

1.22 Please state which:

Supplement Name	Code	How many days in the last 90?	Is It: 1) Tablet 2) Drops 3) Liquid 4) Other? (State)	No. of stated units per day
	☐☐☐☐			
	☐☐☐☐			
	☐☐☐☐			
	☐☐☐☐			

1.23* On average over the last 3 months how many beakers of water has your child drunk each day? Include plain water and drinks made mostly from water, such as squash, tea and coffee.
Number of beakers per day ☐☐ • ☐

1.24* Is this water mainly
1. Tap Water,
2. Filtered Tap Water,
3. Ordinary mineral water, Danone
4. Mineral water with added calcium (eg variety)
5. Other *(Please specify)* _____ ☐

2. SLEEP, ACTIVITY AND EXERCISE

Now I'm going to ask you about your child's sleeping, activity and exercise patterns over the last three months. *[We are trying to get figures that eventually total approximately 24 hours, so rounding to nearest hour is OK – best guess is acceptable]*

2.1 What time does the study child generally go to sleep at night (24 hour clock) ☐☐☐☐

2.2 How many times **per night** does he/she generally wake for any reason? ☐ • ☐ **Per night**
Please answer this in relation to the last month.

If (0) please go to Question 2.4

2.3 In total, how long is he/she awake? ☐☐ **hrs** ☐☐ **mins per night**
(Only record if regularly over 30 mins)

2.4 What time does he/she generally wake up in the morning (24 hour clock) ☐☐☐☐

2.5 **This means that he sleeps for about** ☐☐ **hrs** ☐☐ **mins per night**

2.6 How many **days per week** does he/she take a daytime nap? ☐
Please answer this in relation to the last month
If "0" deduct 2.5 from 24 and insert at 2.9

2.7 On the days he/she naps, what is the **total time** spent napping during the day? ☐☐ **hrs** ☐☐ **mins**

Using responses to 2.6 and 2.7 consult "Daily Averages" grid

2.8 Average daily nap time ☐ **hrs** ☐☐ **mins**

Add 2.5 to 2.8 and deduct from 24

2.9 This would indicate that he/she is awake on average each day? ☐☐ **hrs** ☐☐ **mins**

2.10* On a typical day, how many hours does he/she generally spend watching television/computer games (i.e. DS/iPad or anything requiring 'screen time')?

1. More than 5 hours ☐ 2. 4-5 hours ☐ 3. 3-4 hours ☐
4. 2-3 hours ☐ 5. 1-2 hours ☐ 6.Less than one hour ☐
7. None ☐

YOUR CHILD'S HEALTH

I would like to ask you some questions about your child's skin and any illnesses your child might have had **since we last visited you when he/she was about three years old (since three years old if not seen for the thirty-six month follow-up)**

3 SKIN CONDITIONS

3.1 Has he/she had an itchy skin condition at any time - by itchy we mean scratching or rubbing the skin a lot? *(exclude chicken pox)* ☐

No *go to 2.4* 1. Yes

3.2 *Has thi*s skin condition affected the cheeks, the outer arms or legs, or the skin creases in the past - by skin creases we mean the folds of the elbows, behind the knees, the fronts of the ankles, or around the eyes? ☐

No 1. Yes

3.3 How old was he/she when the rash first appeared? – *Clarify first* ☐ Yrs ☐☐ Mths ☐ Wks ☐ Days

3.4 In the past twelve months, has he/she suffered from a generally dry skin? ☐

0. No 1. Yes 8. To a minor degree

3.5* **In the past twelve months**, has he/she had a **scaly**, or **red and weeping** skin rash affecting any of the following areas: ☐

a) the scalp or behind the ears (including "cradle cap")	0.No	1. Yes	☐
b) around the neck	0.No	1. Yes	☐
c) the cheeks or forehead	0 No	1. Yes	☐
d) either the folds of the elbows or behind the knees	0 No	1. Yes	☐
e) the forearms, wrists, shins or ankles	0 No	1. Yes	☐
f) the shoulders, chest, tummy or back	0 No	1. Yes	☐
g) in the armpits	0 No	1. Yes	☐
h) the nappy area (including nappy rash)	0 No	1. Yes	☐

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4 Has your child had a problem with sneezing or a runny, or blocked nose when s/he did not have a cold or the flu? *If No go to Q6* 0. No 1. Yes ☐

