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The role of epigenetic processes in bone development

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Abstract

Faculty of Medicine

Doctor of Philosophy

The role of epigenetic processes in bone development

by Matthew Hewitt

Osteoporosis is a progressive systemic skeletal disease caused by an imbalance of the bone remodelling process leading to a net loss of bone. Peak bone mass, the amount of bone tissue accrued at the end of skeletal maturation, is a strong predictor of osteoporotic fracture risk in later life. Genotype has been suggested to account for around 10% of the variance in peak bone mass, with increasing evidence suggesting the remaining variance may be explained by environmental exposures in prenatal life, such as maternal vitamin D status. The influence the prenatal environment on the trajectory of skeletal development in postnatal life has been suggested to occur through epigenetic mechanisms. Consistent with this, candidate gene studies have identified epigenetic marks in birth tissues predictive of later bone outcomes.

This project aimed to: (i) identify DNA methylation signatures at birth associated with childhood BMC and BMD, and (ii) determine whether maternal vitamin D supplementation altered DNA methylation in genes linked to improved BMC or BMD in postnatal development. To investigate these aims epigenome-wide association studies (EWAS) were performed using the Illumina Infinium MethylationEPIC Beadchip (EPIC) array to interrogate birth tissue DNA from the Maternal Vitamin D Osteoporosis Randomised Control Study (MAVIDOS RCT) and the Southampton Women's Survey (SWS) studies along with umbilical cord mesenchymal stromal cells (UC-MSCs).

EWAS analysis identified methylation signatures at birth associated with childhood bone outcomes in both the MAVIDOS RCT and SWS studies. Within each study the top dmCpGs at each age group showed overlap between BMC and BMD outcomes, but with less overlap observed between age groups or between studies, indicating a tissue and age specificity to these changes. In addition, EPIC array interrogation of umbilical cord tissue DNA from the MAVIDOS RCT found maternal vitamin D supplementation induced significant differential methylation in three CpGs, including the potentially pro-osteogenic Solute Carrier Family 38 Member 10 and Tenascin C genes. The MAVIDOS RCT bone and intervention-related analyses together indicated differential methylation of multiple CpG sites in Protein Tyrosine Phosphatase Receptor Type N2 (*PTPRN2*), a gene involved in glucose homeostasis which also encodes miR-153, a negative regulator of insulin secretion and an anti-osteogenic factor in human bone marrow mesenchymal stromal cells. In the SWS cohort, differential methylation analysis of SWS umbilical cord blood DNA showed dmCpGs significantly associated with an insufficient maternal vitamin D status in late pregnancy. The most significant dmCpG was within Hexokinase 1, a rate-limiting gene in glycolysis. EWAS analysis of short-term vitamin D treated UC-MSCs also saw enrichment for dmCpGs in cellular respiration and glycolytic pathways.

In this study we identified associations between methylation of birth tissues and postnatal skeletal growth into childhood. Whilst the functional implications of these changes are not yet known, these dmCpGs, after further replication and validation, could potentially act as age and tissue-specific prospective markers of postnatal bone growth, helping to identify those with a lower peak bone mass and so a greater risk for osteoporosis later in life. These findings also suggest maternal vitamin D supplementation induced DNA methylation changes within foetal tissues in prenatal life may have the potential to influence the development of bone in postnatal life through epigenetic mechanisms. The effects of vitamin D on the epigenome may also be indirect through differential regulation of *PTPRN2* expression or through influence on glucose homeostasis, both of which may also have implications for skeletal development.

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Declaration of Authorship

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Title of thesis:	The role of epigenetic processes in bone development

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Abbreviations

Abbreviation	Description
1,25D(OH)2D	1 α ,25-dihydroxyvitamin D3
25(OH)D	25-hydroxyvitamin D3
3'UTR	Three prime untranslated region
5mC	5-methylcytosine
AA	Age acceleration
aBMD	Areal bone mineral density
Akt	Protein kinase B
ALP	Alkaline phosphatase
ALS	Acid-labile subunit
ANCR	Antidifferentiation noncoding RNA
ANOVA	Analysis of variance
AnP	Antarctic phosphatase
ATAC	Assay for transposase-accessible chromatin
ATCC	American type culture collections
ATF4	Activating Transcription Factor 4
AUC	Area under the recovery curve
BA	Bone area
B-cells	B-lymphocyte cells
BER	Base-excision repair
BGLAP	Bone gamma-carboxyglutamate protein
BiNGO	Biological Networks Ontology tool
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMIQ	Beta-mixture quantile
BM-MSCs	Bone marrow mesenchymal stromal cells
BMP	Bone-morphogenic protein
BMU	Basic multicellular units
BRC	Bone resorbing compartment
C/EBPα	CCAAT/enhancer binding protein α
cAMP	Cyclic-adenosine monophosphate
CD	Cluster of differentiation
CD29	Integrin Subunit Beta 1
CD29-PE	Cluster of differentiation 29-phycoerythrin
CD44-FITC	Cluster of differentiation 44-Fluorescein isothiocyanate
CD90	Thy-1 Cell Surface Antigen
CDKN2A	Cyclin-dependant kinase N2A
CFU-F	Colony forming unit fibroblast
ChAMP	Chip analysis methylation pipeline
ChIP-seq	Chromatin immunoprecipitation sequencing
cKO	Conditional knockout
COL10A1	Collagen type X α 1
COL2A1	Collagen type II α 1
COL9A1	Collagen type IX α 1
CpG	Cytosine-phosphate-guanine
CT	Cycle threshold
CV	Coefficient of variation
CX43	Connexin 43

CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1/24-hydroxylase
CYP27A1	Cytochrome P450 family 27 subfamily A member 1/vitamin D3 25-Hydroxylase
CYP27B1	Cytochrome P450 family 27 subfamily B member 1/1 α -hydroxylase
CYP2R1	Cytochrome P450 Family 2 Subfamily R Member 1/vitamin D 25-hydroxylase
DBP	Vitamin D binding protein
DEQAS	UK National Vitamin D External Quality Assurance Scheme
DICER	Cytosolic RNase III
DKK1	Dickkopf-related protein 1
DLX	Distal-less Homeobox
dmCpG	Differentially methylated cytosine-phosphate-guanine dinucleotide
DMEM	Dulbecco's modified eagle medium
DMP1	Dentin matrix acidic phosphoprotein 1
DMR	Differentially methylated region
DNase I	Deoxyribonuclease I
DNMTs	DNA methyltransferases
DOHaD	Developmental origins of health and disease
Dsh	Dishevelled
DXA	Dual energy X-ray absorptiometry
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ENCODE	Encyclopaedia of DNA Elements
eNOS	Nitric oxide synthase
EPIC	Illumina Infinium MethylationEPIC BeadChip
ERK	Extracellular signal-regulated kinase
ERα	Oestrogen receptor α
EWAS	Epigenome-wide association study
EZH2	Enhancer Of Zeste Homolog 2
FBS	Foetal bovine serum
FFSS	Fluid flow shear stress
FGF	Fibroblast growth factor
FGF23	Fibroblast growth factor 23
FGFR	Fibroblast growth factor receptors
FITC	Fluorescein isothiocyanate
FN-BMD	Femoral neck BMD
Fra-1	Fos-related antigen 1
FZD	Frizzled
GAA	Gestational age acceleration
GAD	Gestational age deceleration
GH	Growth hormone
GO	Gene ontology
GR	Glucocorticoid receptor
GSK-3	Glycogen synthase kinase 3
GWAS	Genome-wide association studies
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HES	Hairy and enhancer of split
HEY	HES-related with YRPW motif
HFBC	Human foetal bone cells
HH	Hedgehog
HK1	Hexokinase 1
HMT	Histone methyltransferase
HSC	Haematopoietic stem cell

HUVEC	Human umbilical vein endothelial cells
IDAT	Intensity data
IGF	Insulin-like growth factor
Ihh	Indian hedgehog
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPA	Ingenuity Pathway Analysis
IPTH	Intermittent PTH
ISCT	International Society for Cell and gene Therapy
IU	International units
JNK	c-Jun N-terminal kinase
KDM	JmJc containing histone demethylases
KMT	Histone lysine methyltransferase
LAP	Latency-associated protein
LB	Luria Broth
LMP	Last menstrual period
lncRNA	Long non-coding RNA
LRP	LDL Receptor Related Protein
LS-BMD	Lumbar spine BMD
MADscore	Median absolute deviation score
Maml	Mastermind-like
MAPK	Mitogen-activated protein kinase
MAPKs	Mitogen-activated protein kinases
MAVIDOS	Maternal Vitamin D Osteoporosis Study
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MEPE	Matrix extracellular phosphoglycoprotein
MESP	Maternal early-stage pregnancy
miRNA	MicroRNA
MLO-Y4	Murine long bone osteocyte-Y4
MLSP	Maternal late-stage pregnancy
MMP13	Matrix metalloproteinase 13
MSC	Mesenchymal stem cells
MSX1	Msh Homeobox 1
ncRNA	Non-coding RNA
NES	Normalised enrichment score
NFAT	Nuclear factor of activated T-cells
NFATC1	Nuclear factor of activated T cells 1
NF-kB	Nuclear factor kappa B
NHS	National Health Service
NICD	Notch intracellular domain
OCN	Osteocalcin
OPG	Osteoprotegerin
OSF-1	Osteoblast stimulating factor-1
Osx	Osterix
PAH	Princess Anne Hospital
PBC	Peripheral blood cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PE	Phycoerythrin
PGE2	Prostaglandin E2
PHEX	Phosphate-regulating neutral endopeptidase

Pi	Phosphate
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase a
PKC	Protein kinase C
PKCδ	Protein kinase Cδ
PPAR	Peroxisomal proliferator-activated receptor
PPi	Inorganic pyrophosphate
PS1	Presenilin 1
PTH	Parathyroid hormone
PTH1R	Parathyroid Hormone 1 Receptor
PTHrP	Parathyroid hormone-related protein
QC	Quality control
QQplot	Quantile-quantile plot
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RBPJk	Recombination signal-binding protein for Ig of κ region
RCT	Randomised controlled trial
Rho	Ras homologous
RhoA	Ras homolog family member A
RNA-seq	Ribonucleic acid sequencing
RSK2	Ribosomal protein S6 kinase A3
RT	Room temperature
RUNX2	Runt-related transcription factor 2
RXR	Retinoid X receptor
SAM	S-adenyl methionine
scNMT-seq	Single-cell nucleosome, methylation and transcription sequencing
SMAD	Mothers Against Decapentaplegic Homolog
SNP	Single-nucleotide polymorphism
SOC	Super optimal broth with catabolite repression media
SOST	Sclerostin
SOX9	SRY-Box Transcription Factor 9
SPRING	Southampton Pregnancy Intervention for the Next Generation
SV	Stouffer's value
SVA	Surrogate variable analysis
SVD	Singular value decomposition
SWS	Southampton Women's Survey
TAK1	TGF-β activated kinase 1
TB-BMD	Total body bone mineral density
T-cells	T-lymphocyte cells
TCF1	T cell factor 1
TET	ten-eleven translocation
TF	Transcription factor
TFBS	Transcription factor binding site
TGFβ	Transforming growth factor β
TGFβR	Transforming growth factor-beta receptor
TNF-α	Tumour necrosis factor-α
TSS	Transcription start site
TWIST1	Twist Basic Helix-Loop-Helix Transcription Factor 1
TXNIP	Thioredoxin-interacting protein
UC	Umbilical cord
UCB	Umbilical cord blood

UC-MSCs	Umbilical cord mesenchymal stromal cells
UCT	Umbilical cord tissue
US	United States of America
UVB	Ultraviolet B
VDR	Vitamin D receptor
VDRE	Vitamin D responsive elements
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
WHO	World Health Organisation
Wnt	Wingless-related integration site
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

1 Chapter 1 - Introduction

1.1 Overview

Osteoporosis is a progressive systemic skeletal disease characterised by an imbalance of the bone remodelling process, where osteoclastic bone resorption outpaces the rate of osteoblastic bone formation, leading to a net loss of bone. Factors that can reduce the risk of developing osteoporosis later in life include the ability to maintain bone mass, preventing any loss of bone mass in adulthood and achieving a sufficient peak bone mass in late adolescence. Maximising peak bone mass in early in life is therefore of great importance to reducing the incidence of conditions such as osteoporosis in later life. Twin and family studies have shown 50-85% of peak bone mass to be determined by genotype. A meta-analysis of 30 genome-wide association studies (GWAS) also found 80 loci associated with variation in total body bone mineral density (TB-BMD), however when combining all age groups and risk of fracture these loci only explained around 10% of variation, suggesting a role for the influence of environment in the development of bone (1). Epigenetic processes have been suggested to mediate the influence of the early life environment on later health. Epigenetic marks present at birth have recently been shown to be predictive of measures of both bone mineral content (BMC) and bone mineral density (BMD) into later childhood (2,3), indicating an association between skeletal development and epigenetic processes and perhaps a potential utility for epigenetic marks present at birth as biomarkers. One dietary component which is understood to be required for skeletal development is vitamin D. Whether vitamin D may mediate its effects on bone development through epigenetic processes is unknown, but in understanding the epigenetic changes in early life associated with later bone outcomes we could potentially identify those with a high risk of developing a lower-than-expected peak bone mass and so a higher risk of osteoporosis. Identifying those at risk could therefore potentially allow for the use of early intervention strategies to facilitate skeletal development and improve outcomes later in life.

This introduction will initially provide an overview of the effect of osteoporosis on the architecture of bone and the role of the fundamental bone-forming precursor cell, the mesenchymal stem cell. The factors that influence the development of bone in early life and developmental plasticity, the concept of developmental origins of health and disease (DOHaD) and the epigenetic processes which may mediate the effect of the early life environment on bone development will be introduced. Subsequently the epigenetic processes that can influence osteogenesis of mesenchymal stem cells and the role they play in the early development of bone will then be explored. Finally, the current understanding of the role that vitamin D plays in development of bone and during pregnancy will be established.

1.2 Osteoporosis

Clinically, osteoporosis has been defined by the World Health Organisation (WHO) as a bone mineral density greater than 2.5 standard deviations below the young adult sex-matched mean (5). This reduction in BMD and accompanying micro architectural deterioration of the bone tissue occurs as a result of an imbalance in the bone remodelling process, ultimately leading to an increase in bone fragility and an increased susceptibility to fracture (6). In the United Kingdom there are approximately 536,000 new fragility fractures each year (7). Osteoporosis has been suggested to increase the risk of fragility fractures such that in the UK, 1 in 2 women and 1 in 5 men over 50 will suffer a fracture as the result of the reduction in BMD and increase in bone fragility associated with an osteoporotic bone phenotype. The over-50 population is set to increase from 21.6 million in 2010 to 26.2 million by 2025, therefore the economic burden of this disease is only set to increase. The annual cost of osteoporosis to the National Health Service (NHS) in 2010 was estimated at over £4.4 billion and suggested to increase 24% to £5.5 billion by 2025 (8). Therefore measures for both the prevention and treatment of osteoporosis are going to be increasingly important in managing the incidence of this disease in the future.

The osteoporotic loss of bone means that the ability to maintain bone mass, prevent any loss of bone mass in adulthood and to reach a sufficient peak bone mass, typically achieved between 25 and 30 years of age, are all essential to reducing the risk of fracture later in life (9). Therefore the contribution of any factors that could influence the development of bone may be potential points of intervention for the prevention of this disease in the future.

1.3 Skeletal development

1.3.1 Skeletogenesis

Skeletal formation (skeletogenesis) *in utero* begins with skeletal patterning. Mesenchymal stem cells (MSC) from the neural crest, paraxial and lateral mesoderm migrate and aggregate into mesenchymal condensations taking the shape of skeletal elements at the sites where they will eventually form (Figure 2b) (10). The second phase of skeletogenesis proceeds by either intramembranous ossification or the process of endochondral ossification, two processes which share both distinct similarities and differences. The most significant difference between these processes is that during intramembranous ossification MSCs differentiate directly to osteoblasts via a preosteoblast intermediate to form bone, whereas during endochondral differentiation MSCs initially differentiate into chondrocytes to form a cartilage model (anlagen), which is subsequently ossified to form bone (Figure 1) (11). Ultimately this difference in developmental pathways leads to distinct differences in the signalling pathways required to initiate the processes.

1.3.1.1 *Intramembranous ossification*

Intramembranous ossification is responsible for the development of the scapula, the lateral aspect of the clavicles and sections of the skull including the calvarium of the neurocranium. The calvarium is composed of the frontal and parietal bones, the interparietal section of the occipital bone and the squamous sections of the temporal bone which together, with the chondrocranium, encase the brain and support the sensory organs such as the ear (12). Similarly, of the viscerocranium which forms the structure of the face, the maxilla, temporal, palatine, lacrimal, nasal, zygomatic, zygomatic arch and vomer bones along with the mandible, excluding the mandibular condyle, develop through intramembranous ossification (13–15). In addition, intramembranous ossification-like activity can also be observed in the perichondral and periosteal regions during the development of long bones, where osteoblasts differentiate directly from mesenchymal progenitors, however this is subject to signalling elicited from endochondral differentiation processes (16). Intramembranous ossification begins in the mesenchyme, an embryonic connective tissue containing mesenchymal stem cells. Within this tissue, MSCs arrange together into condensations known as bone blastema. The cells within these blastema differentiate into osteoblasts which begin to secrete osteoid, an uncalcified matrix, establishing a primary ossification centre. The osteoid becomes mineralised forming woven bone. Woven bone is characterised by a disordered network of small, randomly arranged type I collagen fibres deposited rapidly during development resulting in a disorganised latticed structure. This is subsequently later replaced by lamellar bone during the bone-remodelling process in postnatal life, a process suggested to begin from around two to three years of age. In contrast to woven bone, lamellar bone is characterised by collagen fibres more slowly deposited in perpendicular planes in adjacent lamellae forming a mechanically stronger, mature matrix (17–19). Osteoblasts secreting osteoid become entrapped in the developing matrix, mature and terminally differentiate into osteocytes. As osteoblasts differentiate into osteocytes, mesenchymal progenitors at the osteoid surface differentiate into osteoblasts and secrete osteoid which further becomes calcified, forming spicules of bone matrix which radiate out from where ossification first began. The expansion of secreted osteoid around capillaries leads to the formation of a trabecular matrix of spongy, woven bone. These vessels incorporated between the bone trabeculae will eventually form haematopoietic bone marrow. Osteoblasts on the superficial layer of the trabecular bone form the periosteum, where the differentiation of mesenchymal progenitor cells into osteoblasts contributes to the generation of a protective layer of compact bone (20).

Intramembranous ossification continues into postnatal life where at birth, neither the skull or clavicles are fully ossified and neither cranial sutures nor fontanelles closed. Cranial sutures are joins between craniofacial bones, whereas fontanelles are the unossified regions in the skull where sutures intersect, covered by tough membranes to protect underlying soft tissues and the brain. These sites remaining as unossified areas of intramembranous bone growth which serve

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the important purpose of allowing the skull to deform when passed through the birth canal and for the craniofacial bones to expand evenly as the brain grows. Sutures, the joints between the craniofacial bones are composed of two osteogenic fronts with suture mesenchyme between them. Maintaining the functional purpose of these sutures whilst permitting bone growth means there is ultimately a fine balance between bone growth, originating from the proliferation and differentiation of the osteogenic layer of cells lining the bone fronts and apoptosis, seen within sutures and suggested to prevent the number of cells within the suture matrix from becoming elevated (21). The final bones to ossify via intramembranous ossification are the flat bones of the face, occurring in late adolescence, however cranial sutures normally fuse between 20 and 30 years of age and facial sutures after 50 years of age (22–24).

1.3.1.2 *Endochondral ossification*

Endochondral ossification is the mechanism by which the axial skeleton, chondrocranium, mandibular condyle and the bones of the extremities develop (13). Endochondral ossification begins between 6 and 7 weeks of gestation where MSC condensations differentiate into chondrocytes, except at the periphery where MSCs form perichondral cells (25,26). The chondrocytes proliferate rapidly, producing a cartilaginous extra-cellular matrix (ECM) composed of collagen II and aggrecan, forming a cartilage model or primordium of the bone. Rapidly dividing chondrocytes increase the size of the cartilage model longitudinally through interstitial growth and the width of the cartilage primordium increases by appositional growth through the secretion of ECM by chondroblasts in the perichondrium (16). Eventually, chondrocytes at the centre of the cartilage primordium undergo maturation to begin the formation of what will become the growth plates. Chondrocytes form morphologically distinct zones of resting, proliferating, prehypertrophic and hypertrophic chondrocytes. Proliferating chondrocytes arrange into organised, columnar stacks which produce roughly twice their volume in matrix, rich in collagen II. These cells subsequently exit the cell cycle and enter the pre-hypertrophic zone, where chondrocytes express indian hedgehog (Ihh). Ihh acts to promote chondrocyte hypertrophy and stimulates the proliferation of chondrocytes in the proliferative zone, however it also acts on the perichondrium to stimulate synthesis of parathyroid hormone-related protein (PTHrP) which suppresses the transition of proliferative chondrocytes to pre-hypertrophic Ihh expressing cells and so establishes a negative feed-back loop which limits the size and activity of the proliferative zone of chondrocytes. Crucially, Ihh expression also stimulates the osteoblastic differentiation of perichondral progenitor cells which leads to the formation of a periosteal bone collar at the diaphysis. Hypertrophic chondrocytes produce type X collagen which is distinguished by the presence of fibres which confer a stiffness to the matrix which are absent in the collagen II matrix produced by proliferating chondrocytes. The production of collagen X and vascular endothelial growth factor (VEGF) by hypertrophic chondrocytes facilitates the mineralisation of the surrounding ECM and initiates vascular invasion of the model at the diaphysis respectively (Figure

2c) (11,27–29). Vascular invasion at the bone collar brings osteoclasts, blood vessel endothelial cells, bone marrow cells and osteoblast precursors from the perichondrium (30) establishing the primary ossification centre, corresponding to 7-12 weeks of gestation (31). The growth of the primary ossification centre establishes two opposing growth plates at the epiphysis of the bone, responsible for longitudinal bone growth. Chondrocytes in the growth plates continue to proliferate and as such progress toward the articular ends of bone (32). Progression of the growth plates leaves a condensed matrix in which chondrocytes either apoptose or transdifferentiate into osteoblasts (33). The matrix subsequently calcifies and osteoblasts derived from the perichondrium or transdifferentiated chondrocytes are recruited to the calcified tissue surface to form woven, trabecular bone or the 'primary spongiosa' (Figure 2d) (10). Similar to intramembranous ossification, later remodelling will replace woven bone with a mature, lamellar bone matrix, or the 'secondary spongiosa'. Subsequently, secondary ossification centres also form at the epiphyses, typically occurring in the late third trimester or shortly after delivery. The perichondrium subsequently disintegrates from articular ends of the bone, leaving the articular cartilage to differentiate (31,34–36). This leaves a cartilaginous epiphyseal growth plate in between the primary and secondary ossification centres at the epiphyses of the bone (Figure 2e) (37). The cavity between these growth plates is filled by bone marrow.

Postnatally, longitudinal bone growth continues to be driven by activity in the growth plate. As with *in utero* growth, rapidly proliferating chondrocytes on the epiphyseal side of the growth plate develop hypertrophy and secrete an ECM which on the diaphyseal side of the plate matures, calcifies and is replaced by bone. Skeletal maturity is marked by a thinning and eventual complete replacement of the growth plate cartilage with bone, suggested to occur in the distal tibia and fibula by age 16 for females and 19 years for males, however ossification continues up until around 25 years of age (Figure 2f) (26,38). During this time longitudinal growth is also accompanied by bone modelling processes which predominantly occur in childhood and adolescence, leading to the acquisition of peak bone mass (39). Modelling serves to maintain bone shape at the metaphyseal aspect of growing bones and facilitate radial bone growth. This increase in bone diameter occurs through appositional growth where formation modelling on the periosteal surface of bone by osteoblasts and resorptive activity by osteoclasts on the endocortical surface of bone leads to an increase in bone diameter, whilst maintaining a consistent cortical thickness (20).

1.3.1.3 Regulation of intramembranous and endochondral ossification

1.3.1.3.1 Intramembranous ossification

Within mesenchymal condensations undergoing intramembranous ossification runt-related transcription factor 2 (RUNX2), the master regulator of osteogenesis is essential to driving MSC differentiation along an osteogenic pathway (40,41). RUNX2 also induces expression of osterix

(*Osx*), a factor which together with RUNX2 constitute the transcription factors required for the osteogenic commitment of MSCs. Consequently, RUNX2-null mouse models have shown the arrest of osteoblastic differentiation during both intramembranous and endochondral ossification and in *Osx*-null mice, the condensed mesenchyme of membranous skeletal elements is similarly unable to differentiate along an osteogenic pathway (42). During intramembranous ossification RUNX2 expression is driven by enhanced wingless-related integration site (Wnt)/ β -catenin signalling in the mesenchymal condensations which acts to both promote the osteoblastic differentiation of MSCs but also inhibit the expression of SRY-Box Transcription Factor 9 (SOX9), the master determinant of chondrocytic cell fate required for endochondral ossification (29,43–47). The expression of RUNX2 and *Osx* is subsequently followed by the activity and interplay of a number of transcription factors, signalling pathways and systemic factors depicted in Figure 4 and discussed in section 1.3.4.1 which allow for the differentiation of the MSCs along an osteogenic pathway. However, of particular significance to intramembranous ossification is fibroblast growth factor (FGF) signalling. Mutations in the FGF receptors have been seen to lead to syndromes related to craniosynostosis, where sutures can fuse prematurely. Similarly, mutations in twist basic helix-loop-helix transcription factor 1 (TWIST1) has been associated with developing craniosynostosis related Saethre-Chotzen syndrome and mutations in the msh Homeobox 1 (MSX1), MSX2 and distal-less Homeobox (DLX) genes with craniofacial disorders (48,49) indicating their importance in successful intramembranous ossification.

1.3.1.3.2 *Endochondral ossification*

As with intramembranous ossification, the process of endochondral ossification involves the interplay of an array of systemic and local factors, transcription factors and extracellular matrix proteins and as such a subset of the core processes required for endochondral bone development will be highlighted (25,50,51). In contrast to intramembranous ossification, endochondral ossification commences with the chondrogenic differentiation of MSCs through the expression of SOX9 in the precondensing mesenchyme, a factor inhibitory to RUNX2 expression (52,53). SOX9 expression, essential to initiate chondrocytic commitment in the precondensing mesenchyme gives rise to the cartilage primordia consisting of small, rounded chondrocytes which alongside SOX9 produce SOX5 and SOX6 which facilitate the production of the structural proteins collagen type II α 1 (COL2A1) and aggrecan (54). Subsequent maturation into prehypertrophic chondrocytes is marked by the expression of both parathyroid hormone 1 receptor (PTH1R) and *Ihh*. Maturation of chondrocytes into early hypertrophy leads to the expression of collagen type X α 1 (COL10A1), the expression of which, along with PTH1R and *Ihh* leads to a decrease in SOX5, SOX6, SOX9, COL2A1 and aggrecan expression. These cells then lose expression of COL10A1 and progress into later hypertrophy, characterised by expression of vascular endothelial growth factor A (VEGFA). Hypertrophic chondrocytes also produce matrix metalloproteinase 13 (MMP13) which has been suggested to break up the matrix produced by hypertrophic chondrocytes. Hypertrophic

chondrocytes have also been seen to produce osteopontin, which may act to regulate mineralisation, act as a chemoattractant for osteoclasts and as a factor necessary for osteoclasts to function (53,55–61).

Whilst RUNX2 is not required for the initiation of endochondral ossification, the loss of SOX9 expression by chondrocytes in early hypertrophy has been suggested to relieve the inhibitory effect of the factor on RUNX2. RUNX2 is then subsequently upregulated until terminal hypertrophy, at which point it has been suggested to permit chondrocyte-osteoblast transdifferentiation (Figure 1) (62). The role of RUNX2 in the latter stages of endochondral ossification is underlined by models showing RUNX2^{-/-} mice display a lack of chondrocyte maturation in a large proportion of the skeleton excluding the tibia, fibula, radius, and ulna. However these mice also demonstrate a complete lack of vascular invasion into the cartilage through the entire skeleton, potentially due to a lack of VEGF expression from hypertrophic chondrocytes (63–66). Similar to lineage specification during intramembranous ossification, RUNX2 expression has been suggested to be driven by Wnt/ β -catenin signalling and to drive expression of *Osx*, without which endochondral ossification is severely impaired (42,62,67,68). This shows RUNX2 may largely play a role in the latter stages of endochondral ossification, however depending on the skeletal element, RUNX2 alone, or together with either RUNX1 and RUNX3 have also been identified as potentially crucial for earlier stages of chondrogenesis such as for the expression of SOX5/SOX6 or induction of *Ihh* signalling (69,70). When compared to intramembranous ossification this suggests that regardless of developmental route, the expression of RUNX2 during prenatal development is essential for osteogenesis (Figure 4) (62).

Interestingly, a study by *Komori et al.* (2010) presented a mouse model which suggested that whilst RUNX2 triggers the expression of major bone matrix genes during earlier stages of osteoblastic differentiation and indeed induces immature bone formation, it ultimately inhibits osteoblast maturation and is required to be suppressed for mature bone formation (71). However, further research by *Qin et al.* (2020) has identified that whilst this may indeed be accurate for postnatal development, RUNX2-driven transdifferentiation in the later stages of endochondral ossification is required for trabecular bone formation during embryonic and neonatal stages of development (62).

1.3.1.4 Skeletal mineralisation

1.3.1.4.1 The process of skeletal mineralisation

Bone mineralisation occurs through the deposition of crystals of hydroxyapatite, a calcium phosphate mineral within a collagen ECM (89). Bone mineralisation is therefore crucially determined by serum concentrations of both calcium and phosphorous (72–74), albeit potentially more significantly by phosphorous alone (75–77). In the bone microenvironment matrix mineralisation is regulated by a balance between concentrations of both phosphate (Pi) and

inorganic pyrophosphate (PPi). Whilst higher levels of Pi will induce bone mineralisation, an excess of PPi is inhibitory, acting to inhibit the integration of Pi and calcium (Ca^{2+}) into nascent apatite crystals and inhibit their growth (78). Pi concentrations are determined by both dietary intake and the rate of alkaline phosphatase (ALP) mediated hydrolysis of PPi, an ectoenzyme attached to the surface of the osteoblast cell membrane. The activity of ALP therefore shifts the PPi:Pi ratio in favour of Pi, creating a bone microenvironment which favours matrix mineralisation. Whilst levels of Pi can be elevated by the activity of ALP, levels of PPi can also be elevated through synthesis by ectonucleotide pyrophosphatase/phosphodiesterase enzymes from adenosine triphosphate and from the intra- to extracellular transport of PPi from osteoblasts by progressive ankylosis protein. The mechanism by which matrix mineralisation is initiated however has not reached consensus. It has been suggested the structure of the collagen I ECM provides intra and interfibrillar gaps accessible by Ca^{2+} and Pi ions in which mineral nucleation and hydroxyapatite crystal formation can occur, inaccessible to inhibitors of the process (79–81). Alternatively, others have proposed that matrix vesicles released by osteoblasts control localised accumulation of Ca^{2+} and Pi to form hydroxyapatite crystals which are subsequently deposited in the collagen I matrix (82,83). Others have also proposed a hybrid model of the two, where matrix vesicle mediated mineralisation acts simply as an auxiliary method of mineralisation (84). Nonetheless, the process of matrix mineralisation occurs broadly in two stages. After collagen is deposited, within ~5-10 days osteoid undergoes primary mineralisation, where approximately 50-70% of mineral is accrued and then secondary mineralisation, where remaining bone mineral accrues at a slower rate over the course of weeks to years. During secondary mineralisation hydroxyapatite crystals enlarge, carbonate is substituted for phosphate groups in the matrix and collagen becomes more cross-linked and compact (85).

1.3.1.4.2 The regulation of skeletal mineralisation

The steps of mineralisation including initiation, mineral accrual, maturation and maintenance are regulated by the expression and secretion of a number of non-collagenous proteins from both osteoblasts and osteocytes in the bone microenvironment.

1.3.1.4.2.1 Osteoblast-secreted factors

Osteoblasts, differentiated from mesenchymal stem cells play the fundamental role of depositing a type I collagen-rich matrix and then aiding in the mineralisation of this newly-formed bone osteoid (86). Some of the primary factors expressed by osteoblasts which facilitate earlier stages of mineralisation include ALP, the function of which was discussed previously, osteonectin and bone sialoprotein. Osteonectin is located at sites of mineralisation and suggested to bind hydroxyapatite, vitronectin and collagen to promote the formation new crystals. It has also been identified to have a potential role in osteoblastic proliferation, established through knockout in a mouse model which lead to low bone mass or osteopenia (87). Bone sialoprotein is similarly

expressed in early mineralisation and suggested to be involved in the initiation of mineralisation to provide a nucleation point for hydroxyapatite crystals (88). Later in maturation osteoblasts express osteocalcin (OCN), proposed to enhance calcium binding and control mineral deposition. However it has also been suggested to have a potential role in regulating osteoclast activity, as mice with OCN knockout have been shown to have severe osteopetrosis and OCN has been shown to be elevated in postmenopausal osteoporosis (87). A further marker expressed by mature osteoblasts is osteopontin. In contrast to the other factors, osteopontin inhibits mineral formation and crystal growth and as such has a diverse range of potential functions in the mineralisation process, including acting a scaffold between tissues of different compositions, fibre matrix bonding, bridging microcracks in the bone matrix and promoting the adherence of osteoclasts to bone mineral during the bone resorption process (89).

1.3.1.4.2.2 Osteocyte-secreted factors

Osteocytes are also pivotal in regulating the mineralisation process. Osteocytes are the most abundant cell type in mineralised bone (90-95%), differentiated from the 5-20% of mature osteoblasts which become entombed in the osteoid matrix during skeletal development. Osteocytes represent one of the three potential terminal fates for cells of the osteoblastic lineage (Figure 4). Whilst the signalling processes required for osteoblastogenesis are relatively well understood, the processes required for osteocytogenesis are not yet entirely clear (90). Although, *Imai et al.* (1998) demonstrated osteocytes can express osteoblast stimulating factor-1 (OSF-1) to stimulate the recruitment of osteoblasts which differentiate into osteocytes through production and entrapment within an osteoid matrix (91,92). Post-mineralisation of the osteoid, osteocyte cell bodies are embedded within small cavities in the bone matrix termed lacunae. Dendritic projections from these cell bodies extend through canals in the matrix called canaliculi and interconnect at the tip of each process to allow for direct contact with other osteocytes in the bone matrix. Connections between dendritic projections are made via gap junctions to form both an intracellular and extracellular lacuno-canalicular network (93). Osteocytes are master regulators of bone modelling which detect and respond to mechanical and hormonal stimuli to coordinate bone resorption and bone formation through the orchestration of osteoblast and osteoclast activity, a process which is discussed in more detail in section 1.3.2.1.3.1. This response to stimuli is affected through the production and secretion of factors which can influence the activity of other bone cells by paracrine or autocrine mechanisms and the production hormones that affect other tissues by endocrine mechanisms. The function of osteocytes can be explored through their capacity as highly secretory cells. Broadly, osteocytes produce factors related to i) osteocytogenesis, ii) phosphate metabolism and matrix mineralization, iii) bone formation and iv) bone resorption. Here, we will explore the role of osteocytes in phosphate metabolism and matrix mineralisation.

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As mentioned previously, levels of ionic calcium and inorganic phosphate are critical determinants for bone mineralisation and indeed, disruption to circulating concentrations of these factors has been shown to lead to impairment in bone mineralisation (72–74). Osteocytes facilitate both calcium and phosphate homeostasis through orchestration of the bone-remodelling mediated resorption process which liberates mineral from the skeleton (section 1.3.2.1.3.1) and potentially more directly through perilacunar remodelling (94,95). However osteocytes also have a more direct role in regulating phosphate homeostasis and so the process of matrix mineralisation in an endocrine manner.

Fibroblast growth factor 23 (FGF23) is a hormone expressed and secreted by osteocytes which acts to inhibit mineralisation through the modulation of phosphate homeostasis, the physiological mechanism of which is further detailed in section 1.3.4.1.3.4. FGF23 is a member of the parathyroid hormone (PTH)-vitamin D-FGF23 axis which is essential in regulating calcium and phosphate homeostasis. However whilst PTH and vitamin D act to elevate systemic concentrations of both calcium and phosphorous, FGF23 acts as a counter-regulatory phosphaturic hormone to remove excesses of phosphate (96). These factors exist in a negative feedback loop, where both $1,25\text{D}(\text{OH})_2\text{D}$ and PTH are able to induce the release of FGF23 from osteocytes and FGF23 has the capacity to inhibit the synthesis or release of $1\alpha,25$ -dihydroxyvitamin D3 ($1,25\text{D}(\text{OH})_2\text{D}$) and PTH. Once secreted, FGF23 acts on the parathyroid glands to reduce PTH expression and secretion, whereas in the kidneys it suppresses phosphate resorption, increases breakdown and reduces formation of vitamin D, which results in a concomitant decrease in phosphate uptake in the small intestine (97–100). As a result of this interrelationship, a number of heritable disorders characterised by pathologically high blood concentrations of FGF23 also exhibit abnormal renal phosphate wasting and bone mineralisation leading to osteomalacia and rickets, a finding mirrored in animal models (101–103). Conversely, mice deficient in FGF23 exhibit excessive plasma concentrations of $1,25\text{D}(\text{OH})_2\text{D}$, calcium and phosphate, leading to severe tissue calcification, deranged bone mineralisation and a range of other non-skeletal conditions (104–106).

Alongside FGF23, osteocytes express and secrete a number of other proteins which contribute to regulating phosphate homeostasis and matrix mineralisation including dentin matrix acidic phosphoprotein 1 (DMP1), phosphate-regulating neutral endopeptidase (PHEX) and matrix extracellular phosphoglycoprotein (MEPE). Similar to FGF23, MEPE is an inhibitor of mineralisation, the deletion of which has been seen to increase BMD in a mouse model (107). In contrast, deletion or loss of function of PHEX has been shown to lead to X-linked hypophosphatemic rickets, an effect similarly seen with deletion of DMP1 (103,108). However these factors do not act in isolation, for example the roles of PHEX and DMP1 in promoting mineralisation have been suggested to be secondary to their role in inhibiting FGF23 and so

indirectly promoting levels of circulating phosphate. As such, humans without functional DMP1 or PHEX exhibit high levels of FGF23 and hypophosphatemia (101,109,110).

Interestingly, the expression of both DMP1, PHEX and MEPE have also been seen to increase in response to loading and the expression of sclerostin (SOST), a negative regulator of Wnt/ β -catenin signalling and osteoblastogenesis has also been shown to decrease (111–113). Whilst the expression of DMP1, PHEX, MEPE and SOST has been suggested to be regulated by mechanical forces, the influence of mechanical force on the regulation of FGF23 expression is not yet clear. Therefore, whilst much attention is typically drawn to the effects of mechano-sensing by osteocytes on bone remodelling, this demonstrates proteins known to regulate mineralisation and mineral homeostasis can also be affected.

1.3.2 The postnatal skeleton

1.3.2.1 Bone modelling and remodelling

1.3.2.1.1 Bone modelling

Over the course of life, the skeleton goes through the process of both bone modelling and remodelling. Modelling is distinguished by from bone remodelling by the uncoupled activity of osteoblasts and osteoclasts on separate bone surfaces, acting to increase bone mass and maintain or alter bone shape. However, despite being locally independent the activities of these cells are not globally independent, as both processes, whilst occurring simultaneously must be coordinated to shape bone. Modelling primarily occurs in childhood and adolescence, leading to the acquisition of peak bone mass (39). After this point, in the absence of a pathology bone modelling largely occurs in response to fractures and the process of bone remodelling predominates (114). Modelling primarily occurs in response to either physiological influences or mechanical strain encountered by the skeleton where if local strain exceeds a certain threshold, formation modelling is initiated to add new bone, whereas if strain is low then resorptive modelling is stimulated and bone is removed until strain in the area is normalised. Bone modelling processes maintain metaphyseal morphology during longitudinal growth and drive increases in bone diameter by appositional growth as described in section 1.3.1.3.2, again driven by mechanical loading, but also factors such as PTH and sclerostin (20,115).

1.3.2.1.2 Bone remodelling

In contrast to bone modelling, bone remodelling is characterised by the tightly coupled, sequential activity of osteoblasts and osteoclasts for the replacement of older, damaged bone with newly formed bone at the same spatial location and as a mechanism for rapid access to calcium and phosphate to maintain mineral homeostasis (116,117). Whilst bone remodelling predominates in adulthood after achievement of peak bone mass, it is a process which occurs throughout life for the maintenance of bone mass rather than an increase in bone mass as with

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bone modelling (20). Anatomically, bone remodelling occurs in structures known as basic multicellular units (BMUs) which contain the requisite osteoblasts, osteoclasts and capillary blood supply required for the process to take place (118). However, the BMU lasts longer than the lifespan of these cells and so requires replenishment, a process controlled by osteocytes.

1.3.2.1.2.1 Initiation of bone remodelling

Bone remodelling can be regarded as either targeted or stochastic, where targeted remodelling occurs in response to a specific, local signalling event which leads to the recruitment and activation of osteoclast precursors at a particular location of damaged or old bone to begin the remodelling process. Targeted remodelling can be initiated by direct mechanical strain, leading to disruption of osteocyte canaliculi as a result of bone matrix microdamage or osteocyte apoptosis, although these processes are often linked. This has been suggested to lead to the release of paracrine factors, increasing local angiogenesis and the recruitment of osteoclast and osteoblast precursors but also to instigate signalling between neighbouring osteocytes and other osteoblast lineage cells to change the expression of pro and anti-osteoclastogenic genes (117,119–125). Targeted remodelling has also been suggested to be stimulated by high mineralisation or alterations in collagen cross linking (123,124). Alternatively, stochastic or non-targeted remodelling can occur without a location-specific signalling event, such as a systemic change in the concentration of hormones such as PTH for the purposes of calcium homeostasis or oestrogen. These triggers initiate what is known to be the activation phase of the bone remodelling process (section 1.3.2.1.2.2).