5 Has this nose problem been accompanied by itchy-watery eyes? 0. No 1. Yes ☐

6 Has your child had an allergic reaction when in contact with animals? 0. No 1. Yes ☐

7 Has your child had wheezing or whistling in the chest 0. No 1. Yes ☐
If no, go to Question 11a

If yes, when did it start? ☐ Yrs ☐☐ Mths ☐ Wks ☐ Days

8 Does your child wheeze in association with chest infection? (chesty cough and fever) 0. No 1. Yes ☐

9 Has your child had wheezing in association with a cold? (nasal congestion and discharge) 0. No 1. Yes ☐

10 Does your child wheeze in between colds or a chest infection? 0. No 1. Yes ☐

11a Has your child had a dry cough at night, apart from a cough associated with a cold or chest infection? 0. No 1. Yes ☐

If yes, when did it start? ☐ Yrs ☐☐ Mths ☐ Wks ☐ Days

11b Has your child had chest infection/s (chesty cough and fever) 0. No 1. Yes ☐

If so, how many since last visit? ☐☐

12 Has your child had any colds (nasal congestion and discharge) 0. No 1. Yes ☐

If so, how many since last visit (or in the last 12 months if seen for the first time)? ☐☐

13 Has a doctor ever diagnosed asthma in your child? 0. No 1. Yes ☐

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14* Have any of the following medications been used? 0. No 1. Yes ☐

a) Bronchodilators (e.g. Ventolin/Salbutamol) 0. No 1. Yes ☐

If yes, how long ago was it last given? ☐☐ Mths ☐ Wks ☐ Days

b) Antihistamines 0. No 1. Yes ☐

If yes, how long ago was it last given? ☐☐ Mths ☐ Wks ☐ Days

c) Corticosteroids 0. No 1. Yes ☐

Oral 0. No 1. Yes ☐

If yes, how many courses? ☐☐

If yes, how long ago was it last given? ☐☐ Mths ☐ Wks ☐ Days

Inhaled 0. No . 1. Yes ☐

If yes, age when prescribed? ☐☐ Mths ☐ Wks ☐ Days

Any other medication for wheeze? (*Please specify below)*

_____

_____

15 Does your child take any regular medicines (either from the chemist, doctor, or alternative therapies)? *Please include inhalers for asthma.* 0. No . 1. Yes ☐

If no, please go to Question 21.
If yes, please list them in the table below

Medicine Name	**Code**	**How many days in the last 90?**	**Is it: 1) Tablet 2) Drops 3) Liquid 4) Other *(specify)***	**Dose per day**
	☐☐☐☐			
	☐☐☐☐			

16* How is the study child's health in general? Would you say it was:-

1. Very good ☐ 2. Good ☐ 3. Fair ☐
4. Bad ☐ 5. Very bad ☐

17 Does he/she have any long-standing medical conditions? By long standing I mean anything that has troubled him/her over a period of time, or that is likely to effect him/her over a period of time. 0. No 1. Yes ☐
If no, go to Q21

18 What is this condition?

_____

19 Does this condition limit his/her activities in any way? 0. No 1. Yes ☐
If no, go to Q20

20 If yes, in what way does it limit his/her activities?

_____

_____

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FOOD ALLERGIES

21 Does your child have adverse reactions to any foods, such as eczema, breathing problems or gastrointestinal problems? 0. No . 1. Yes ☐

22* *Has your child suffered from a food allergy? 0. No . 1. Yes ☐

If no, go to question 23

Suspected food 1

_____

How many reactions to this food? ☐☐

a) How long did it take to develop the symptoms?

1. 30 mins 2. 30 mins – 2 hours 3. > 2 hours ☐

b) What symptoms? Please tick (✓)

Urticaria/ hives ☐ Eczema ☐ Angio-oedema ☐ Oral symptoms ☐ Wheezing/ SOB ☐

Vomiting ☐ Diarrhoea ☐ Colic ☐ Sysemic ☐

Other (Please specify)

_____

c) ***Suspected food 2***

_____

How many reactions to this food? ☐☐

d) How long did it take to develop the symptoms?