Our understanding of the role of osteocytes in particular as cells with the ability to regulate bone homeostasis in response to mechanical load has evolved over time. In 1892, Julius Wolff suggested bone growth and remodelling occur in response to forces placed on the bone in response to mechanical stimulation in a healthy person, also known as Wolff's law (126,127). Since then multiple *in vivo* studies have shown strong support for significant bone formation on the application of force to bone, and significant loss with unloading (112,128,129). Osteocytes were suggested to be the cells able to sense and orchestrate a response to mechanical loading or unloading, and indeed this was later supported in an experiment by Tatsumi *et al.* (2007) where mice with osteocyte ablation were resistant to unloading-induced bone loss (125,130). As such osteocytes have become understood to serve a critical function as principal regulators of bone mechanosensation and mechanotransduction, functions critical in enabling the skeleton to alter the architecture of bone and regulate bone homeostasis, density and strength in response to the mechanical demands placed upon it (131). Upon sensing mechanical stimulation osteocytes orchestrate the activation of both osteoblasts and osteoclasts through the production and secretion of a range of signalling proteins and cell to cell communication (132). Typically in

response to mechanical loading the activity of osteoblasts is stimulated, whereas the activity of osteoclasts is partially suppressed and the process reversed for mechanical unloading.

The mechanical forces imposed on the bone matrix are 'sensed' by osteocytes through the generation of biophysical stimuli in the ECM in which osteocytes are embedded. Osteocytes experience a range of biophysical stimuli including strain, stress, shear, osmotic pressure, fluid flow, streaming potentials and acceleration, however the shear stress as a result of fluid flow (FFSS) through the lacuna-canalicular spaces has been suggested as the main force responsible for the stimulation of osteocytes (133–135). The manner by which osteocytes sense the external mechanical environment, convert this to a biochemical signal and transduce these signals into biological functions is still an active area of ongoing research. However, it is currently suggested osteocytes may potentially utilise up to seven different mechanosensory pathways to respond to physical stimulation, including through the cytoskeleton, the dendrites, primary cilia, integrins within focal adhesions, connexins within gap junctions, ion channels and the glycocalyx, a pericellular matrix between the osteocyte and ECM, reviewed in Qin *et al.* (2020) (136). Connexin 43 (CX43), one of the family of connexins which form gap junctions is particularly significant in bone development, where CX43 null mice have been shown to exhibit delayed ossification, craniofacial abnormalities and osteoblast dysfunction (137). Gap junctions have also been suggested to contribute to mechanotransduction in bone and indeed FFSS has been shown to stimulate gap-junction mediated intercellular communication and increase the expression of CX43 (138). When osteocytes experience mechanical stimulation, the CX43 protein is phosphorylated, opening the connexon or channel of connexins, allowing for the transfer of effectors such as calcium, adenosine triphosphate (ATP), prostaglandin E₂ (PGE₂) and cyclic adenosine monophosphate (139). Subsequently, CX43 deficiency in a number of mouse models has been shown to desensitise bone to the effects of mechanical loading and unloading, but also potentially to the effects of hormonal stimulation from factors such as PTH and oestrogen (140–143).

Downstream of these physical 'sensors' are signalling pathways important affecting a response from these stimuli. The Wnt/ β -catenin pathway, already understood to be the major regulator for the transition of mesenchymal stem cells into mature osteoblasts activates through a coreceptor complex consisting of LDL receptor related protein 5 (LRP5) or LRP6 and the frizzled (FZD) family of receptors. LRP-5-null mice, when challenged with cyclic mechanical loading or disuse associated unloading showed reduced responses after loading and a greater loss of cancellous bone after disuse (144–147). Similarly, mechanical stimulation has been seen to induce extracellular signal-regulated kinase (ERK) activation through integrins and the cytoskeleton in osteocytes, supporting osteocyte survival (148). Once the osteocytes perceive the mechanical stimulus this can activate a cascading series of events leading to the differential regulation of mechanosensitive genes, discussed in section 1.3.2.1.3.1.

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1.3.2.1.2.2 Remodelling cycle

The bone remodelling cycle occurs in five overlapping steps of activation, resorption, reversal, formation and termination. As discussed previously, both mechanical and systemic factors can initiate the activation phase of bone remodelling, during which bone lining cells separate from the surface of the bone and form a raised canopy over the site to be resorbed. Mononuclear cells recruited from the circulation form multinucleated preosteoclasts which bind to the bone matrix, forming a sealing zone around the bone resorbing compartment (BRC) or BMU, where remodelling activities take place isolated from the rest of the surrounding bone. Differentiation and activation of osteoclasts leads to the rearrangement of the osteoclast cytoskeleton, allowing for adherence to the bone surface and the generation of a ruffled border which creates a far greater surface area to facilitate resorption (149,150).

The next stage of the bone remodelling cycle, resorption, constitutes osteoclast mediated bone resorption activity lasting approximately 2-4 weeks where osteoclasts secrete hydrogen ions which act to lower the pH within the bone resorbing compartment to a pH of around 4.5 to mobilise the bone mineral. The subsequent secretion of enzymes such as matrix metalloproteinases and cathepsin K degrades the collagen-rich bone matrix. The finalisation of the resorption phase is marked by the apoptosis of osteoclasts, ensuring that excess resorption does not occur (151–153)

Following the resorption phase, the reversal phase approximately 4-5 weeks in duration occurs where bone resorption switches to formation, broadly through employing two key processes. Firstly, the bone surface is prepared by cells of an osteoblastic lineage, where an unmineralised collagen matrix 'cement-line' is deposited to enhance osteoblastic adherence. Signalling also occurs that couples cellular resorption and formation activities to ensure there is no net loss of bone (154–156). Whilst the exact signal that couples formation to resorption is not yet fully understood, it has been suggested that osteoclasts may be the source of the coupling factor, either secreting cytokines such as interleukin 6 (IL-6), or through interactions between Ephrin family receptors on osteoclastic surfaces and their respective membrane bound ligands, Ephrins, present on the surface of osteoblasts, discussed in section 1.3.2.1.3.3 (157–160). Transforming growth factor β (TGF β) has also been proposed as a coupling factor as concentrations of TGF β in the bone matrix have been seen correlate with bone mineral turnover indices such as serum osteocalcin and bone-specific alkaline phosphatase. Liberation of TGF β also inhibits the production of receptor activator of nuclear factor kappa-B ligand (RANKL), a stimulator of osteoclastic differentiation by osteoblasts and so decreases osteoclastic resorption (145,161–163).

The penultimate step, formation, typically takes around 4-6 months to complete. Initially, osteoblasts synthesise and secrete a type I collagen-rich osteoid matrix to fill the cavities left by

osteoclasts. Osteoid is then subsequently mineralised in two distinct phases to form bone. Primary mineralisation involving the incorporation of calcium and phosphate into the collagen matrix occurs rapidly over 2-3 weeks and accounts for around 70% of the final mineral content, whereas secondary mineralisation, the final addition and maturation of hydroxyapatite crystals occurs can occur over weeks or potentially years (20). Osteoblasts continue to form bone until they transform into quiescent bone lining cells which completely cover the newly formed bone surface. After completion of bone formation, roughly 50%-70% of osteoblasts undergo apoptosis with the rest becoming encased within the bone matrix to terminally differentiate into osteocytes or bone lining cells, the latter of which maintain the capacity to redifferentiate into osteoblasts and produce matrix once more under the correct stimuli (82,164).

The final stage of bone remodelling is termination, a process roughly four months in duration. In this stage mineralisation of the deposited osteoid completes and bone remodelling enters a quiescent phase where the amount of bone formed equals the amount resorbed (165).

Osteocytes play a key role in inducing this concluding stage through the secretion of antagonists of the Wnt/ β -catenin signalling pathway such as sclerostin (166), however they also contribute more broadly to the overall regulation of the process along with further local, systemic factors and other physiological systems.

1.3.2.1.3 Regulation of the bone remodelling cycle

The maintenance of physiological bone remodelling and systemic mineral homeostasis requires a fine balance between both bone formation and bone resorption. The regulation of this process not only involves direct communication between cell types within the BMU, but the coordinated activity of a number of locally and systemically released factors (167,168).

1.3.2.1.3.1 Osteocytes in bone remodelling

Locally within the bone microenvironment, osteocytes influence the bone remodelling process through the finely balanced production and secretion of factors with roles in regulating both osteoblast and osteoclast activity. Osteoclastic differentiation and survival are modulated by the osteocyte-derived cytokines RANKL, macrophage colony-stimulating factor (M-CSF) and the anti-osteoclastogenic osteoprotegerin (OPG). Conversely, the generation, survival and function of osteoblasts is determined by osteocyte-derived antagonists of Wnt/ β -catenin signalling including SOST and dickkopf-related protein 1 (DKK1).

The RANKL–RANK–OPG system is crucial for the formation of osteoclasts and regulation of bone resorption. The system is composed of receptor activator of nuclear factor kappa-B (RANK), the receptor for RANKL expressed by osteoclast precursors and OPG, which alongside RANKL is expressed by osteoblasts, stromal cells and osteocytes. RANKL binds the RANK receptor, activating intracellular signalling, whereas OPG acts as a soluble decoy receptor to RANKL to

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neutralise its activity and prevent binding to the RANK receptor, therefore making the ratio of RANKL to OPG critical for bone remodelling. However whilst all these cell types express these factors, deletion of RANKL from osteocytes alone in a mouse model has been seen as sufficient to render mice osteopetrotic due to a reduction in osteoclast number, decreased bone resorption and a progressive increase in bone mass suggesting osteocytes are the primary source of RANKL in bone (169,170). The binding of RANK and RANKL has been suggested to both drive the differentiation of mononuclear precursors into multinucleated active osteoclasts but also promote osteoclast activation and survival through activation of the tyrosine protein kinase SRC (171).

RANKL expression has also been suggested as sensitive to mechanical regulation. Previous studies in murine long bone osteocyte-Y4 (MLO-Y4) cells has indicated FFSS reduces the capacity for osteocytes to support osteoclastogenesis and indeed in a mouse model Xiong *et al.* (2011) showed deletion of RANKL genes from osteocytes prevented an increase in RANKL production and cortical bone loss typically induced as a result of hind limb unloading (169,172). This RANK-RANKL-OPG ratio can be further modulated by the mechanosensitive expression of MEPE, a factor expressed by osteocytes which upregulates the expression of OPG, skewing the RANKL/OPG ratio in favour of OPG and so inhibiting osteoclast development (173). Another factor produced by osteocytes pivotal in the regulation of bone resorption is M-CSF. M-CSF alongside RANKL is critical for osteoclastogenesis, demonstrated by its absence within osteocytes in mouse models leading to both reduced osteoclast numbers and osteopetrosis. In addition, M-CSF also plays role in maintaining osteocyte viability, identified by deletion of the M-CSF receptor in osteocytes which lead abnormal osteocyte morphology, increased production of reactive oxygen species and an increase in apoptosis (174–176).

Conversely, a range of factors expressed and secreted from osteocytes also have central role in controlling bone formation during the bone remodelling process through the regulation of osteoblast development and activity. The Wnt/ β -catenin signalling pathway, as discussed previously is a central factor in directing the differentiation of mesenchymal stem cells along various cell lineages. With respect to the bone remodelling cycle, Wnt/ β -catenin signalling can promote osteoblastic maturation, the survival of osteoblasts and osteocytes and inhibit osteoclastogenesis through increasing the expression of OPG in osteoblasts and osteocytes. Wnt/ β -catenin signalling functions through the binding of Wnt proteins to a receptor complex composed of FZD and LRP5 and 6 which stabilises β -catenin, permitting its translocation to the nucleus to activate gene transcription (177). Osteocyte secreted DKK1, a Wnt antagonist binds both LRP5 and 6, prevents the binding of Wnt ligands and so negatively regulate bone formation. Subsequently mice overexpressing DKK1 in osteoblastic cells exhibit decreased bone formation and low bone mass due to reduced osteoblast number (171,178). Sclerostin, produced primarily by mature osteocytes also acts to inhibit canonical Wnt signalling through binding to both LRP5

and 6 but additionally also to LRP4, a further modulator of Wnt/ β -catenin signalling. Subsequently deletion, or the introduction of neutralising antibodies against LRP4 or sclerostin in mice has been found to produce a high bone mass phenotype, an effect similarly seen in humans lacking sclerostin or LRP4 activity (177,179–184).

Mechanical loading has been identified to activate Wnt/ β -catenin signalling, potentially through the PGE₂ induced translocation of β -catenin via a phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)-mediated mechanism. Furthermore, deletion of osteocyte β -catenin has been identified to result in severe bone loss and an impaired response mechanical loading, highlighting the essential nature of osteocytes in mediating the response to mechanical loading within bone. As may be expected, the expression of both DKK1 and sclerostin have been shown to decrease with loading, but sclerostin has been suggested to mediate a larger proportion of the changes in bone formation instigated by mechanical loading (185). A study by Robling *et al.* (2008) showed increased loading of the mouse ulna led to reduced expression of sclerostin in osteocytes whereas unloading in the hind limb elevated expression (112). This was further corroborated by studies which identified both the suppression of bone formation caused by tail suspension could be prevented in sclerostin-null mice and load-induced bone formation could be blunted in mice over-expressing sclerostin (186,187).

1.3.2.1.3.2 Systemic factors in bone remodelling

Alongside local factors, bone remodelling is regulated by a milieu of systemic factors. The variety of endocrine and paracrine factors capable of influencing the bone remodelling process are reviewed in Kenkre and Bassett (2018) (188). Amongst this group two factors, PTH and vitamin D are by necessity major hormonal regulators of the remodelling process due to their role in maintaining calcium homeostasis by in part through the liberation of calcium stores from the mineralised skeleton when dietary sources are limited (189).

PTH acts to stimulate the catabolic resorption of bone, promote renal calcium retention and the production of the active form of vitamin D, 1,25(OH)₂D. Interestingly, PTH can have directly opposing effects in bone remodelling depending on the period of exposure. Continuous PTH exposure stimulates bone resorption as a key mechanism necessary to maintain calcium homeostasis. Continuous PTH exposure can induce cortical and trabecular bone loss through monocyte chemoattractant protein 1 (MCP-1), but it largely occurs through the modulation of the RANKL-RANK-OPG system in both osteoblasts and osteocytes where it increases RANKL production and inhibits OPG to stimulate osteoclastogenesis. In contrast, intermittent PTH has an anabolic effect on bone, acting to reduce expression of osteocyte-derived Wnt/ β -catenin inhibitors SOST and DKK1, but increasing the expression of Wnt ligand Wnt10b. This leads to increased osteoblastogenesis, target gene expression and bone formation (190–196).

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As mentioned previously vitamin D acts in an endocrine function to facilitate the regulation of calcium and phosphate uptake in the small intestine and as such is a factor integral to bone mineralisation. Similar to PTH, $1,25(\text{OH})_2\text{D}$ has been suggested to potently induce the expression of RANKL in osteoblasts and osteocytes and also act to downregulate expression of osteoprotegerin in osteoblasts (197–200). In addition, cytochrome P450 family 27 subfamily B member 1 (CYP27B1), which converts vitamin D to its active form $1,25(\text{OH})_2\text{D}$ has been shown to be expressed in osteoblast, osteoclast precursors and osteocyte cell types, suggesting possible local regulation of vitamin D synthesis and a potential autocrine role for vitamin D in skeletal cells (201,202). In support of this, the metabolism of vitamin D by CYP27B1 in osteoclast lineage cells has been shown to promote osteoclastic differentiation, but interestingly ameliorate their resorptive capacity and promote the coupling of bone resorption to formation (203,204). Furthermore, $1,25(\text{OH})_2\text{D}$ treatment has been seen to enhance the mineralisation activity of osteoblasts and the expression of osteocytic markers DMP1 and FGF23 within induced pluripotent stem cell derived osteoprogenitors, suggesting vitamin D may promote osteoblast to osteocyte differentiation (205).

1.3.2.1.3.3 Osteoblast-osteoclast interaction in bone remodelling

Beyond both the expression of osteocyte-secreted and systemic factors in the regulation of bone remodelling, direct osteoblast-osteoclast interaction is also integral in this highly regulated process. An example of this interaction is ephrin-ephrin receptor signalling. There are two families of ephrins, ephrin-A proteins (A1-A6) and ephrin type-A (EphA1–A10) receptors along with ephrin-B (B1–B3) and ephrin type-B receptors (EphB1–EphB6). As a bidirectional signalling pathway, signalling can occur ‘forward’ through the receptor into the receptor-presenting cell and in ‘reverse’ through the ligand presenting cell. The ephrin-B-EphB family play a potentially crucial role in maintaining bone homeostasis by coupling the resorption of bone to bone formation. The binding of the ephrin-B2 protein ligand, expressed on the cell surface of osteoclast lineage cells to the EphB4 receptor on osteoblast lineage cells triggers the reverse signalling of ephrin-B2 to inhibit the expression of the main osteoclastogenic factors nuclear factor of activated T cells 1 (NFATC1) and c-Fos and so inhibiting osteoclast differentiation. Simultaneously, forward signalling through ephB4 receptor can stimulate the differentiation of osteoblasts through the inhibition of the small GTPase ras homolog family member A (RhoA) and the upregulation of transcription factors crucial for osteoblast differentiation, such as RUNX2, DLX and Osx (160,171,206). However notably, direct contact between osteoblasts and osteoclasts within the BMU is not always possible and bone formation can be sustained long after osteoclasts vacate the resorption site, highlighting the presence of both contact dependent and contact-independent signals in bone remodelling.

1.3.2.1.3.4 The role of osteoimmunology in bone remodelling

The skeletal and immune systems share a close and reciprocal relationship where bone cells can influence immune biology and vice versa. The interaction between the two systems was first identified by Horton *et al.* (1972) in the context of periodontitis, where bacterial antigen-stimulated immune cells were seen to produce osteoclast-activating factors (207). The term 'osteimmunology' was later coined in the year 2000 and the field has developed rapidly, finding factors typically considered as primarily immune-related such as the IL-6, IL-11, IL-17, and IL-23, tumour necrosis factor- α (TNF- α), RANK and its ligand RANKL, NFATC1 as significant in bone cell biology (208–210). The immune system contains a diverse range of cell types descended from haematopoietic stem cell (HSC) niches within the bone marrow. A significant proportion of immune cells influence skeletal biology (211), for example, T-lymphocyte cells (T-cells), a key component of adaptive immunity have been found to be potent inducers of osteoclastogenesis. T helper 17 cells in particular, through the expression of select cytokines can induce M-CSF and RANKL in osteoclasts and stromal cells, produce both RANKL and TNF- α and in parallel increase RANK expression in osteoclast precursors leading to the enhancement of osteoclastogenesis (212–214). B-lymphocyte cells (B-cells), similarly part of the adaptive immune system have also been shown to share a common precursor with osteoclasts and regulate their activity through the production of RANKL (215). Additionally, immunoreceptor tyrosine-based activation motif signals, critical in the activation of signalling in immune cells and RANKL signalling have both been identified to cooperate to facilitate osteoclastogenesis (216,217).

The interactions of bone with the immune system, along with the aforementioned influences of osteocytes, systemic factors and direct cell-cell interactions highlights the inherent complexity of the bone remodelling process and the potential for dysregulation to occur through a number of potential processes.

1.3.2.1.4 *Dysregulation of the bone remodelling cycle*

Postnatally, through childhood and adolescence the skeleton continues to go through a process of bone growth and mineralisation. Around 95% of adult bone mass will be accumulated by the ages of 16 in girls and 18 in boys, with peak bone mass typically attained around the age of 30. Peak bone mass is the point at which the age-related changes in bone outcome are no longer positive and the bone tissue accrued during adolescence has plateaued (9), marking the end of skeletal maturation. After peak bone mass is achieved the growth of the skeleton largely ceases and begins to go through a process of remodelling based on a balance between osteoblastic bone formation and the osteoclastic resorption of bone. This allows for the repair, renewal and removal of old bone to maintain strength, allow for adaptation to physical stresses and to maintain calcium homeostasis (218). However, with age, the bone remodelling process begins to progressively favour resorption. This imbalance can eventually lead to a structural deterioration of cortical bone, characterised by cortical thinning and increased intracortical porosity which has

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been suggested to be an indicator and contributor to increased fracture risk (219–223). This imbalance in the bone remodelling process has been suggested to be primarily due to the reduction in osteoblast number seen with aging which leads to an overall reduction in bone formation relative to bone resorption (224–226). However additionally these affects have been attributed, in part, to an increase in osteocyte apoptosis with age, leading to a decrease in the number of osteocytes which has been seen to correlate with microdamage and microcrack accumulation in cortical bone (227,228). This reduction in osteocyte numbers in the bone matrix have been suggested to be due to a decline in physical activity with older age, an accumulation of reactive oxygen species in the tissue, increased levels of endogenous glucocorticoids with age and can also be influenced by a loss of sex steroids, most profoundly from a loss of oestrogen which acts to inhibit osteoblast and osteocyte apoptosis to maintain bone formation and limit bone remodelling (229–232). However in addition to changes in osteocyte viability with age, the expression profile of these cells has been seen to change with age, leading to potential alterations in both bone formation and resorption (233,234). As discussed previously, osteocytes facilitate bone resorption in part through the expression of RANKL, the expression of which is seen to increase with age, accompanied by an increase in endocortical bone resorption. Indeed apoptotic osteocytes have been seen to release factors which stimulate RANKL production in neighbouring osteocytes. In female mice with apoptosis-resistant osteocytes unable to complete the apoptotic death program osteocytes showed increase productions of alarmins, RANKL, and other factors that ultimately increase intracortical remodelling and loss of cortical mass (235). In contrast to RANKL, concentrations of SOST in cortical bone have been seen to decrease with age (234), a change which should promote osteoblast formation with age, yet ageing bone is characterised by a decrease in osteoblast number and so an area which requires further investigation. Whilst there are treatments which can ameliorate the processes related to the age-related deterioration of skeletal tissue (reviewed in (236)), maximising peak bone mass in the earlier stages of life, maintaining bone mass and minimising bone loss is therefore ultimately critical for delaying the onset of these age-related changes (237) and reducing the risk of fracture in later life.

1.3.3 *Assessing skeletal growth*

Both total bone mineral content (BMC) and total bone mineral density (BMD) have been used as assess skeletal development within the context of the studies used in this dissertation. Both measures were assessed by dual energy X-ray absorptiometry (DXA). DXA is a technique which uses x-ray radiation and the inherent ability of both soft tissue and bone to attenuate x-ray radiation to allow for the assessment of BMD (g/cm^2) in the skeleton. BMD is measured in a segmented area, or region of interest and BMC (g) then derived from this measurement as $\text{BMD} \times \text{area}$, meaning BMD can also be defined as BMC/area . Therefore, whilst these measures are inextricably linked, they can still provide slightly different information. For example, within a given study if an association was identified between a variable and BMC but not BMD, we could make a

differential inference that this relationship or association may be due to skeletal size. Therefore, within this dissertation measures of both BMC and BMD were used for differential methylation analyses.

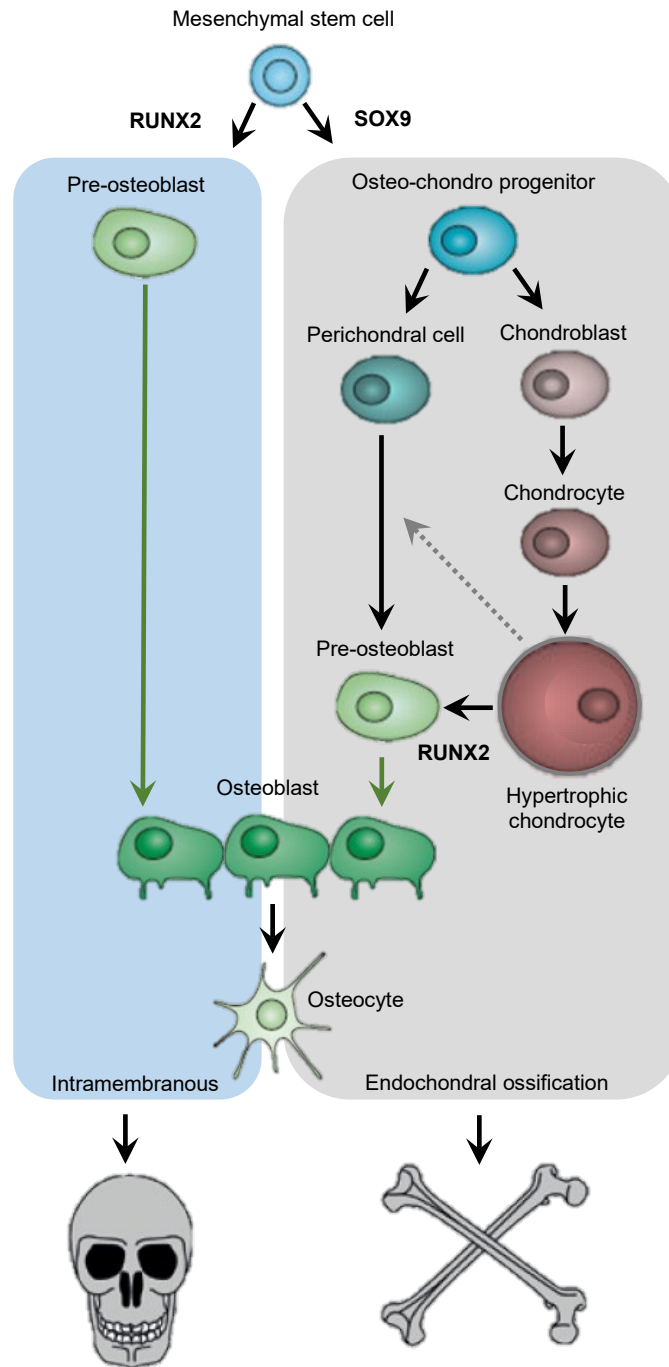


Figure 1. A flow-chart depicting the differentiation pathway for MSCs during both intramembranous and endochondral ossification

Intramembranous ossification of the clavicles, calvaria, maxilla and mandible occurs through the RUNX2-mediated differentiation of MSCs into pre-osteoblastic and finally osteoblastic cells. Whereas endochondral ossification of the axial skeleton, section of the clavicle and limbs involves the SOX9-mediated condensation of MSCs into chondrogenic progenitors and perichondral cells. Hypertrophic chondrocytes have been suggested to transdifferentiate into preosteoblastic cells and stimulate the osteogenesis of perichondral cells into preosteoblastic cells, which subsequently differentiate into osteoblasts. Adapted from *Rutkovskiy et al. (2016) (13)*.

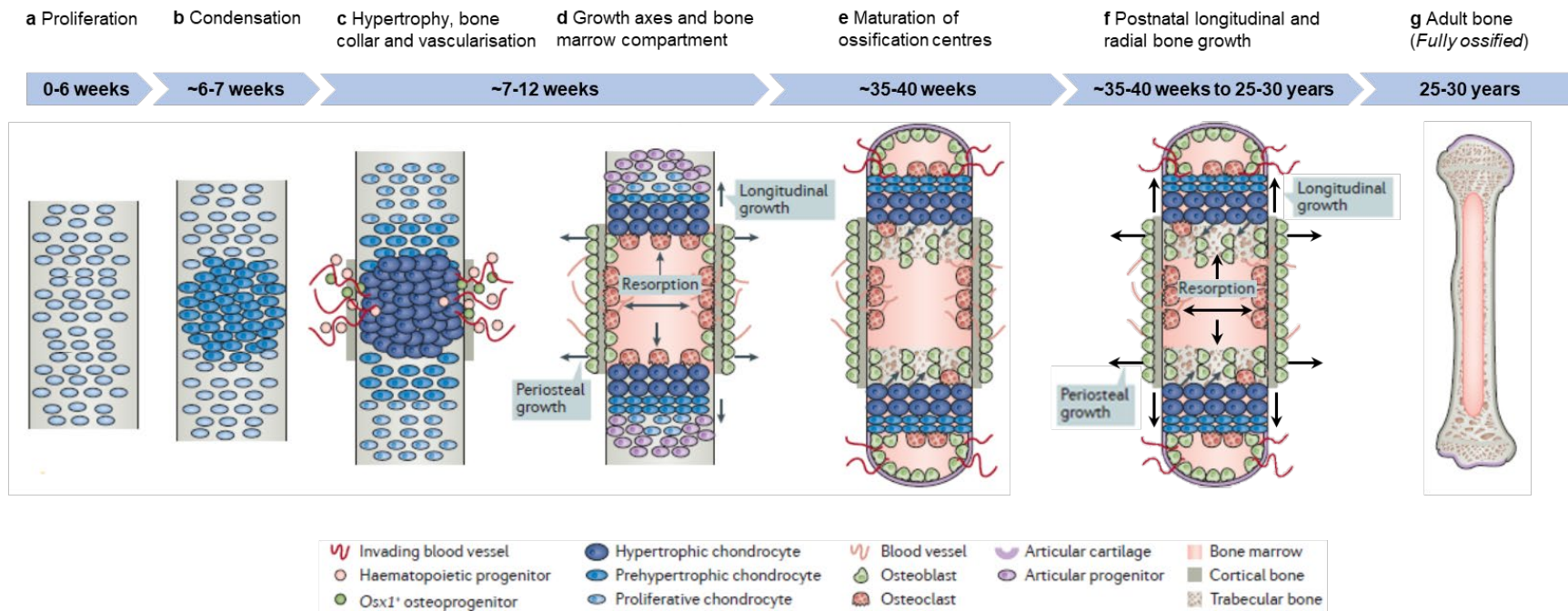


Figure 2. A temporal view of bone development during skeletogenesis depicting key stages in endochondral bone development

The stages of endochondral ossification, including a) proliferation of progenitor MSCs, b) condensation of MSCs, c) hypertrophy of chondrocytes, generation of the bone collar and vascularisation, d) generation of growth plate and growth along bone axes and bone marrow compartment, e) maturation of the ossification centres and f) postnatal longitudinal and radial bone growth, where longitudinal growth ceases at 16-19 years with closure of the epiphyseal growth plate, but bone modelling process continue until the achievement of peak bone mass between g) 25 and 30 years, where the adult bone is fully formed and remodelling processes predominate (fully ossified, growth plates closed). Adapted from *Salazar et al.* (2016) (36).

1.3.4 Mesenchymal stem cells

Mesenchymal stem cells are the fundamental precursor of the bone-forming osteoblast with the capacity to differentiate into multiple cell types such as chondrocytes and adipocytes in addition to osteoblasts. In developing bone, mesenchymal stem cells exhibit an inverse relationship between adipogenesis and osteogenesis (238). This can be seen in conditions such as osteoporosis where there is not only an imbalance in the bone remodelling process, but an increase in marrow adiposity at the expense of bone formation (239). This increase in marrow adiposity has also been seen to inhibit osteogenesis (240) and decrease osteoprogenitor proliferation capacity (241) as a result of the increased potential of MSCs to differentiate into adipocytes over osteoblasts (239). Therefore, understanding the mechanisms which influence the lineage commitment of MSCs towards an osteoblastic lineage in early development could aid in the understanding of pathologies where insufficient bone formation occurs, such as osteoporosis.

1.3.4.1 *Regulating the commitment and differentiation of mesenchymal stem cells*

The multi-stage process of the differentiation of MSCs into osteoblasts involves the confluence of transcription factors, signalling pathways and systemic factors among others. A temporal overview of the activity of the respective factors over the course of osteogenic differentiation of MSCs is shown in Figure 4.

1.3.4.1.1 *Transcription factors*

1.3.4.1.1.1 SRY-Box Transcription Factor 9

SOX9 is a transcription factor essential for endochondral bone development. SOX9 acts as a master regulator of the chondrogenic lineage and is expressed in all osteochondroprogenitor cells (242,243). Inactivation of SOX9 before the formation of mesenchymal condensations was shown in a mouse model to result in the complete absence of mesenchymal condensations, cartilage or bone formation. However, the inactivation of SOX9 after the formation of mesenchymal condensations was shown to lead to osteoblastogenesis, but almost completely lack of cartilage in the endochondral skeleton (47). This suggests SOX9 may be required to establish mesenchymal condensations but is not required for further osteoblast differentiation. This finding may be further explained by studies which have shown SOX9 is expressed in all regions of the growth plate, except the hypertrophic zone (Figure 2d) where it is downregulated (244). A study by *Dy et al.* (2012) found in a mouse model SOX9 acts to maintain chondrocyte proliferation, direct hypertrophic maturation and suppress the expression of osteoblastic transcription factors (245). Further work by *Lui et al.* (2019) corroborated this finding in a similar model, showing that the overexpression of SOX9 in the hypertrophic zone leads to expansion of the region, delaying ossification, whereas deletion of SOX9 shortened the region and promoted osteogenesis (33). This suggests during endochondral ossification SOX9 acts to inhibit the transition of proliferating

chondrocytes into pre-hypertrophic and hypertrophic regions which may inhibit the progression of endochondral ossification.

1.3.4.1.1.2 Runt-related transcription factor 2

RUNX2 is the fundamental, indispensable master regulator of osteoblast differentiation for both intramembranous and endochondral ossification. During development RUNX2 is expressed throughout the chondrogenic mesenchyme, later becoming confined to the perichondrium. Both *Otto et al. (1997)* and *Komori et al. (1997)* showed in a mouse model that in the absence of RUNX2 the perichondrium becomes hypoplastic or underdeveloped, suggesting RUNX2 may be necessary for the production or maintenance of these progenitor cells (40,246). The homozygous knockout of RUNX2 in mice results in a complete absence of osteoblasts and no ossification, leaving only a cartilaginous model. Mice with a homozygous knockout of RUNX2 are therefore non-viable at birth (40,246). In humans it has also been shown RUNX2 haploinsufficiency leads to a delayed closure of fontanelles, hypoplastic clavicles or cleidocranial dysplasia, a condition primarily affecting the development of the bones and teeth (247,248). In a transgenic mouse model *Ducy et al. (1999)* also found that once differentiated, RUNX2 is required for the proper functioning of mature osteoblasts including the synthesis of bone matrix (249).

1.3.4.1.1.3 Osterix

Osx is a transcription factor necessary for the osteoblastic differentiation of MSCs that lies downstream of the *RUNX2* gene. *Osx*-knockout mice are born with short, non-mineralised limbs, demonstrating its pivotal role in skeletal development (42). *Nakashima et al. (2002)* demonstrated that a loss of *Osx* in mice lead to an increase in ectopic cartilage formation beneath a thickened perichondrium, due to an apparent switch in the lineage commitment of perichondral cells from osteoblasts to chondrocytes (42). Additionally, conditional deletion of *Osx* in mouse models disrupted osteoblast differentiation and severely disrupted the functioning of osteocytes and osteoclasts (250). This demonstrated along with its role in development, *Osx* plays an essential role in the development and homeostatic maintenance of bone later in life.

1.3.4.1.1.4 Activating transcription factor 4 (ATF4)

ATF4 plays a significant role during the early development of bone, the homeostatic maintenance of bone and the development of mature osteoblastic cells. In humans Coffin-Lowry syndrome, a condition characterised by delayed bone formation during embryonic development and low bone mass throughout postnatal life has been attributed to a lack of ATF4 phosphorylation by the ribosomal protein S6 kinase A3 (RSK2), which subsequently hampers osteoblast differentiation (251). In a mouse model, *Wang et al. (2009)* showed ATF4 may act in chondrocytes during endochondral ossification to stimulate the induction of pro-osteoblastic *Ihh* signalling and

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therefore the specification of the osteoblast lineage in development (252). Additionally, ATF4 is thought to regulate RANKL which promotes osteoclast differentiation and function (251).

1.3.4.1.2 Signalling pathways

There are a number of signalling pathways which act both independently and in concert to contribute to the osteoblastic differentiation of MSCs including hedgehog (HH), Wnt, TGF β , bone-morphogenic protein (BMP), notch and FGF signalling pathways.

1.3.4.1.2.1 Hedgehog signalling pathway

The hedgehog signalling pathway is essential for embryonic development. The mammalian hedgehog signalling pathway acts through three hedgehog ligand proteins, Desert hedgehog, Ihh and Sonic hedgehog (253). These proteins act through binding to the Patched homologue 1 receptor, signalling through the seven-pass transmembrane protein to regulate gene transcription through the activation and depression of Gli transcription factors (Figure 3a) (254). Of particular importance during skeletal development is Ihh which is required for endochondral ossification. Ihh is expressed by prehypertrophic and early hypertrophic chondrocytes in the endochondral cartilage primordium, signalling to chondrocytes and adjacent perichondral cells to promote the differentiation of potential progenitor cells into osteoblasts. Consequently, Ihh knockout mice die just after birth due to a lack of RUNX2 expression in the perichondrium and subsequently a complete absence of osteoblasts. This indicates Ihh signalling is directly required in the perichondrium for the commitment and initialisation of MSC osteoblastogenesis (28,29,255,256). Ihh also has a potential role in postnatal bone development. A study by *Deng et al.* (2017) demonstrated post-differentiation, Ihh knockdown in an osteoblastic cell line resulted in cell-cycle arrest, decreased alkaline phosphatase activity and mineral deposition of osteoblasts (257).

1.3.4.1.2.2 Wingless-related integration site signalling

Wnt signalling is classified into either canonical (Wnt/ β -catenin dependant) or noncanonical (β -catenin independent) based on the ability of the pathway to prevent the degradation of the transcription factor β -catenin (258). In canonical Wnt signalling, Wnt binds to the Frizzled receptor and LRP5 or LRP6 co-receptor to stabilise cytosolic β -catenin (Figure 3c). β -catenin enters the nucleus and stimulates transcription of Wnt target genes through interaction with T cell factor 1 (TCF1), TCF3, TCF4 and lymphoid enhancer-binding factor 1 (259). Wnt signalling has roles in both the prenatal and postnatal accrual of bone. It has been shown *in vivo* that the prenatal deletion or inactivation of β -catenin in osteochondroprogenitor cells within the perichondrium blocks osteoblastic differentiation and instead favours chondrocytic differentiation, a finding that has also replicated *in vitro*. These cells expressed both alkaline phosphatase and RUNX2 but failed to express *Osx*, a factor essential to osteoblast lineage commitment (260,261). This suggested canonical Wnt/ β -catenin signalling is essential for osteoblastic lineage specification. Additionally,

a postnatal loss of function mutation in the Wnt co-receptor LRP5 leads to a form of juvenile-onset osteoporosis, whereas mutations that render it resistant to extracellular inhibitors such as DKK1 cause high bone mass syndrome (262–264). This indicates the active and integral role Wnt signalling plays in both the pre- and postnatal development of bone.

The non-canonical or β -catenin-independent pathway can be divided further into the planar cell polarity pathway and the Wnt/ Ca^{2+} pathway. The planar cell polarity transduction cascade occurs through FZD receptor independent of LRP5/6, leading to the activation of dishevelled (Dsh). Dsh, through Dishevelled-associated activator of morphogenesis 1 can activate both the Ras homologous (Rho) pathway or the actin binding protein Profilin. Dsh can also alone activate c-Jun N-terminal kinase (JNK) signalling through Rac activation. Alternatively, as per the Wnt Ca^{2+} pathway, binding of Wnt to Fz can through the activation of Dsh recruit and activate guanine nucleotide-binding proteins (G-proteins). Dsh can then activate phosphodiesterase which inhibits protein kinase G and in turn inhibits Ca^{2+} release or through phospholipase C activates inositol triphosphate, which leads to release of intracellular Ca^{2+} . This can go on to activate protein kinase C (PKC) signalling, calcineurin which activates nuclear factor of activated T-cells (NFAT) and finally calcium/calmodulin dependent protein kinase II which will lead to activation of both Transforming growth factor beta-activated kinase 1 and NF-kappa-B essential modulator, factors which will inhibit β -catenin and TCF function (265). With respect to the regulation of the osteoblast lineage, deletion of Frizzled-9, one of the 10 FZD receptors within a mouse model led to a decreased bone mass, partly through the downregulation of ISG15 Ubiquitin Like Modifier, without impairing β -catenin signalling. Furthermore, Wnt5A, a non-canonical Wnt ligand has been suggested to inhibit adipogenesis and so promote osteogenesis by inducing suppressive histone methylation at the promoter of the peroxisome proliferator-activated receptor- γ (PPAR γ) gene. Finally, Wnt-induced G protein-coupled phosphatidylinositol signalling and induction of protein kinase C δ (PKC δ) has been shown in a mouse model to promote the progression of osteoblastic differentiation from RUNX2 positive, to both RUNX2 and OSX positive cells, albeit by an as of yet unknown mechanism (266–268).

1.3.4.1.2.3 TGF β superfamily signalling

The TGF β superfamily is a collection of 33 cytokine ligands which based on both structure and function are split into subfamilies which include TGF β s, BMPs, activin, inhibin and others. Both TGF β s and BMPs play significant and diverse roles in skeletogenesis including condensation of the mesenchyme, skeletal morphogenesis, growth plate development and osteoblastic differentiation (269).

1.3.4.1.2.3.1 TGF β signalling

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TGF β cytokines exist in three isoforms, TGF β 1, TGF β 2 and TGF β 3. TGF β signalling can occur through a canonical, smad-dependant pathway or through a non-canonical, smad-independent pathway. Canonical TGF β signalling is classically induced by the binding of a TGF β ligand to transforming growth factor-beta receptor 2 (TGF β R2), one of the three TGF β receptors (TGF β R1, 2 and 3). The binding of a ligand to TGF β R2 leads to the recruitment and phosphorylation of TGF β R1 which phosphorylates the downstream Mothers Against Decapentaplegic Homolog 2 (SMAD2) and SMAD3. SMAD 2 and 3 will further bind with SMAD4, forming a complex which translocates to the nucleus affecting transcription of TGF β target genes. Alternatively, depending on the proteins recruited to the ligand-receptor complexes binding of TGF β s can activate non-canonical signalling. Non-canonical signalling can stimulate a range of possible kinases, including the mitogen-activated protein kinases (MAPKs), ERKs, P38, JNK, PI3K/Akt, or rho-associated protein kinase which can have effects on a range of processes including proliferation, differentiation, apoptosis and epithelial-mesenchymal transition (270).

The pleiotropic TGF β cytokines have been suggested to play a role in regulating cellular proliferation, migration and differentiation during development and tissue homeostasis in adults. TGF β 1 is the most abundantly expressed, found in cartilage, both endochondral and intramembranous bone and the skin (271–274). This abundance of TGF β 1 in bone is highly significant in the bone remodelling process. TGF β 1 is synthesised and deposited by osteoblasts during bone matrix production as an inactive, large precursor molecule composed of TGF β 1 and latency-associated protein (LAP). During osteoclastic bone resorption the acidic microenvironment created by osteoclasts leads to the release of TGF β 1 from the ECM into the bone microenvironment and cleavage from LAP. Once released in its newly-activated state, TGF β 1 acts as a chemoattractant, inducing the migration of MSCs to resorption sites where they are induced to differentiate into osteoblasts and as so facilitate bone deposition (275).