1. 30 mins 2. 30 mins – 2 hours 3. > 2 hours ☐

e) What symptoms? Please tick (✓)

Urticaria/ hives ☐ Eczema ☐ Angio-oedema ☐ Oral symptoms ☐ Wheezing/ SOB ☐

Vomiting ☐ Diarrhoea ☐ Colic ☐ Sysemic ☐

Other (Please specify)

_____

f) ***Suspected food 3***

How many reactions to this food? ☐☐

g) How long did it take to develop the symptoms?

1. 30 mins 2. 30 mins – 2 hours 3. > 2 hours ☐

h) What symptoms? Please tick (✓)

Urticaria/ hives ☐ Eczema ☐ Angio-oedema ☐ Oral symptoms ☐ Wheezing/ SOB ☐

Vomiting ☐ Diarrhoea ☐ Colic ☐ Sysemic ☐

Other (Please specify)

YOU AND YOUR FAMILY

23 Do you (mother) smoke?
0. No
1. Yes, Occasionally
2. Yes Daily ☐

24 Does anyone else smoke <u>inside</u> your home?
0. No
1. Yes, Occasionally
2. Yes Daily ☐

25 Is your child exposed to tobacco smoke outside the home? (for example at grandparents or other relatives, baby sitter)
0. No
1. Yes, Occasionally
2. Yes Daily ☐

26 Do you work in paid employment at the moment?
0. No
1. Yes, but currently on maternity leave
2. Yes ☐

27 How old was your child when you first went back to work? ☐☐ Mths ☐ Wks ☐ Days ☐ n/a

OR

On what date did you go back to work? d d m m y y ☐☐ ☐☐ ☐☐

CHILD CARE QUESTIONS

NURSERY

28 Has your child attended day care, a nursery, pre-school, or childminder? (Day care could include going to Grandparents) 0. No *(go to Q37)* 1. Yes *(go toQ29)* ☐

29 How old was your child when she/he first started day care? ☐☐ Mths ☐ Wks ☐ Days ☐ n/a

30 Has your child now stopped attending day care, a nursery, pre-school or childminder? 0. No (go to Q32 1. Yes (go toQ31) ☐

31 How old was your child when she/he stopped attending day care? ☐☐ Mths ☐ Wks ☐ Days ☐ n/a

32 How many hours a week on average does/ did your child attend day care, a nursery, pre-school or childminder? ☐☐ hrs ☐☐ mins

33 What type of day care does your child attend? *Please tick (✓)*

Child minder ☐ Nursery/Creche ☐ Pre-school ☐ Grandparents ☐

34 Approximately how many other children are/ were cared for by the childminder or attend/ attended the nursery (number in child's nursery/ pre-school room)/crèche ☐☐ children

35* Does/ did the childminder OR nursery/crèche/ pre-school have a pet(s)? 0. No 1. Yes ☐

36* If yes, please specify what pet(s) and where they are allowed
0. No,
1.Yes, allowed in bedroom (room where child sleeps)
2. Yes, not allowed in bedroom,
3. Yes, not in the house

Dog ☐ Cat ☐

Other *Please specify* ..... ☐

Other Please specify ..... ☐

Other Please specify ..... ☐

HOME

37* What main type of flooring is in the room where your child sleeps?

1. Carpet ☐ 2. Wooden, laminate,parquet ☐ 3. Linoleum or vinyl tiles ☐

4. Ceramic/terracotta tiles or stone ☐ 5. Sea-grass or coir-type matting ☐ 6. Other (Please specify) ☐

---

38 Does your child's mattress have a plastic surface or cover? 0. No . 1. Yes ☐

PETS

39 Do you have a pet at home? 0. No . 1. Yes ☐

40* If yes, please specify what pet(s) and where they are allowed
0. No,
1.Yes, allowed in bedroom (room where child sleeps)
2. Yes, not allowed in bedroom,
3. Yes, not in the house

Dog ☐

Cat ☐

Other *Please specify*..... ☐

Other Please specify..... ☐

Other Please specify..... ☐

41a* Do you have regular *(i.e. more than once a week)* exposure to animals elsewhere? ☐

0. No . 1. Yes

1. Dog ☐ 2. Cat ☐ 3. Other *(Please specify)* ☐

41b* Does your child have regular *(i.e. more than once a week)* exposure to animals elsewhere? ☐