TGF β signalling has also been identified to promote early proliferation, differentiation and matrix production by osteoprogenitors, but inhibit later differentiation and matrix mineralisation (276–279). Indeed deletion of TGF β R2 in early mouse limb bud mesenchyme by *Seo and Serra* (2007), resulted in severe skeletal defects and embryonic lethality (280). Furthermore *Wang et al.* (2013) showed deletion of TGF β R2 in the dental mesenchyme resulted in reduced mineralisation of the mandible due to reduced osteoblast differentiation (281,282). TGF β has also been identified to modulate the lineage specification and commitment of MSCs. Deleting TGF β in mouse BM-MSCs has been shown to lead to a marked increase in adipogenic commitment and differentiation, reflected by increases in the expression of increase in PPAR γ and fatty acid binding protein 4, but decreases in the number of osteoblasts (283).

1.3.4.1.2.3.2 Bone morphogenic protein signalling

BMP proteins are part of the TGF β superfamily. TGF β and BMP signalling both have fundamental roles in the embryonic development of bone and postnatal bone homeostasis. BMPs exert their effects through heterotetramer transmembrane type I and type II serine/threonine kinase receptor complexes via either a canonical smad-dependant, or non-canonical smad independent signalling pathway (269,284–286). In the canonical pathway BMPs bind to the transmembrane complexes, activating SMADs 1, 5 or 8 through site-specific phosphorylation which bind with SMAD4 and translocate to the nucleus to regulate gene expression (Figure 3d) (285,287). In prenatal life BMPs are required for the formation of mesenchymal condensations during endochondral ossification (288). In particular, BMP2 and BMP4 are highly involved in bone development. BMP2 alone is strongly osteoinductive (289) and *Bandyopadhyay et al.* (2006) found during development a threshold level of both BMP2 and BMP4 signalling is required for osteoblastic differentiation. Additionally, the knockout of BMP2 and BMP4 in the limb buds results in the severe impairment of osteogenesis (290). BMP signalling has also been suggested to have a regulatory role in the function of mature osteoblasts. When overexpressed in a mouse model, the BMP inhibitor noggin caused a reduction in osteoblast function and a subsequent 23–29% reduction in total, vertebral and femoral BMD (291,292).

BMPs have been shown to be able to activate a number of non-canonical, Smad-independent signalling pathways implicated in skeletal development and osteogenic differentiation including MAPK, PI3K/Akt, PKC, Rho-GTPases and others. BMP4 has been identified to activate TGF β activated kinase 1 (TAK-1), a serine-threonine kinase of the mitogen activated protein kinase kinase pathway. MSC or chondrocyte-specific deletion of TAK-1 mice has been shown to result in a disorganised growth plate, whereas in a mouse model postnatal deletion in chondrocytes alone resulted in severe defects of growth plate and articular cartilage development. Whereas osteoblast-specific deletion of TAK-1 in osteoblasts has been shown to result in clavicular hypoplasia and delay fontanelle fusion, a phenotype which mirrors cleidocranial dysplasia in humans, a condition caused by RUNX2 deficiency (293–295).

1.3.4.1.2.4 Fibroblast growth factor signalling

The FGF family is currently known to encode 22 genes, 18 of which signal through binding to cell surface tyrosine kinase FGF receptors (FGFRs). This binding can lead to the phosphorylation of a range of signalling proteins and the activation of a number of signalling pathways (Figure 3e). Mutations in FGFs and FGFRs 1-3 have been associated with a range of skeletal diseases including osteoporosis, osteomalacia and rickets (296–299). FGF2 is a potent mitogen for osteoblasts and osteoblast precursors, expressed in the growth plate of long bones during the process of endochondral ossification (300–303). Postnatally, FGF2 knockout mice have normal limbs but exhibit a significant reduction in mineral apposition, trabecular bone volume and rate of bone formation (304,305). The role of FGF signalling during differentiation is dynamic, where FGFR

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expression and functional control of osteoblastogenesis depends on the stage of differentiation. In a mouse model, *Jacob et al.* (2006) showed the inactivation of FGFR1 in osteochondroprogenitor cells increased proliferation and delayed osteoblast differentiation, whereas inactivation in differentiated osteoblasts accelerated differentiation and maturation (306). Additionally, FGFR2 has been seen to promote the proliferation of preosteoblasts and the anabolic function of mature osteoblasts, whereas an alternatively spliced form has been seen to upregulate RUNX2 expression, promoting osteoblast differentiation (307–309). Therefore FGF signalling has a diverse and multi-faceted role in regulating preosteoblastic and osteoblast differentiation and the function of mature osteoblasts.

1.3.4.1.2.5 Notch signalling

Notch signalling acts through direct contact with neighbouring cells. Notch receptors bind to their respective ligands on neighbouring cells, leading to proteolytic cleavage catalysed by the γ -secretase complex which also requires the activity of presenilin 1 or 2 (PS1, PS2). This activity leads to the release of the notch intracellular domain (NICD) from the plasma membrane which translocates to the nucleus. Here, it interacts with recombination signal-binding protein for Ig of κ region (RBPJk) and mastermind-like (Maml) to regulate transcription of genes such as the hairy and enhancer of split (*HES*) and HES-related with YRPW motif (HEY) transcription factors (310–314) (Figure 3b). Converse to the previous pathways, notch signalling has been suggested to suppress the differentiation of osteoblast progenitors in part through the induction of HEY1 and HEY-like activity, which act to reduce RUNX2 transcriptional activity. Consequently, the overexpression of HEY1 in transgenic mice leads to osteopenia and gain of function mutations in notch 2 cause a disorder characterised by severe progressive bone loss (315–317). Conversely, knockout of PS1/2 or notch 1/2 in the limb mesenchyme results in high bone mass in adolescent mice, indicating the inhibitory role of notch signalling in bone development.

1.3.4.1.2.6 Cross-talk between pathways

Whilst each of the aforementioned pathways have specific and significant roles in bone biology, crosstalk between these and other pathways is essential for the coordination of osteogenesis and skeletal development. BMP for example can act to stimulate osteoblast and chondrocyte differentiation through synergistic activity with the Wnt pathway. BMP2 has been identified to promote Wnt signalling through the induction of Wnt3a, Wnt1 and LRP5 expression, whilst inhibiting Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase and F-box E3 ligase, factors involved in the degradation of β -catenin in osteoblasts (318,319). However BMP signalling can also inhibit Wnt activity via Bone Morphogenetic Protein Receptor Type 1A to activate Smad and MAPK signalling which increases expression of both DKK1 and SOST expression to negatively regulate bone mass (96, 98). Similarly, Smad4 can also competitively bind β -catenin and prevent binding with the TCF/LEF complex such that ablation has accordingly been seen to promote bone

formation (320,321). Both BMP and FGF signalling processes show significant interdependence during osteogenesis that both act in synergy to promote osteogenesis, but play opposing roles in mineralisation, a process promoted by BMP2 (322,323). BMP signalling has been shown to be essential for FGF-induced osteoblastic differentiation and similarly, disruption of FGF2 in mice has been shown to impair the expression BMP2 and reduce bone mass (324,325) highlighting the co-ordinated action of the pathways. It has also been suggested Notch signalling facilitates BMP-induced osteoblastic differentiation where inhibition has been seen to inhibit expression of BMP target genes. In addition, alongside BMP2, TGF β has been shown to regulate expression of signalling proteins in the Notch pathway, including HEY1 and HES1 (326–329). TGF β has also been shown to interact with PTH signalling, where osteoblast-specific TGF β R2 conditional knock out mice have shown increased bone mass due to the hyperactivation of the Parathyroid Hormone 1 Receptor (PTH1R), an effect ablated by disruption of the receptor. Furthermore, BMP signalling has also been shown to be essential for Ihh-induced osteoblast differentiation and promote HH, PTHrP signalling, pathways which similarly promote BMP signalling. TGF- β also has similar interactions with Wnt, HH, FGF, Notch pathways (330,331).

Here, we have addressed some of the interactions of both BMP and TGF β signalling with other signalling pathways significant in osteoblastic differentiation. However, this remains only a small subset of the interactions occurring between pathways to facilitate osteoblastic differentiation. Furthermore, this omits the effect interactions between these pathways has for chondrocytic differentiation, underscoring the complexity and critical nature of the interactions of these pathways during skeletal development.

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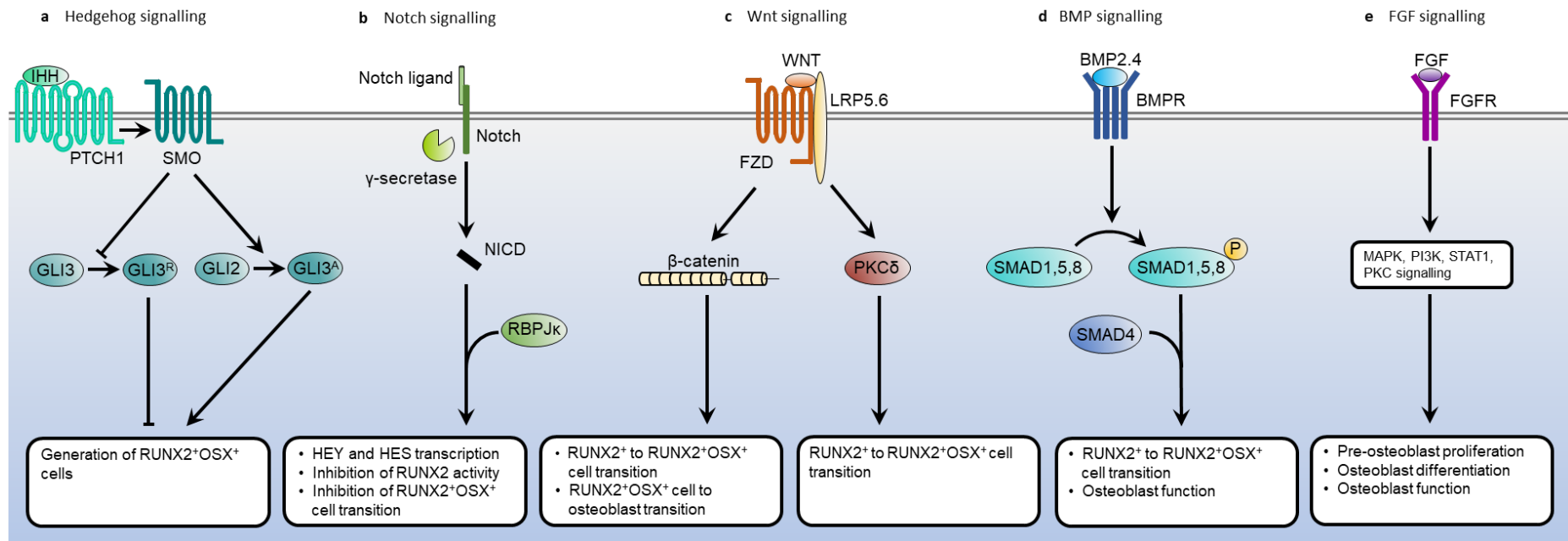


Figure 3. Key signalling pathways regulating the osteoblastic differentiation of mesenchymal progenitor cells

The key signalling pathways involved in influencing the osteoblastic differentiation of mesenchymal progenitor cells including a) hedgehog signalling, b) notch signalling, c) Wnt signalling, d) BMP signalling and e) FGF signalling. IHH, Indian hedgehog; PTCH1, Patched 1; SMO, Smoothened; GLI3, GLI Family Zinc Finger 3; NICD, Notch intracellular domain; RBPJk, Recombination Signal Binding Protein For Immunoglobulin Kappa J Region; Wnt, Wingless-related integration site; LRP5/6, Low-density lipoprotein receptor-related protein 5/6; FZD, Frizzled; PKCδ, Protein kinase C-delta; BMP, Bone morphogenetic protein; BMPR, Bone morphogenetic protein receptor; HEY, HES-related with YRPW motif; HES, Hairy and enhancer of split; Adapted from Long *et al.* (2012) (256).

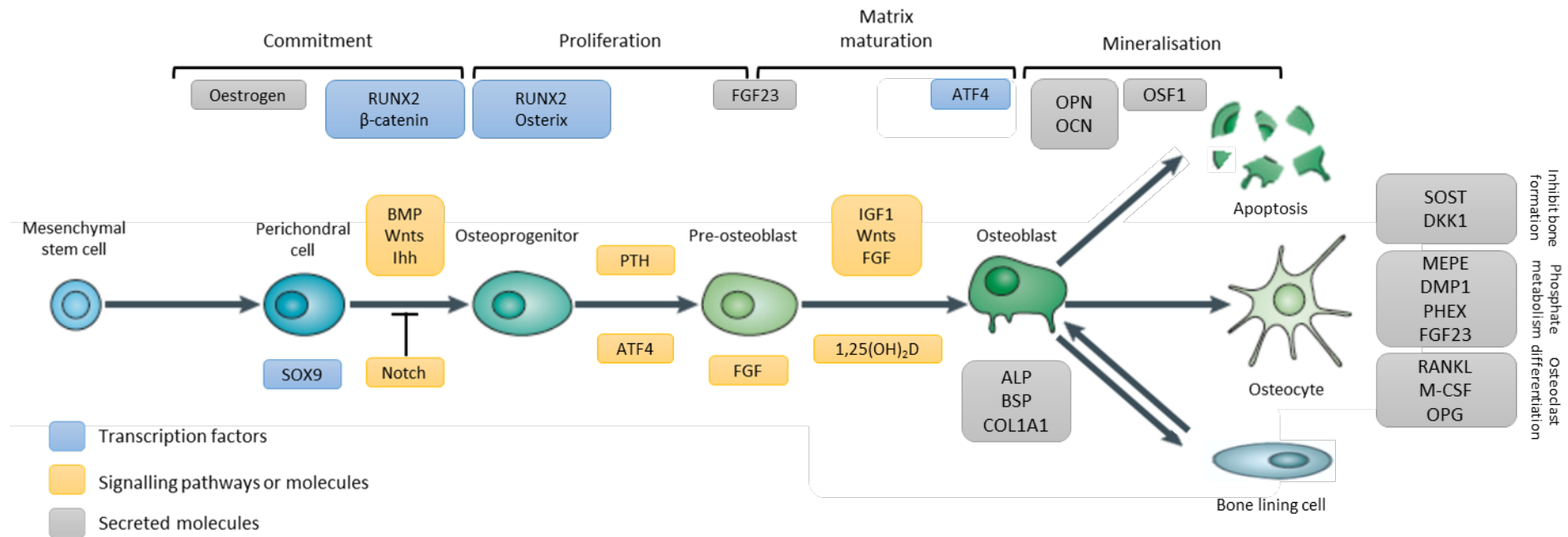


Figure 4. Stages in the osteoblastic differentiation of mesenchymal progenitor cells

The interplay of transcription factors, signalling pathways or molecules and secreted molecules involved in the regulating the osteoblastic differentiation of mesenchymal stem cells. When cells of an osteoblastic lineage reach maturity, the cells produce osteoid and undergo one of three possible cell fates: 1) Cell death through apoptosis; 2) becoming embedded in the osteoid and terminally differentiating to form osteoid osteocytes; or 3) remaining on the bone surface as flattened bone-lining cells, not actively producing osteoid, a cell type which can be 're-activated' to form osteoblasts once more. RUNX2, Runt-related transcription factor 2; Fra-1, Fos-related antigen; ATF4, Activating transcription factor 4; ALP, Alkaline phosphatase; BMP, Bone morphogenic protein; BSP, Bone sialoprotein; COL1A1, Collagen, Type I, Alpha 1; DKK1, Dickkopf-Related Protein 1; DMP1, Dentin Matrix Acidic Phosphoprotein 1; FGF23, Fibroblast growth factor 23; Ihh, Indian hedgehog; MEPE, Matrix Extracellular Phosphoglycoprotein; M-CSF, Macrophage Colony Stimulating Factor 1; OPG, Osteoprotegerin; PTH, Parathyroid hormone; FGF, Fibroblast growth factor; IGF1, Insulin-like growth factor 1; OCN, Osteocalcin; OPN, Osteopontin/bone sialoprotein I; OSF1, Osteoblast stimulating factor-1; PGE2, Prostaglandin E2; PHEX, Phosphate Regulating Endopeptidase Homolog X-Linked; RANKL, Receptor Activator Of Nuclear Factor Kappa-B Ligand; SOST, Sclerostin; Wnt, Wingless-related integration site; 1,25(OH)₂D, 1,25-Dihydroxyvitamin D₃. Adapted from *Crocket et al.* (2011) (243).

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1.3.4.1.3 Systemic regulation

In addition to both the action of transcription factors and the activation signalling pathways, there are systemic factors that can significantly influence the differentiation of osteoblast progenitors and the development of bone.

1.3.4.1.3.1 Parathyroid hormone

PTH is synthesised and secreted by the parathyroid glands and essential for the maintenance of calcium and phosphate homeostasis (332–334). PTH acts through the G-protein-coupled receptor PTH1R, present on the surface on a number of cells including osteoblasts, osteoclasts and stromal cells. The activation of PTH1R leads to the induction of cyclic-adenosine monophosphate (cAMP) and protein kinase a (PKA) signalling pathways, the subunits of which translocate to the nucleus to modulate gene expression (335,336). Persistent exposure to PTH has been found to have catabolic effects on bone, whereas intermittent PTH (IPTH) exposure has converse, anabolic effects on bone. IPTH acts to increase bone mass in a number of ways. IPTH has been shown to increase the proliferation and differentiation of osteoblasts *in vitro* and *in vivo* (337,338). It also acts to decrease the rate of apoptosis of osteoblasts in the earlier stages of differentiation and increase the rate of apoptosis for more mature osteoblasts (339). Additionally, IPTH stimulates Wnt signalling activity to promote proliferation and differentiation of osteoblast precursors (340) and finally, IPTH activates quiescent periosteal lining cells to form osteoblasts, increasing osteoblast number and the rate of bone formation (341).

1.3.4.1.3.2 Insulin-like growth factors

The insulin-like growth factor (IGF) regulatory system is composed of the ligands IGF-1 and IGF-2, their respective receptors IGF-R1 and IGF-R2 and the proteins regulating their activity, acid-labile subunit (ALS) and the IGF binding proteins (342). Both IGF-1 and 2 have a strong association with osteogenesis, secreted by osteoblasts to stimulate bone formation, growth, metabolism, prevent apoptosis and modulate skeletal activity in an autocrine and paracrine manner (343,344). IGFs are in circulation and extremely abundant within the skeleton, bound to IFGBPs in the bone matrix and liberated during bone modelling and remodelling to stimulate the osteoblastic differentiation of MSCs, making IGFs essential to maintaining skeletal homeostasis (345). IGF-1 is one of the central anabolic factors for bone growth, required for the terminal differentiation of osteoprogenitors and positively associated with bone mass, peaking with acquisition of peak bone mass and reducing with any age-related bone loss (344,346). Overexpression of IGF-1 has been associated with accelerating the rate of bone formation and matrix mineralisation, whereas IGF-1 and IGF-R1 null-mice show underdeveloped short bones, low bone mineral density and delayed calcification (347). An IGF-2 mutation in humans has been associated with early postnatal growth restriction, potentially due to uncoordinated growth plate development (348). Therefore the IGF system has a fundamental role the regulation of bone development.

1.3.4.1.3.3 Vitamin D

1.3.4.1.3.3.1 Synthesis, metabolism and signalling

Vitamin D, a biologically inactive prohormone exists in two main isoforms. Vitamin D₂ is produced through ultraviolet B (UVB) irradiation of plants and fungi whereas vitamin D₃ (D₃) is obtained from sources such as fatty fish and endogenous synthesis within the skin (349). D₃ is synthesised via UVB irradiation of the precursor 7-dehydrocholesterol, forming pre-D₃ that is subsequently thermally isomerised to D₃. When ingested D₃ is incorporated into chylomicrons, passed into the lymphatic system and enters the circulation where it travels bound to vitamin D binding protein (DBP) and lipoproteins. To become its active form D₃ is metabolised in three major steps. Initially D₃ is 25-hydroxylated in the liver via a cytochrome P450 mixed function oxidase (CYP) with 25-hydroxylase activity, cytochrome P450 family 2 subfamily R member 1 (CYP2R1) and cytochrome P450 family 27 subfamily A member 1 (CYP27A1), to 25(OH)D₃. 25(OH)D₃ is the main circulating form of vitamin D that is typically used to determine an individual's vitamin D status. In the kidney 25(OH)D₃ is then 1- α hydroxylated by cytochrome P450 family 27 subfamily B member 1 (CYP27B1) to become the active form of vitamin D, 1,25(OH)₂D. Additionally both 25(OH)D and 1,25(OH)₂D can be metabolically inactivated in the kidney and intestine through 25-hydroxylation by cytochrome P450 family 24 subfamily A member 1 (CYP24A1) to form either 24,25(OH)₂D or 1,24,25(OH)₃D, excreted via the bile and urine (349–354) (Figure 5).

The active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ acts through binding to the vitamin D receptor (VDR) (354,355), a steroid hormone receptor located largely in the cytoplasm. The binding of 1,25(OH)₂D to VDR induces a conformational change which allows this complex to heterodimerise with the retinoid X receptor (RXR) which translocates to the nucleus. This complex can then subsequently bind to vitamin D responsive elements (VDRE) in the promoter region of target genes, affecting transcription (356,357).

1.3.4.1.3.3.2 Systemic effects

Vitamin D both directly and indirectly influences the development of the skeleton. A major function of 1,25(OH)₂D, in conjunction with PTH, is to regulate calcium and phosphate homeostasis to maintain cellular function and subsequently promote the mineralisation of bone (Figure 5) (358,359). Therefore mice lacking VDR will typically manifest with vitamin D resistance, resulting in severe growth impairment, retarded growth, immune abnormalities and premature death at 15 weeks of age due to hypocalcaemia (360–362). However, these effects can be ameliorated with a rescue diet indicating that if calcium homeostasis is regulated appropriately, there is only an indirect effect on bone growth. Direct effects of 1,25(OH)₂D have also been observed. Decreased 1,25(OH)₂D during development can lead to abnormal development of the growth plate, partly due to reduced vascular invasion, reduced chondroclast and osteoclast activity (363). Treatment of ovariectomised mice with 1,25(OH)₂D or a 1,25(OH)₂D analogue has

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been shown to have an anabolic effect on bone without changes in blood calcium (364,365). Furthermore, in mouse models mature osteoblasts overexpressing VDR showed an increases in cortical, periosteal and trabecular bone with concomitant reductions in bone reabsorption (366,367).

1.3.4.1.3.3.3 Cellular effects

Experimentation *in vitro* from a number of studies has suggested $1,25(\text{OH})_2\text{D}$ treatment enhances the osteogenic differentiation of MSCs, pre-osteoblastic cells and bone mineralisation in osteoblasts (368–374), suggesting the pro-osteogenic role of vitamin D goes beyond that of maintaining systemic calcium and phosphate homeostasis. This effect has been observed in studies which have proposed the vitamin D mediated enhancement of the osteogenic differentiation of MSCs may occur through synergistic activity with either BMP-2 or TGF β (375). *Liu et al.* (1998), in human BM-MSCs showed TGF β as a factor inhibitory to alkaline phosphatase expression (ALP) a marker of early osteoblastic differentiation. However, a combined treatment of both TGF β and $1,25(\text{OH})_2\text{D}$ synergistically enhanced ALP activity, producing a six to eight-fold increase in ALP expression over controls and a two to four-fold increase relative to cells treated with $1,25(\text{OH})_2\text{D}$ alone. It was also found TGF β would abrogate $1,25(\text{OH})_2\text{D}$ induced osteocalcin expression, a marker of later osteoblast maturation. Overall this suggests a role for both TGF β and $1,25(\text{OH})_2\text{D}$ in facilitating the differentiation of early osteoprogenitors along an osteogenic pathway, but the factors likely diverge in their function in the later stages of osteoblastic maturation where TGF β is largely inhibitory to osteogenic maturation as discussed earlier (section 1.3.4.1.2.3.1) (376).

In line with the reported pro-osteogenic effects of $1,25(\text{OH})_2\text{D}$ and the reciprocal inhibitory nature of osteoblastic and adipogenic differentiation, several studies have identified $1,25(\text{OH})_2\text{D}$ as anti-adipogenic in BM-MSCs and pre-adipocytic cells (377). In a study by *Kong and Li* (2006), treatment of pre-adipocytic cells with $1,25(\text{OH})_2\text{D}$ has been shown to antagonise the activity of PPAR γ , a master regulator of adipogenesis the activity of which was restored after treatment with a PPAR γ agonist (378). Unliganded VDR alone has also been suggested to inhibit PPAR γ activity through competitive binding to their common heterodimerisation partner, RXR (378,379). As such, studies have shown treatment of BM-MSCs with $1,25(\text{OH})_2\text{D}$ leads to the downregulation of PPAR γ in human BMSCs, whereas the treatment of mice with a PPAR γ antagonist upregulated VDR expression in bone (380,381). Conversely in a study by *Cianferotti et al.* (2007) BM-MSCs cultured from VDR-null mice showed increased PPAR γ expression, adipogenesis and expression of DKK1 and Secreted Frizzles Related Protein 2, inhibitors of the pro-osteogenic canonical Wnt signalling pathway. Furthermore, expression of these factors was blunted in wild-type BM-MSCs by treatment with $1,25(\text{OH})_2\text{D}$ in the presence or absence of adipogenic inducers (382). However, other studies have suggested the inhibitory effects of $1,25(\text{OH})_2\text{D}$ on adipogenesis may vary with

concentration of vitamin D and may possibly act in a stage-dependant manner, where it acts to inhibit commitment and the early stages of pre-adipocyte proliferation and differentiation, but enhances the later stages of differentiation and maturation (378,382–386). Nonetheless, this research highlights the significant role vitamin D may play in the differentiation of MSCs and consequently the development of the skeleton.

1.3.4.1.3.4 FGF23

The fibroblast growth factors are a family of proteins with a diverse set of functions, including embryonic development, organogenesis and metabolism. There are currently understood to be seven subfamilies of canonical FGFs, one subfamily of intracellular factors and one endocrine subfamily of FGFs. FGF23 is a member of the FGF19 endocrine subfamily which function as circulating hormones (106,387–389). Functionally, FGF23 is a factor which participates in the PTH-vitamin D-FGF23 axis, which acts to regulate both calcium and phosphate homeostasis (96). However, whilst PTH and $1,25(\text{OH})_2\text{D}$ act to increase uptake of both calcium and phosphate, FGF23 is a counter regulatory phosphaturic hormone which acts to remove the excess phosphate that accompanies PTH-stimulated bone resorption during calcium mobilisation. Proper phosphate homeostasis is required a range of important processes, including the normal development growth and functioning of bone. An endocrine feedback loop, including the combined action of the intestines, kidneys, parathyroid glands and the skeleton acts to maintain systemic phosphate control, critical for hydroxyapatite formation. Following an increase in serum phosphate or $1,25(\text{OH})_2\text{D}$, FGF23 is produced from osteocytes (102) which acts on the kidneys to increase phosphate excretion by downregulating the expression of cell surface sodium-dependant phosphate transporters NaPi-2a and NaPi-2c in the proximal tubule (390–395). FGF23 also acts to reduce circulating levels of active vitamin D by reducing the formation and increasing the degradation of the active form of vitamin D, $1,25(\text{OH})_2\text{D}$, preventing vitamin-D-mediated phosphate absorption in the gut (392). The final action of FGF23 is to reduce PTH secretion by the parathyroid glands which would otherwise stimulates osteoclastic bone resorption to liberate calcium and phosphate from the skeleton and increase the concentration of active $1,25(\text{OH})_2\text{D}$.

1.3.4.1.3.5 Oestrogen

Oestrogen is the major female sex hormone essential for sexual and reproductive development. Endogenous oestrogen exists in four forms, estrone, estradiol, estriol and estetrol, however estriol and estetrol are produced by the placenta and foetal liver respectively, therefore not detectable in non-pregnant women. Estrone is the primary sex hormone in post-menopausal women whereas estradiol has a significant role as the most potent female sex hormone in the development of female secondary sex characteristics (396–398). The physiological effects of the oestrogens on bone metabolism are mediated through oestrogen receptor α ($\text{ER}\alpha$) and beta ($\text{ER}\beta$) (399).

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The first indication of the importance of oestrogen for bone development in women originated from the work of Fuller Albright in the 1940s who identified that oestrogen deficiency as a result of either natural menopause or surgical ovariectomy resulted in bone loss which could be reversed by administration of estradiol (400). Oestrogen was subsequently found to be critical for the development and maintenance of the skeleton over the life course. As a period where a significant proportion of final peak bone mass is accumulated, puberty is a critical time for skeletal development. During puberty oestrogen has been seen in both males and females to promote growth hormone (GH) and IGF-1 activity, both of which play a central role in pubertal bone growth and closure of the growth plates (401). In females, oestrogen acts to maintain peak bone mass primarily through suppressing bone resorption through inhibition of RANKL-dependent osteoclast differentiation, corroborated by the rise of RANKL seen at the onset of menopause (402). Furthermore, oestrogen has been shown to protect against the autophagy or apoptosis of both osteocytes and osteoblasts and knockdown in a mouse model has been shown to compromise both bone mass and strength (403–405). The role of oestrogen in both the development and maintenance of the skeleton means that the postmenopausal drop in oestrogen can have significant effects on bone biology. Whilst ageing related bone loss contributes significantly to the cause osteoporotic fracture in both men and women, the rapid decline in oestrogen levels during the menopause leads to an accelerated resorption of bone which outpaces the rate of bone formation, leading to structural deterioration, increased fragility and fracture risk in women (406). This is borne out by the incidence of osteoporotic fractures in the United States of America (US) where 20% of men and 33% of women will expect to have an osteoporotic fracture in their lifetime (407,408).

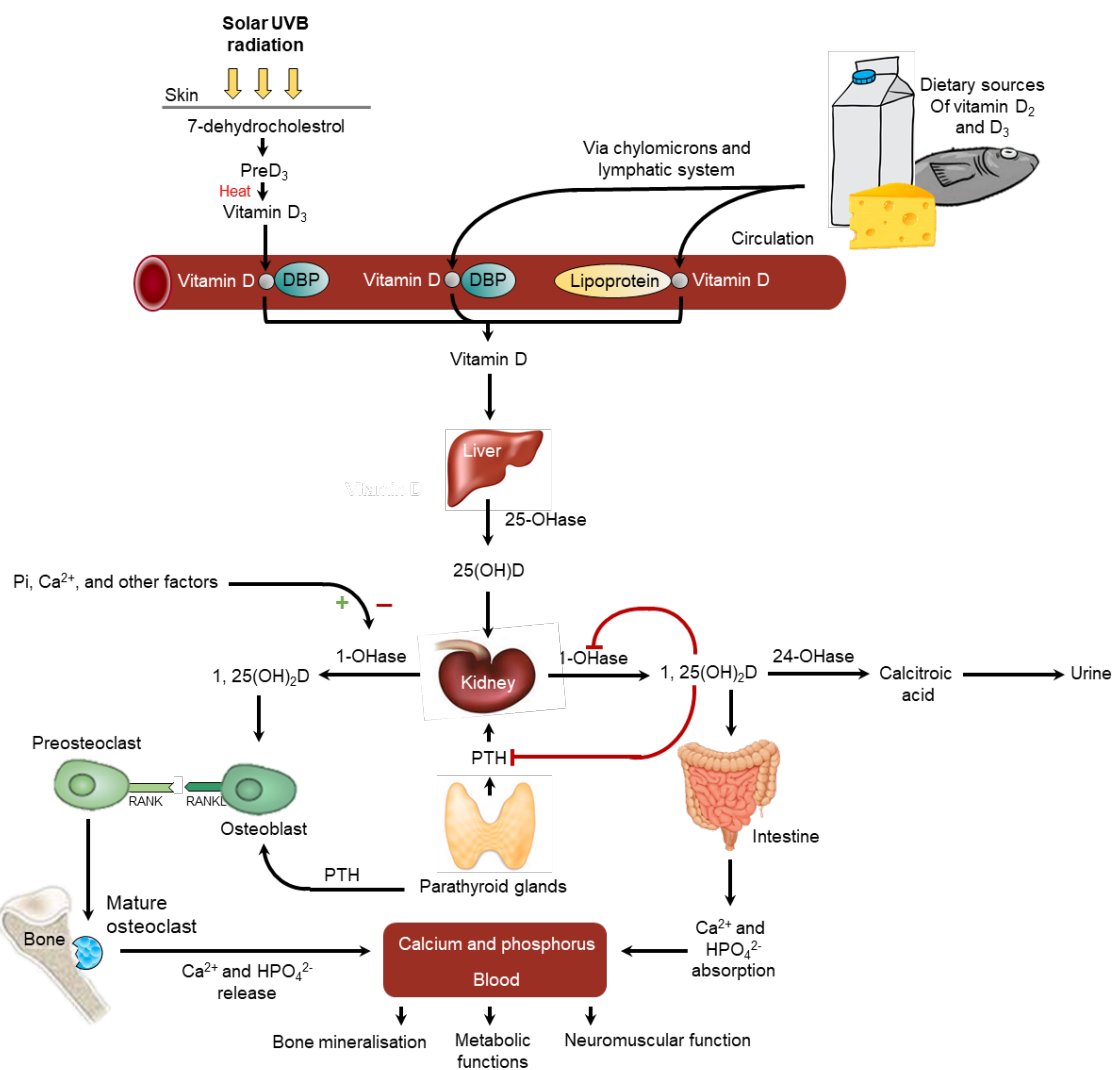


Figure 5. The combined actions of PTH and 1,25(OH)₂D in the maintenance of phosphorous and calcium homeostasis
 DBP, vitamin D binding protein; 1-OHase, 1 α -hydroxylase/CYP27B1; 24-OHase, 24-hydroxylase/CYP24A1; 25-OHase, 25-hydroxylase/CYP2R1; 25(OH)D, 25-hydroxyvitamin D₃; 1,25(OH)₂D, 1,25-dihydroxyvitamin D₃; PTH, parathyroid hormone; RANK, Receptor activator of nuclear factor- κ B; RANKL, Receptor activator of nuclear factor- κ B ligand.
 Adapted from Holick *et al.* (2006) (354).

1.4 Umbilical cord mesenchymal stromal cells

1.4.1 Development of the umbilical cord

The umbilical cord is the conduit which allows for the transport of nutrient rich, oxygenated blood from the placenta to the foetus and the return of deoxygenated, nutrient-depleted blood from the foetus to the placenta.

Formation of the umbilical cord begins between the 4th and 8th weeks of gestation wherein the expanding amnion envelops tissue from the yolk stalk (vitelline duct), the umbilical coelom and body stalk which is composed of extraembryonic mesoderm. By the 5th week blood flow is established through two umbilical veins which carry oxygenated blood to the foetal heart and two umbilical arteries which return oxygen-depleted blood back to the placenta. By the end of the 6th week the right umbilical vein is obliterated, resulting in a single persisting left umbilical vein. The allantois arises as a diverticulum of the yolk sac, extending from the early foetal bladder into the body stalk and contributes to umbilical vessel development. Between the 6th and 8th weeks the regression of both the allantois occurs, leaving the remnants between the two arteries within the cord. Between weeks 10 and 12 the extraembryonic mesoderm develops an extracellular matrix called the Wharton's jelly. The umbilical cord continues to lengthen in the second trimester to become approximately the crown to rump length of the foetus (409,410). Whilst it is not yet fully understood what determines the length of an umbilical cord, it has been suggested foetal movements *in utero* may be a determining factor (411). At term, the umbilical cord comprises two umbilical arteries and one umbilical vein embedded in the mucoid, proteoglycan-rich gelatinous Wharton's jelly, surrounded by a single layer of amniotic epithelium (412,413). Whilst the cross-sectional classification of the structure of the umbilical cord has gone through multiple iterations, *Davies et al.* (2017) attempted to pose a unifying model (413). The amnion epithelium, a layer 1-3 cells thick and the subamnion, a thin layer of Wharton's jelly below the subamnion together form the cord lining. Below the cord lining is the intermediate region, composed of a sparse matrix and cells and surrounding the vessels is the perivascular region which has been suggested to contain almost 45% of the cells in the Wharton's jelly (413,414) (Figure 6). The Wharton's jelly itself is a rich gelatinous extracellular matrix derived from the extraembryonic mesoderm composed largely of proteoglycans, specifically hyaluronic acid and chondroitin sulphate. The Wharton's jelly acts primarily as a structural support to umbilical vessels, protecting the cord from twisting or compressive forces which could potentially restrict blood supply to the foetus whilst also providing the necessary flexibility to allow for foetal movement *in utero*. In addition, when exposed to a significant temperature change such as at birth, the structure of the Wharton's jelly collapses, serving as a contributor to the physiological clamping of the cord (415–422).

This umbilical cord has been increasingly understood a rich source of experimental cell types. Hematopoietic, endothelial and endothelial progenitor cells and mesenchymal stromal cells have

been extracted from umbilical cord blood (423–426). From the umbilical cord tissue, epithelial stem cells have been extracted from the amnion epithelium, endothelial cells from the tunica media of the umbilical cord vein (HUVEC), smooth muscle cells from the walls of the three vessels and mesenchymal stromal cells from the Wharton's jelly (427–430). A study by *Wu et al.* (2018) showed from both human leukocyte antigen typing and karyotype analysis that mesenchymal stromal cells are genetically derived from the foetus, which in the context of this study may allow us to potentially gain a reflection of the environment *in utero* on the foetal methylome (431). Whilst HUVEC cells could also serve this purpose, Wharton's jelly MSCs have the same capacity for trilineage differentiation as BM-MSCs, meaning interrogation of this cell type would additionally allow us to explore the potential functional implications of methylation changes in this cell type *in utero* within tissues such as bone.

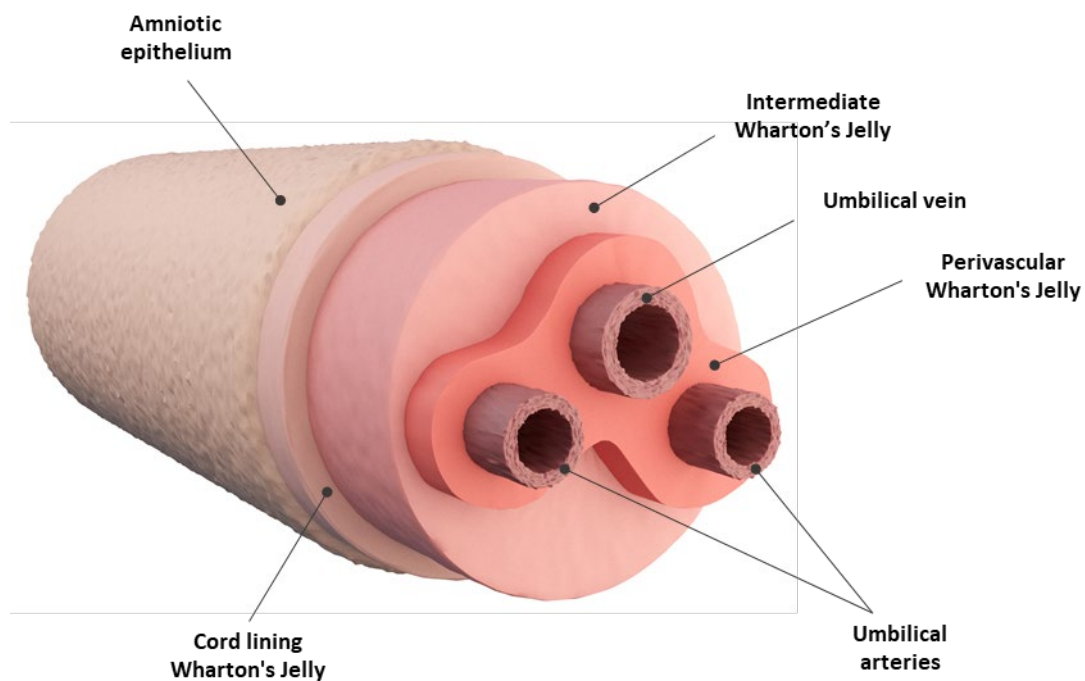


Figure 6. A three-dimensional cross-sectional of the human umbilical cord

A three-dimensional cross-sectional structure of the human umbilical cord based on a consolidated model proposed by and adapted from *Davies et al. (2017) (413)*.

1.4.2 Identification and classification of mesenchymal stem and stromal cells

Mesenchymal stem cells were first identified in 19th century experiments by *Goujon* (1869) where the heterotopic transplantation of bone marrow resulted in the *de novo* generation of both ectopic bone and bone marrow, an experiment later replicated by *Tavassoli et al.* (1968) (432,433). Subsequently in a series of experiments during the 1960s and 1970s (reviewed in (434)) Alexander Friedenstein found the osteogenic potential of bone marrow was associated with a minor sub-population of bone marrow cells. These cells were plastic-adherent, with colony forming unit fibroblast (CFU-F) capacity that held the ability to differentiate into multiple tissues including bone, cartilage and adipose tissue *in vivo* from the progeny of a single BM stromal cell (434–438). *Owen and Friedenstein* (1988) later proposed the concept of these cells as clonogenic, multipotent, self-renewing cells as part of the stromal microenvironment (439), later defined as “mesenchymal stem cells” for their ability to differentiate into various mesenchymal tissues (440,441). However the use of ‘stem cell’ when describing these cells is contentious (146).

A position paper from the International Society for Cell & gene Therapy (ISCT) by *Horowitz et al.* (2005) suggested that whilst mesenchymal stem cells do exist, the term should be reserved specifically for a population of cells with demonstrable progenitor cell functionality of self-renewal and differentiation supported by rigorous evidence from both *in vitro* and *in vivo* data. The ISCT has clarified in a past (442) and more recent position statement (443) the current criteria of plastic-adherent cells defined by the expression of a particular set of cell-surface makers and a tri-lineage differentiation potential (appendix 9.1) can be more accurately described as multipotent mesenchymal stromal cells. This population may include a heterogeneous mixture of fibroblasts, myofibroblasts and a small population of stem cells (438,443–446). Whilst heterogeneity can refer to a mixture of cells in a culture, cellular heterogeneity largely refers to differences in morphology and function among cell populations. Even in seemingly ‘pure’ cell populations, gene expression profiles can vary between cells due to variation within intrinsic regulatory systems and the extrinsic microenvironment. Heterogeneity in MSC populations has been reported between donors, between tissues from which the cells are extracted and within cell populations. Functionally, these differences can be reflected through reported differences in morphology, proliferation capacity, differentiation and transdifferentiation potential, immunophenotype and paracrine activity (447–455). Therefore it is suggested cells isolated under ISCT guidelines be prefixed with their tissue source and undergo a comprehensive *in vivo* and *in vitro* characterisation using if possible, a quantitative RNA analysis of select genes, flow cytometry of cell surface markers and protein analysis of the MSC secretome (456). Therefore from this point onwards, the abbreviation MSC will refer to mesenchymal stromal cells.