0. No . 1. Yes

1. Dog ☐ 2. Cat ☐ 3. Other *(Please specify)* ☐

_____

42 How many adults live in the household? ☐☐

43 How many children live in the household (including this child)? ☐☐

44 How many bedrooms does your home have, including the child's room and guest room? ☐☐

QUESTIONS ABOUT YOUR HOME

45* Is your home

1. Owned Privately ☐ 2. Rented Privately ☐

3. Rented–Council/Housing Association ☐ 4. Other ☐

46* Do you cook on

1. Gas ☐ 2. Electricity ☐ 3. Other (specify) ☐

_____

47 Does your home have damp spots on the walls or ceilings? 0. No 1. Yes ☐

48 Does your home have visible moulds or fungus on the walls or ceiling? 0. No 1. Yes ☐

49* How often do you keep the windows open in your home?

1. Every day 2. Once a Week 3. Once a Month 4. Seldom 5. Never ☐

50* How often do vehicles pass your house or on the street less than 100 metres away?

1. ≥10 per hour 2. 1-9 per hour 3. 10 per day 4. Seldom 5. Never ☐

51* What term best describes where you live now?

1. Farm *(Go to Q59)* 2. Small town e.g. Romsey
3. City Centre e.g. Southampton
4. Rural village e.g. Exbury 5. Suburbs of a city e.g. Bassett ☐

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52 If you live on a smallholding, is there contact with farm animals (including animals kept in the garden)? (0. No. 1. Yes) please mark each box

Dairy Cattle . ☐ Beef Cattle ☐ Poultry ☐

Sheep ☐ Pigs ☐ Horse ☐

Other *(Please specify)* _____ 0. No 1. Yes ☐

53 Has your child received any vaccinations? 0. No 1. Yes ☐

Since the 3 year visit, *(or since the age of 3 years if the 3 year visit was omitted)*

54 **VACCINATIONS**

What vaccinations has your child received and dates received? (from vaccination record)
(Insert date if "Yes", otherwise tick under "No" or "Don't know")

	Routine Immunisations	Yes – Date given	0. No	9. Don't know
3 years 4 months	Diphtheria, tetanus, pertussis and polio Measles, mumps and rubella	d d m m y y	☐	☐

Non-Routine Immunisations	Yes – Date given	0. No	9. Don't know
Tuberculosis	d d m m y y	☐	☐
Hepatitis B	d d m m y y	☐	☐
Other (please specify) _____	d d m m y y	☐	☐
Other (please specify) _____	d d m m y y	☐	☐

55 In the past twelve months has your child broken any bones? 0. No 1. Yes ☐

If Yes which bone was broken? *(Please specify)* _____

56 Has your child had any illnesses requiring admission or assessment in hospital? 0. No 1. Yes ☐

Details:

57 Assessed (including outpatient appointments, and GP assessments)? 0. No 1. Yes ☐

Details:

58 Does your child do any organised physical activities e.g. swimming? 0. No 1. Yes ☐

(Please specify)

59 On an average day how many hours does your child spend outdoors:
a) in the winter ☐☐.☐☐

b) in the summer ☐☐.☐☐

60 Does your child use sunblock in sunny weather? 0. No 1. Yes ☐

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61 CHILD EXAMINATION

61.1 Measurement Date d d m m y y

61.2 Time (24 hr clock)

61.3 Measurer

61.4 Helpers (Parent 90)

61.5 Occipito-frontal circumference

. cm . cm

. cm . cm

. cm

Wriggling 0. No, 1. Yes

61.6 Left mid-upper arm circumference (arm straight)

. cm . cm

. cm . cm

. cm

Wriggling 0. No, 1. Yes

61.7 Chest circumference

. cm . cm

. cm . cm

. cm

Wriggling 0. No, 1. Yes

61.8 Waist circumference (standing)

. cm . cm

. cm . cm

. cm

Wriggling 0. No, 1. Yes

61.9 |Hip circumference (standing)

. cm

Wriggling 0. No, 1. Yes

Skinfold thicknesses

61.10 Triceps skinfold

☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm
☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm
☐☐•☐ cm			

Wriggling 0. No, 1. Yes ☐

61.11 Subscapular skinfold

☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm
☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm
☐☐•☐ cm			