1.4.3 Extracting UC-MSCs

Using umbilical cord mesenchymal stromal cells (UC-MSCs) for the investigation of MSC function provides multiple benefits over that of other MSC types. Obtaining MSCs from a foetal source typically has ethical issues and obtaining MSCs from adult bone marrow typically provides a limited cell number and diminishing stemness *in vitro*. UC-MSCs however are obtained from umbilical cord, a sustainable source tissue typically regarded as medical waste with few ethical issues (457). Umbilical cord blood and tissue has also been identified as a good surrogate for tissues of mesodermal origin (458). With respect to the methylome, UC-MSCs have been identified as being derived from the foetus and also provide comparatively high yield from extraction from tissue, they have a high proliferative capacity and a greater differentiation potential when compared to bone marrow mesenchymal stromal cells. The umbilical cord has multiple compartments from which UC-MSCs can be extracted (412,459,460) (Figure 7), however cells are typically preferentially extracted from Wharton's Jelly, the tissue surrounding umbilical vessels for a number of reasons. These cells have shown the greatest differentiation potential, the fewest non-stem cell contaminants, they are rich in stemness characteristics and can be isolated in an extraction process which can be standardised (412,461). The presence of multiple cell types in the UC means the extracted cells need to be characterised carefully. The ISCT classifies stromal cells by the possession of three characteristics, firstly, the ability to adhere to plastic in standard culture conditions, secondly, $\geq 95\%$ of the MSC population express three specified positive markers and $\leq 2\%$ of the population express five specified negative markers (appendix 9.1). Finally, the cells require the multipotent differentiation potential to differentiate in to chondrocytes, adipocytes or osteoblasts as demonstrated by staining (446). These criteria have been suggested to produce heterogeneous, non-clonal stromal cultures with different multipotential properties based on donor site, isolation protocol and passage (462–464). This would be ameliorated by the ISCT recommendation of comprehensive *in vitro* and *in vivo* characterisation, however this may also be beyond the ability of this study. Within the context of this study, a novel selection of markers will be used to identify an UC-MSC population, similar other groups studying this cell type (459). Within the context of this dissertation, both Integrin Subunit Beta 1 (CD29) and CD44 were employed for the characterisation of MSCs isolated from umbilical cord and Thy-1 Cell Surface Antigen (CD90) for the enrichment MSCs isolated from umbilical cord. CD90 was selected due to the suggestion this marker is highly expressed on all MSCs irrespective of source, its capacity as a marker recommended to identify a positive fraction of MSC cells by the ISCT and its expression being correlated most strongly with an undifferentiated MSC cell type (446,465–467). Similarly, CD44 has been seen to strongly identify undifferentiated UC-MSCs and together with CD29 represent markers commonly used to identify MSC cells extracted from the Wharton's jelly of the umbilical cord (457,468–471).

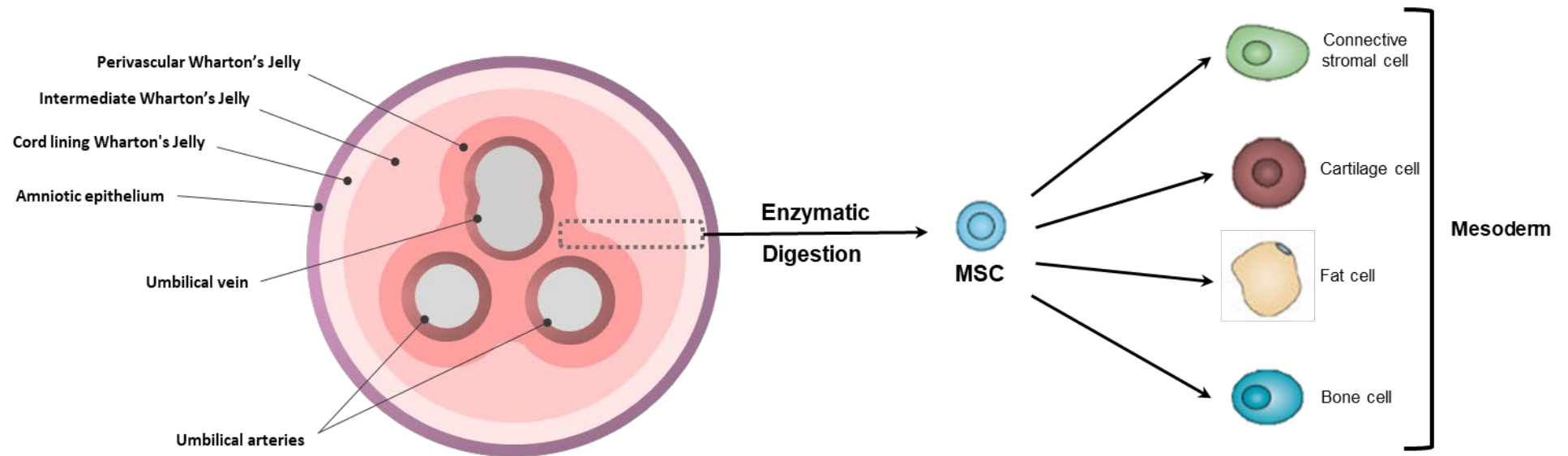


Figure 7. The differentiation potential of MSCs extracted from the Wharton's jelly area of the human umbilical cord

Depicted is a cross section of the human umbilical cord as proposed by *Davies et al. (2017) (413)*, the location from which UC-MSCs are typically extracted, the Wharton's jelly (WJ), or the interstitial area between veins and arteries. Adapted from *Arutyunyan et al. (2016) (459)*, *Davies et al. (2017) (413)* and *Uccelli et al. (2008) (460)*.

1.5 Genotype and environment

Bone strength and fracture risk in older adults is affected by the peak bone mass achieved in late adolescence, the ability to maintain that bone mass over the course of life and any subsequent loss of bone mass in adulthood (Figure 8) (219,220,472). Maximising peak bone mass is understood to protect against age-related bone loss and also acts as one of the best predictors of fracture risk later in later life. As such, a model developed by *Hernandez et al.* (2003) suggests a 10% increase in peak BMD could delay the onset of osteoporosis by up to 13 years (237). Between 50 and 85% of variance in peak bone mass density has been found to be genetically determined in twin and family studies (473–476). However, a meta-analysis of five genome wide association studies by *Rivadeneira et al.* (2009) showed that in a combined total of 19,195 European participants, only 3% of the variance in BMD was shown to be accounted for by genetic variation (477) and further studies by *Makovey et al.* (2007) showed subsequent loss of bone was not genetically determined (478). Additionally, meta-analysis of 30 genome-wide association studies identified 80 loci associated with variation in TB-BMD, but when combining all age groups and risk of fracture these loci only explain around 10% in variation (1). Therefore whilst there is some genetic influence, these findings together imply that the studied variations in BMD have not been explained by genetics alone (479,480).

Environmental influences such as physical activity and diet have been suggested to influence between 20%-40% of accumulated adult peak bone mass (9). Three-week old rats and eight-week old mice fed a diet supplemented with calcium over 30 days and vitamin D₃ over five weeks showed a concomitant increase in peak bone mass (481,482), however the effect of calcium and vitamin D supplementation in human adolescents is less clear (483–486). Animal models also show that caloric restriction in the diet of young, growing mice is associated with impaired skeletal acquisition and high marrow adiposity (487,488). Conversely, rats fed a high-fat diet experience down-regulation of homeobox A10, a factor essential in skeletal patterning (489). Collectively this suggests environmental factors play a role in determining the development of healthy bone.

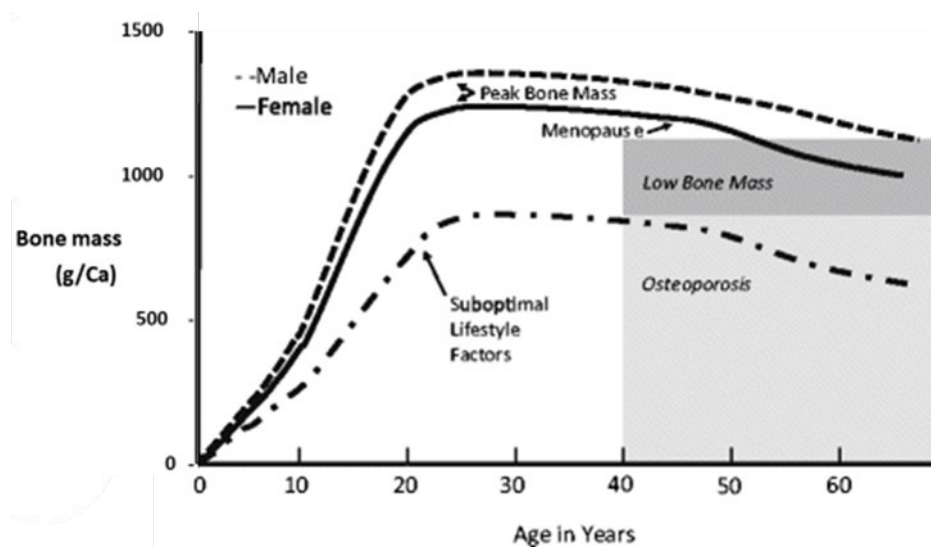


Figure 8. A theoretical, schematic bone mass in both males and females across the lifespan with optimal and sub-optimal life choices

Taken from *Weaver et al. (2016)* (9)

1.6 The Developmental Origins of Health and Disease

When examining epidemiological data from across England and Wales *Barker et al.* (1986) found that upon division of the country into 212 local authority areas, there was a strong, positive geographical correlation between the standardised rates of for infant mortality from 1921 to 1925 and the incidence of ischaemic heart disease around 50 years later. However, paradoxically whilst the rates of ischaemic heart disease in the UK were increasing with rising prosperity in the country, the incidence of the disease in the poorest areas and in the lowest income groups was twice that of more prosperous regions. This led *Barker et al.* (1986) to hypothesise that a poor nutritional environment in prenatal and early life increased the susceptibility of an individual to developing cardiovascular disease as the result of a more nutrient-rich affluent diet in later life (490–497). This was later confirmed by *Barker et al.* (1989) in a cohort of men from Hertfordshire, where those with the lowest birthweights had the highest death rates and conversely, those with the highest birthweights had the lowest death rates (490). This area of research developed rapidly, leading to the suggestion that foetal tissues and organs go through critical periods of developmental plasticity *in-utero* where the maternal environment potentially programmes the foetal tissue, altering patterns of hormonal secretion and metabolic activity (498–503). This theory was further developed to form the idea of the Predictive Adaptive Response, that if the postnatal environment was different from the environment predicated by the exposure *in utero* then a mismatch would occur, leading to maladaptation and an increase in disease risk (499,504). This work collectively helped to establish the developmental origins of health and disease (DOHaD) hypotheses which postulates that the environment during early development, including pre-conceptual, *in utero* and early postnatal periods may be consequential for offspring health later in postnatal life.

1.6.1 DOHaD & bone outcomes

Peak bone mass is a factor crucial to reducing the risk of diseases such as osteoporosis later in life, and while there is evidence from twin and family studies that peak bone mass is partly inherited, GWAS studies have shown that heritability only accounts for a small proportion of variation in bone mass and fracture risk. A meta-analysis of five GWAS studies of femoral neck BMD (FN-BMD) and lumbar spine BMD (LS-BMD) encompassing 19,195 subjects of Northern European descent showed that of 20 loci that reached genome-wide significance with respect to BMD. However, upon further examination combinations of these loci only explained ~2.9% and ~1.9% of the variance in LS-BMD and FN-BMD in the population respectively (477,505). Furthermore, a meta-analysis of 30 GWAS encompassing 66,638 individuals from across America, Europe, and Australia found 80 loci associated with TB-BMD, however similarly when combining all age groups and risk of fracture, these loci together explained around 10% of the variance in TB-BMD seen in the population (1). This suggests a potentially significant role for environment and indeed studies

have suggested lifestyle factors such as physical activity, smoking and nutritional intake may account for 20-40% variance in peak bone mass (9,506,507). In addition to this, there is growing evidence that both the maternal environment as well as exposures in early infancy also play a role in the observed variations in peak bone mass (508–510).

1.6.1.1 Human models

The earliest epidemiological evidence for the influence of the early-life environment on postnatal bone growth originated from a study examining the birth and school records of a cohort of 153 women from Bath UK, born between 1968 and 1969. This study observed statistically significant associations between weight at one year of age and BMC at 21 years of age, independent of both adult weight and BMI. These findings were subsequently replicated in a larger cohort of both men and women from Hertfordshire, UK (510–512). Significantly, upon adjustment of these models for lifestyle factors that may have influenced bone mass later in life such as physical activity, dietary calcium intake, cigarette smoking and calcium consumption these associations remained, indicating these associations may account for some of the variance seen in postnatal bone growth irrespective of lifestyle. These initial observations were replicated by later studies that further characterised the associations between birthweight, BMC and BMD in later life and fracture risk (513–519), including twin studies (520).

Subsequent work initially focused on possible physiological determinants of bone growth later in life which may have been defined at birth. These studies examined the concentration of both growth hormone and cortisol with bone mineral density in adult men and women. GH was selected for its role in the promoting the secretion of IGF-1, a major regulator of growth in late infancy. Cortisol however is an endogenous glucocorticoid and was selected for its potential similarity to exogenous glucocorticoids which are known to have deleterious effects of on bone cell function, bone remodelling and rate of bone loss. Investigation by *Fall et al. (1998)*, *Dennison et al. (1999)* and *Phillips et al. (1998)* found birthweight to be predictive of the circulating concentrations of GH and cortisol in adulthood and suggested they may have been factors in determining the rate of bone loss, with the associations seen between cortisol concentration and bone density in remaining significant after adjustment for lifestyle factors such as cigarette smoking, alcohol consumption, dietary calcium intake and physical activity (521–523). Together, these studies provided evidence that the trajectory of bone growth into adulthood may already be partially determined in early postnatal life, irrespective of the influences of lifestyle in later life.

A later study by *Godfrey et al. (2001)* links between maternal lifestyle and offspring postnatal skeletal development, identifying associations between neonatal bone mass, birth weight, birth length and placental weight with DXA measures of bone mass at birth, maternal parity, fat stores and walking speed in late pregnancy, independent of the infants gestational age and gender. Maternal smoking and a lower energy intake at 18 weeks was found to be negatively associated

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with neonatal whole-body BMC and BMD (524,525). This work was subsequently replicated in children at 9 years of age, which similarly found reduced maternal height, maternal smoking, reduced maternal fat stores in late pregnancy, lower preconception weight and reduced maternal height were all associated with a reduced whole-body BMC in children at the age of nine years (526). A study by *Harvey et al.* (2012) in the Southampton Women's Survey (SWS) cohort found that while late pregnancy foetal abdominal circumference was related to bone mass at birth, early pregnancy growth was more strongly related to bone mass at four years of age (524,527). An adverse environment in during pregnancy and in early postnatal life has also been linked to an increased risk of fracture, with maternal height and a low rate of childhood growth found to be two important determinants for hip fracture risk, controlling for age and gender (514). Taken together, these studies demonstrate the important role that the prenatal environment can play on postnatal skeletal development.

1.6.1.2 *Animal models*

Whilst human studies had provided the first suggestions that the prenatal and early life environment could be influential for postnatal skeletal development, later experimental animal models allowed for a corroboration and further exploration of these findings. One of the earliest models established by *Mehta et al.* (2002) showed that when pregnant rats were fed a low-protein diet, the offspring exhibited a reduced bone area and BMC, but not BMD and an altered growth plate morphology in adulthood. The authors posed that the changes in BMC and bone area (BA) but not BMD in the offspring of protein-restricted dams suggested that bone size, rather than volumetric bone density is the primary outcome of the protein restricted diet, corroborating previous findings in initial human studies (510,511,528). Later, much larger sequential studies by *Lanham et al.* (2008) found in a similar, protein-restricted rat model specifically had a deleterious effect on the bone environment manifesting as significant differences in bone structure and density at various sites (529–531). Similar structural changes were also seen in the offspring of a microswine model subjected to a maternal low protein nutritional challenge (532). In contrast, a study within a mouse model where dams were fed a high fat diet lead to increased adiposity and an altered trabecular structure within the femurs of the offspring (533). This not only corroborates the finding in human studies that maternal influences during pregnancy can influence postnatal bone growth of offspring, but identifies that the intrinsic trabecular structure of the bone can be changed and that different nutritional divergences can lead to alterations in the postnatal development and structure of the offspring skeleton.

1.6.2 *Epigenetics*

Underpinning DOHaD is epigenetics, which is heritable, stable, but potentially reversible changes in gene expression without changes of the underlying DNA nucleotide sequence (59). Epigenetics

has been suggested to be potentially influenced by environment, genotype and stochastic perturbations.

1.6.2.1 *Epigenetic mechanisms*

1.6.2.1.1 *DNA methylation*

DNA methylation is a process essential for silencing retroviral elements in the genome, regulation of tissue-specific gene expression, genomic imprinting and X chromosome inactivation. During development, the genome goes through dynamic periods of both demethylation and methylation, establishing largely stable and unique DNA methylation patterns in differentiated tissues.

However, the stability of these marks is subject both to developmental mutations or environmental influences. Around 60-80% of cytosine-phosphate-guanine (CpG) dinucleotides are heavily methylated in somatic cells, particularly within intergenic regions of the genome which acts to stop expression of transposable and viral elements potentially deleterious to the genome. In contrast, approximately 7% of CpGs in the genome are located in CpG islands which are clusters of largely unmethylated CpGs associated with approximately 70% of annotated gene promoters. CpG methylation in a gene promoter is typically associated with transcriptional repression of gene expression as a result of methylated cytosines blocking transcription machinery from binding to the promoter site, however the effect of gene body or intragenic methylation on transcriptional activity is less clear (534–536).

1.6.2.1.1.1 *Regulating DNA methylation*

DNA methylation involves the transfer of a methyl group from S-adenyl methionine (SAM) to the 5-position of cytosine to form 5-methylcytosine (5mC) in a cytosine-phosphate-guanine dinucleotide, a process catalysed by DNA methyltransferases (DNMT). All members of the DNMT family are required in development. DNMT1 is largely involved in the maintenance of DNA methylation, repairing DNA methylation marks and acting during DNA replication to transfer methylation marks from the parental DNA strand onto the newly synthesised daughter strand to maintain the original pattern of DNA methylation in a cell lineage (537–539). In contrast, both DNMT3A and DNMT3B establish methylation patterns on unmodified DNA and as such are known as *de novo* DNMTs. Knockout of DNMT1 has shown embryonic lethality in mice at days 8-10.5, at which point embryos exhibit a two-thirds loss of methylation in addition to apoptotic cells in a number of tissues. Similar to DNMT1, DNMT3b knockout is embryonically lethal, but DNMT3a mice survive until around 4 weeks after birth, highlighting the crucial role of these factors during development (540–542).

Demethylation of these methylated CpG sites can occur in both an active and passive manner. DNA can become passively demethylated by dilution of the 5mC mark through several rounds of DNA replication due to the absence of DNA methylation maintenance activity following

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replication. Alternatively, active demethylation can occur through the iterative oxidation of 5mC by ten-eleven translocation (TET) dioxygenase enzymes through multiple intermediates to 5-carboxylcytosine which is excised by the base-excision repair (BER) pathway (543).

1.6.2.1.2 Histone modification

At the most basic level DNA wraps around and complexes with a histone protein octamer to form a nucleosome, a single unit of chromatin. Chromatin conformation is important in determining transcriptional regulation of DNA expression. The compacted form of chromatin, heterochromatin physically blocks access of transcription factors and therefore gene expression, whereas euchromatin is loosely packaged allowing for access and so subsequently gene transcription to occur (544). Histone modifications control the transition between these two states based on post-translational modification of N-terminal tails protruding from the nucleosomes. This causes changes in chromatin structure and so regulates the access of transcription factors to the target gene promoters. Among other modifications, histones can be post translationally ubiquitinated, phosphorylated, sumoylated, methylated and acetylated which depending on location can each have a different effect on DNA transcription.

1.6.2.1.3 Non-coding RNAs (ncRNAs)

Despite only 1-2% of the genome encoding for proteins, the Encyclopaedia of DNA Elements (ENCODE) project found 74.7% of the human genome has functional activity (545). The portion of these functional elements transcribed into RNA without coding for proteins are termed ncRNAs. This group is further sub-divided into two categories, small and long ncRNAs (lncRNA). Small ncRNAs are shorter than 200 nucleotides and include among others, microRNAs (miRNAs), short interfering RNAs and short hairpin RNAs.

1.6.2.1.3.1 MicroRNAs

MiRNAs are short, single stranded ncRNAs around 20 nucleotides long that post-transcriptionally regulate gene expression by binding to mRNAs. Briefly, mature miRNAs regulate gene expression through association with an Argonaute protein inside a miRNA-induced silencing complex, which binds to the 3'-untranslated region of mRNAs. Depending on the degree of complementarity between the two, the mRNA will be either translationally suppressed or targeted for degradation (546,547). MiRNA have been seen to play roles in cell proliferation, differentiation, programmed apoptosis and cell death. It has also been suggested that over 60% of human protein coding genes are targets for miRNAs, therefore these ncRNAs have the potential to be involved in the regulation of expression for a wide array of genes (548).

1.6.2.1.3.2 Long non-coding RNAs

Long noncoding RNAs are non-protein-coding transcripts longer than 200 nucleotides that typically play a role in cellular proliferation, differentiation, migration and development (549,550). The exact mechanism by which the majority of lncRNAs act is not yet clear, but lncRNAs have been suggested to regulate transcription in *cis* and *trans*, organise nuclear domains, regulate protein and RNA and initiate enhancers or promoters. However they may not perform sequence specific functions (551,552).