Wriggling 0. No, 1. Yes ☐

61.12 Skinfold callipers used ☐☐☐☐

61.13 Height (barefoot) Leicester H/M

☐☐☐•☐ cm	☐☐☐•☐ cm	☐☐☐•☐ cm	☐☐☐•☐ cm
☐☐☐•☐ cm	☐☐☐•☐ cm	☐☐☐•☐ cm	☐☐☐•☐ cm
☐☐☐•☐ cm			

Wriggling 0. No, 1. Yes ☐

61.14 Sitting height Leicester H/M

☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm
☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm	☐☐•☐ cm
☐☐•☐ cm			

Wriggling 0. No, 1. Yes ☐

61.15 Stadiometer used ☐☐

61.16 Child's weight ☐☐•☐☐ kg
(preferably in underwear only, with no nappy)

61.17 Weight of any clothes/nappy ☐•☐☐ kg

61.18 Weighing scales used ☐☐☐☐

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61.19 **RIGHT SIDE** **LEFT SIDE**

GRIP STRENGTH
(Record to nearest 0.5 kg)

☐☐ • ☐ ☐☐ • ☐
☐☐ • ☐ ☐☐ • ☐
☐☐ • ☐ ☐☐ • ☐

61.20 Which hand does your child mostly use to write or hold a pencil with?

Left ☐ Right ☐

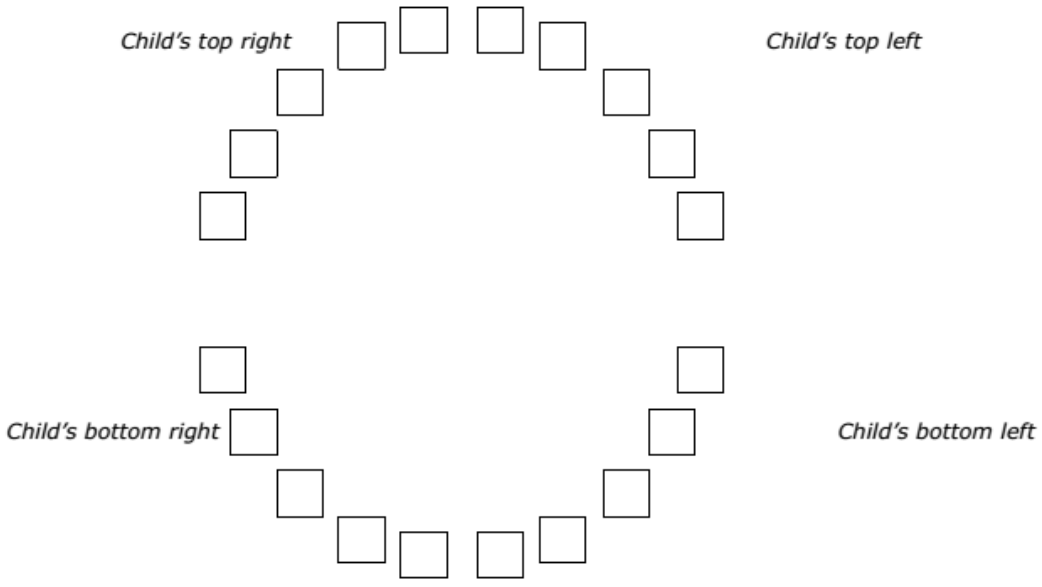
Ambidextrous ☐

(Writes with both hands)

62 TEETH

62.1 Number of teeth ☐☐

62.2 Position of teeth ☐☐
(Mark with a cross for each tooth present)



62.3 Has your child lost any teeth? ☐
0. No.
Yes – number of teeth

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63 SKIN EXAMINATION

Eczema = poorly defined redness with scaling, crusting, vesicles or accentuated skin markings (lichenification).

		Eczema Please tick box 0. No 1 Yes
63.1	Is/are there any?	☐
63.2	Scalp/Behind ears	☐
63.3	Face – cheeks and forehead	☐
63.4	Face – around the mouth	☐
63.5	Neck	☐
63.6	Arms – palms of the hands	☐
63.7	Arms – antecubital fossae	☐
63.8	Arms – remainder (backs of hands, forearms, upper arms)	☐
63.9	Arms – axillae	☐
63.10	Trunk – back	☐
63.11	Trunk – front (chest and abdomen)	☐
63.12	Legs – soles of feet	☐
63.13	Legs – popliteal fossae (behind knees)	☐
63.14	Legs – remainder of (i.e. thighs, lower leg, dorsa feet)	☐
63.15	Nappy area (including nappy rash)	☐