1.6.3 Epigenetics and the in-utero environment

1.6.3.1 Human models

The effect of environment on postnatal phenotype may in part be mediated by epigenetic processes, one of the most commonly studied of which is DNA methylation (324,325). One of the initial studies indicating the influence of epigenetic modifications *in utero* comes from the Dutch hunger Winter. The Dutch Hunger Winter was a famine which arose in the Netherlands in the Winter of 1944-45 during which registries and health care remained intact, meaning those who were exposed to this famine prenatally could be traced and as such provide an insight into the effects of a nutritional insult *in utero* on the epigenome of offspring born to women pregnant during this period. The availability of data such as this not only allows us to examine trends in the health of those exposed to the famine in prenatal life over time, but also to build statistical models that can account and adjust for factors that may otherwise confound an analysis, such as sex or age.

In humans, IGF-2 is a paternally imprinted hormone promoting growth and development during gestation. *Heijmans et al.* (2008) aimed to compare the methylation of 5 CpG sites within the *IGF-2* gene in 60 famine-exposed individuals to their unexposed, same-sex siblings. The authors found all but one CpG sites significantly differentially methylated when exposed to famine during the periconceptual period when compared to their same-sex sibling almost 60 years later (553). This study provided significant evidence that transient environmental conditions early in human gestation can be recorded by persistent changes in epigenetic state.

Similarly, a study by *Wang et al.* (2020) investigated 1959–1961 Chinese great famine and whether DNA methylation within the insulin receptor (*INSR*) gene, activated by IGF-1 and IGF-2, mediated the association of prenatal exposure to the Chinese great famine with adulthood waist circumference and BMI. The authors randomly selected subjects from provinces severely and moderately affected by the famine and indeed found prenatal exposure to famine was associated with higher overall methylation of *INSR* and a greater waist circumference. However more significantly methylation of a single CpG site was found to mediate 32% of the association between prenatal exposure to the Chinese great famine and adulthood waist circumference (554). Further to previous studies, this study not only shows a distinction in the methylation profile of the *INSR* gene for those severely and moderately impacted by famine *in utero*, but

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manages to identify the contribution of a single methylation change to a phenotypic outcome potentially induced by an adverse environment *in utero* to a phenotypic outcome later in life.

1.6.3.2 Animal models

Animal models have been critical in demonstrating a causal relationship between changes in the foetal environment and epigenotype. DNA methylation is an epigenetic modification that can significantly influence the expression or repression of genes and subsequently phenotype. It has also been increasingly shown to be influenced by the early life environment. A classic demonstration supporting the role of the epigenetic regulation of gene expression via nutrition *in utero* is the agouti mouse model. Agouti yellow mice are typically obese and yellow as the result of a retrotransposon upstream of the agouti gene, promoting constitutive ectopic expression of a gene not typically expressed. It was found when fed dietary methyl donors and co-factors such as vitamin B12, choline, betaine and folic acid before, during and after pregnancy the mice gave birth to thin, brown offspring whereas control animals' offspring remained obese and yellow. This was associated with methylation of a retrotransposon at the 5' end of the Agouti allele correlated inversely with coat colour, glucose tolerance and tumour susceptibility among littermates (555–557). This model provides one of the first indications that epigenetic modifications in the mother could be passed along the female germ line to be inherited by the offspring.

Environmental constraint was already understood to be associated with an increased risk of disease later in life, suggesting the occurrence of a persistent alteration in gene transcription. *Lillycrop et al.* (2005) aimed to investigate the causal mechanism by which unbalanced prenatal nutrition may result in altered gene expression, and the potential role of methylation in the process. The authors investigated the effect of an unbalanced maternal nutrition on methylation status of both the peroxisomal proliferator-activated receptor (*PPAR*) genes and glucocorticoid receptor (*GR*) gene in rat offspring after weaning. In doing so, they found peroxisomal proliferator-activated receptor alpha (*PPARα*) gene methylation to be 20.6% lower and expression 10.5x higher in pups born to dams on a protein restricted diet and similarly GR methylation to be 22.8% lower and expression 3x higher in pups born to dams on a protein restricted diet (558). Further work also established the capacity for phenotypes induced as a result of these changes to be transmitted across generations (559). This work established gene-specific epigenetic regulation of gene transcription *in utero* as a mechanism for foetal programming.

1.6.4 Epigenetics and bone

Epigenetic influences *in utero* been shown to be potentially significant in the development and regulation of bone development in postnatal life (560,561). A study by de *Andrés et al.* (2013) extracted a range of cell types including human foetal bone cells (HFBC) from developing foetal femurs to examine the DNA methylation and the expression of genes important in the formation and remodelling of bone, such as inducible nitric oxide synthase (*iNOS*) and collagen type IX

(*COL9A1*). Knockout studies in the mouse calvarial preosteoblastic MC3T3-E1 cell line have shown nitric oxide may play a role in bone formation and resorption and other studies have implicated the factor in bone healing, bone development and bone loss. In addition, it has been shown that the lack of collagen IX seriously affects growth plate organization, especially in young animals. Over the course of foetal development, the authors saw a decrease in the methylation of CpGs in the promoter region *iNOS* within HFBC DNA correspond with an increase in expression, a change which inversely correlated with foetal foot length as a determinant for age. Similarly, *COL9A1* gene expression was seen to correlate with loss of methylation within the *COL9A1* promoter in HFBC DNA during foetal femur development, a change that correlated with foetal foot length (562). This showed these genes required for proper skeletal development may be at least in part regulated by epigenetic mechanisms over the course of development *in utero*. This suggests environmental influences *in utero*, determined by maternal lifestyle, diet, body composition amongst other factors may play a significant role in the development of foetal bone.

1.6.4.1 *Epigenetics and the regulation of MSC commitment*

As discussed previously, the early life environment has been seen to effect change on the epigenome through epigenetic processes such as DNA methylation. Epigenetic processes such as DNA methylation have also been seen to play a significant role in regulating the commitment and differentiation of MSC cells, so may provide a plausible mechanism by which the early life environment may also influence bone development. DNA methylation, non-coding RNAs and histone modifications have all been seen to play a role in the epigenetic regulation of lineage commitment and differentiation of MSCs (546).

1.6.4.1.1 *DNA methylation*

DNA methylation is an epigenetic modification that can significantly influence the expression or repression of genes and subsequent phenotype. *Zhou et al.* (2009) found that treatment of MSCs with the demethylating agent 5-Azacytidine for 24 hours prior to culturing in osteogenic media lead to a significant upregulation in osteogenic differentiation and the expression of osteogenic genes such as *DLX5*, a factor essential for the BMP2 mediated osteogenesis of BM-MSCs (563,564). Significantly, *Zhang et al.* (2011) showed during the osteogenic differentiation of adipose tissue derived MSCs that upregulation in the expression of both *RUNX2* and *Osx* was significantly correlated with a decrease in the methylation of the promoter regions of these genes (565). Furthermore, inhibition of DNA demethylase enzymes blunted the expression of both genes, suggesting a significant role for DNA methylation in the osteogenic differentiation of MSCs (565).

1.6.4.1.2 *ncRNAs*

1.6.4.1.2.1 Long non-coding RNAs (lncRNAs)

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A study by *Qiu et al.* (2017) showed the osteogenic differentiation of BM-MSCs led to the upregulation of 433 lncRNAs and the downregulation of 232 lncRNAs, one of which was seen to promote osteogenic differentiation when downregulated and suppress osteogenic differentiation when upregulated (549). The lncRNAs, maternally expressed gene 3 (MEG3) and H19 imprinted maternally expressed transcript (H19) have both been seen to inhibit osteogenic differentiation of MSCs through interaction with the BMP4 and BMP9 respectively, both of which play a significant role in the regulation of bone development (566,567). Furthermore, osteogenic differentiation of a foetal osteoblastic cell line led to a decrease in the lncRNA antidifferentiation noncoding RNA (ANCR), whereas overexpression of ANCR promoted osteoblast differentiation through modulating the expression of enhancer of zeste homolog 2 (EZH2) and the RUNX2 expression (568).

1.6.4.1.3 MicroRNA (miRNA)

MiRNAs have been shown to play a significant role in osteoblastogenesis. Cytosolic RNase III (DICER) is responsible for the maturation of pre-miRNAs to mature miRNAs. Inactivation of DICER leads to irregularities in miRNA cleavage resulting in mice with skeletal defects and premature death. This suggests miRNAs are essential for skeletal development (569). Additionally, miR-20a has been seen to facilitate osteodifferentiation of MSCs by its action on the *BMP-RUNX2* pathway and miR-204 and 138 have been seen to inhibit osteogenesis by the repression of *RUNX2* and *Osx* genes (546). MiRNAs have also been shown to have direct interactions with the pathways previously discussed as pivotal to osteoblastogenesis (section 1.3.4.1.2). MiRNA activity exerts both positive and negative influences on these pathways through the post-transcriptional control of their specific target genes. Examples of these interactions include both the Wnt and BMP signalling pathways, both integral to skeletal development. In a mouse model, inhibiting glycogen synthase kinase 3 (GSK-3), an inhibitor of the Wnt canonical signalling pathway has been shown to enhance Wnt signalling and increase bone mass in the mice after 14 days of treatment with an inhibitor (570). GSK3 exists in two isoforms, GSK-3 α and GSK-3 β . A study by *Wang et al.* (2013) found miR-346 can bind to the three prime untranslated region (3'UTR) of GSK-3 β mRNA and as such overexpression of miR-346 both increased Wnt signalling activity and promoted osteoblastogenesis in human BM-MSCs (571). Additionally, miR-21 was later shown by *Meng et al.* (2015) to promote the phosphorylation of GSK-3 β in human UC-MSCs, leading to both an increase in the stabilisation and accumulation of a high concentration of β -catenin in the cytoplasm and enhanced expression of *RUNX2* (572). In contrast, miR-214, identified as significantly downregulated during osteogenic differentiation in a number of studies has been seen to inhibit BMP-driven osteogenic differentiation of human BM-MSCs. MiR-214 does so by binding to the 3'UTR of BMP-2 to inhibit its expression, a finding further validated through the discovery that the lncRNA KCNQ1OT1-mediated upregulation of BMP2 expression occurs through the inhibition of miR-214 (573). Later studies also identified miR-214 overexpression in BM-MSCs

inhibited FGF and TGF- β signalling, along with a number of factors integral to the osteoblastic differentiation of MSCs such as RUNX2, COL1A1, ALP and OCN (574,575). This demonstrates the role and potent effect miRNA activity can have on the signalling and regulatory pathways required for osteoblastic differentiation and highlights nature of the process as one with many interacting components.

1.6.4.1.4 Histone modifications

Histone acetylation involves histone acetyltransferase (HAT) or histone deacetylase (HDAC) mediated addition or removal of an acetyl group to the lysine residues in the N-terminal tails of histones. This leads to a change in the overall charge of the histone, weakening the interaction with DNA and allowing greater access to transcription factors (463,576). Acetylation has been seen to play a significant role in osteogenic differentiation of MSCs, with H3K9 acetylation required for osteodifferentiation and the acetylation of H3 and H4 required for osteocalcin induction (577,578).

Histone methylation generally involves the transfer of methyl groups to lysine residues of histone proteins. The lysine residues can be either mono-, di- or tri- methylated by the transfer of methyl groups from S-adenosyl methionine to lysine or arginine residues via a histone methyltransferase (HMT) (579). Histone modification has been shown to be of particular importance in the lineage commitment of MSCs and therefore the differentiation into osteoblastic cells, making these enzymes key for the study of bone loss related diseases such as osteoporosis. Of particular interest for bone are SET-domain containing histone lysine methyltransferases (KMTs) and JmJc containing histone demethylases (KDMs), specifically KDM6A/B and EZH2 (579).

Lesser studied but important histone modifications are both histone phosphorylation and ubiquitination. Phosphorylation of H2B, one of the four main histone proteins supporting chromatin structure has been shown to lead to the recruitment of EZH2 and facilitate adipogenesis through increases in H3K27me3 marks in the promoters of Wnt genes and subsequent repression of Wnt gene expression (580). Conversely, histone ubiquitination has recently been identified as having a role in the differentiation of osteoblasts *in vitro*. *In vivo*, conditional deletion of the histone 2B ubiquitin ligase Ring Finger Protein 40 (RNF40) in a mouse model led to impaired bone formation and revealed RNF40 is required for the earlier stages of osteoblastic commitment but dispensable for the development of mature osteoblasts. However interestingly RNF40 was required for the active expression of the gene encoding RANKL, Tumour Necrosis Factor Superfamily Member 11 (*Tnfsf11*) in mature osteoblasts. Cells of an osteoblastic lineage in the mouse model failed to express *Tnfsf11* and as such failed to activate osteoclasts, leading to reduced bone resorption and overall increases in bone mass (581). Whilst both may be less frequently studied histone modifications, this does suggest both histone phosphorylation and

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ubiquitination as potentially significant factors in regulating the differentiation of MSCs and mature osteoblast biology.

1.6.4.2 Differential methylation as a marker of postnatal bone growth

A number of significant methylation changes have been found in birth tissues associated with postnatal bone development. Initially, studies by *Godfrey et al.* (2011) and *Lillicrop et al.* (2017) identified perinatal differential methylation within regions within the retinoid X receptor- α (*RXR α*), endothelial nitric oxide synthase (*eNOS*) and Cyclin-dependant kinase N2A (*CDKN2A*) genes in birth tissues which associated with postnatal adiposity of the offspring (582,583). Given the reciprocal relationship between fat and bone development seen in both epidemiological and *in vitro* studies, further investigation was conducted into the potential association between the differential methylation of all three genes with offspring postnatal bone measures (584,585). In umbilical cord tissue from 332 SWS mother-baby pairs *Curtis et al.* (2017) identified perinatal methylation at the *CDKN2A* locus was inversely associated with BMC, BMD and whole body minus head bone area in offspring at both 4 and 6 years of age (2). Further to this, a study by *Harvey et al.* (2012) in umbilical tissue from 66 Princess Anne Hospital cohort (PAH) mother-baby pairs showed a strong positive association between perinatal methylation status of a CpG site within *eNOS* and offspring whole body bone area, BMC and areal bone mineral density (aBMD) at 9 years of age (586). Analysis of umbilical tissue from 230 SWS mother-baby pairs also found higher methylation of 4 out of 6 CpG sites in the *RXR α* promoter associated with lower BMC, with one CpG also negatively correlating with maternal free 25-hydroxyvitamin D₃ (25(OH)D) status (3,488). The mechanistic relationship of these methylation changes with bone development have yet to be determined. However, *RXR α* is a cofactor required for the nuclear activity of vitamin D, a factor essential for proper skeletal development. Furthermore, a study by *Sabani et al.* (2008) showed the genetic disruption of all three nitric oxide synthases, one of which is required for the expression of *eNOS*, led to an increased BMD and bone turnover when knocked out in 12-week old mice (587). Finally and there is evidence cyclin-dependent kinases such as *CDKN2A*, plays a role in skeletal development, homeostasis and bone cell activity (2). Therefore there is some indication of a biological relationship with skeletal development, however if functionally independent from skeletal development and simply methylation changes occurring as the result of a bystander effect, these marks still provide promise as biomarkers. A potential prospective biomarker indicating trajectory of bone growth in early life may allow for an intervention to be taken in early life, which may prevent a deficit in peak bone mass and as such reduce the incidence risk for osteoporosis in the future.

1.7 Vitamin D

Vitamin D has been suggested to directly or indirectly interact with around 3-10% of the genome. One of the cofactors required for the nuclear activity of 1,25(OH)₂D, VDR, has been estimated to

have 2000-8000 chromosomal target sites in at least 38 cell types in the human body and has been suggested to regulate between 100-1250 genes (588–593). Physiologically, maternal vitamin D insufficiency during pregnancy has been related to adverse outcomes for both the neonate and the mother. Adverse outcomes for offspring can include pre-term birth, low birthweight, higher risk of being small for gestational age, respiratory tract infections, asthma, low immunity and autism. Maternal repercussions of vitamin D insufficiency during pregnancy have been seen to include obstetric complications such as an increased risk of gestational diabetes mellitus, preeclampsia, caesarean section delivery and bacterial vaginosis (594).

1.7.1 *The role of vitamin D in pregnancy*

Maternal lifestyle influences such as diet, smoking and physical activity during pregnancy have been shown to have the capacity to impact on the postnatal skeletal development of offspring (595). With respect to a maternal diet during pregnancy, a study by *Cole et al.* (2009) of 198 mother-children pairs showed an 11% increase in BMC for children at 9 years of age whose mothers were in the upper quartile of those with the most 'prudent' diet during the late stages of pregnancy, a diet characterised by an elevated intake of fruit, vegetables, wholemeal bread, rice, and pasta and a low intake of processed foods (595). Further work by *Yin et al.* (2010) in a cohort of 216 adolescents also showed maternal diet, including intake of milk, fat and magnesium during the third trimester of pregnancy was predictive of BMD at age 16 (596). It has also been long known that a sustained deficiency of nutrients such as calcium in pregnant women can produce skeletal defects in their offspring at birth (103). In addition, vitamin D has been long established to have a relationship with bone growth. During pregnancy maternal 25(OH)D readily crosses the placenta (597–599) to allow the foetus to synthesise its own 1,25(OH)₂D (597,600,601) meaning maternal vitamin D status will have direct implications for vitamin D bioavailability for the foetus. A study of 1200 children showed neonates can be born vitamin D deficient, which may have significant consequences for health in early life (602). Subsequently, depending on the severity of the insufficiency or deficiency of vitamin D in the mother, maternal vitamin D status has been implicated in a range of conditions including rickets, foetal growth restriction and metabolic conditions such as excess adiposity and insulin resistance in the offspring in both human and animal models (600,603–606). However, alarmingly a meta-analysis has shown 54% of pregnant women and 75% of new-borns worldwide have a vitamin D insufficiency (<50 nmol/l), whereas 18% of pregnant women and 29% of new-borns worldwide show a severe deficiency (<25 nmol/l) (607) indicating the need for a deeper mechanistic understanding of the role of vitamin D *in utero*.

Significantly in animal studies, a study by *Wen et al.* (2018) demonstrated that in a rat model where mothers were vitamin D deficient, the offspring of the vitamin D-deficient rats had a higher weight when compared to control offspring (608). Combined DNA methylation and ribonucleic

acid sequencing (RNA-seq) showed that of the genes that were differentially methylated, 141 genes were differentially expressed in the offspring of the vitamin D deficient group (608).

1.7.2 Maternal vitamin D and bone

The biologically active form of vitamin D, 1,25(OH)₂D, is responsible for the maintenance of calcium and phosphorous homeostasis, the support of metabolic functions such as bone development and mineralisation, neuromuscular function, cellular proliferation and differentiation. 1,25(OH)₂D exerts its effects through the vitamin D receptor. Vitamin D receptor loss of function mutation in humans and VDR-null mice develop skeletal deformities, hypocalcaemia, hypophosphatemia and osteomalacia (609). A chromatin immunoprecipitation sequencing (ChIP-seq) and RNA-seq study by *Meyer et al.* (2016) also demonstrated on a genomic level that the addition of 1,25(OH)₂D to MSCs can induce a large, genome-wide increase in VDR binding events from 1,752 to 10,557 and a statistically significant upregulation of 173 genes when compared to vehicle (610). Vitamin D therefore has a significant effect on bone biology on a physiological and transcriptomic level.

Maternal 25(OH)D status in both early and late pregnancy has been linked to offspring bone outcomes, including in later childhood. Whilst vitamin D deficiency is commonly linked to conditions of the skeleton such as rickets, congenital rickets typically only occurs in infants born to mothers with severe vitamin D deficiencies and osteomalacia (611,612). However, studies have shown a correlation between impaired foetal bone ossification in full term infants with maternal vitamin D deficiency (613,614). In a cohort of 424 pregnant women from the SWS study, *Mahon et al.* (2010) used non-invasive, high-resolution 3D ultrasound to measure the ratio of foetal femoral metaphyseal cross-sectional area to femur length (femoral splaying index) at 19 weeks gestation. Foetal femoral splaying, which can indicate a deficiency in mineralisation was shown to be highest in the mothers vitamin D deficient and lowest in those vitamin D sufficient, indicating maternal vitamin D insufficiency may influence foetal femoral development as early as 19 weeks' gestation (615). However during late pregnancy when the most significant mineralisation of the foetal skeleton has been suggested to take place, *Javaid et al.* (2006) showed an association between low maternal 25(OH)D in late stage pregnancy and reduced whole body BMC and BMD in offspring at 9 years of age independent of social class, body size and diet (616). A study of 198 mother-offspring pairs found maternal vitamin D status resulted in improved bone outcomes at 9 years of age (616) and the longitudinal RAINE study investigating 341 mother-offspring pairs similarly found positive associations between maternal vitamin D status and offspring bone mass at 20 years of age (488). Furthermore, as the predominant source for vitamin D worldwide, estimated maternal exposure to sunlight or more specifically UVB radiation during pregnancy has shown a positive correlation with offspring BMC at 9 years (617,618) and exposure during late pregnancy along with vitamin D supplementation predicted maternal 25(OH)D concentration and

childhood bone mass. This work collectively suggests a potentially significant influence of maternal vitamin D status during pregnancy on postnatal offspring bone development.

However significantly, studies have also demonstrated no association between maternal 25(OH)D status and offspring bone outcomes. A 3960 mother-offspring pair study found no relationship between maternal vitamin D status during pregnancy and offspring BMC in late childhood (619). Additionally a study by *Morales et al.* (2015) of 1498 mother-child pairs found no association between maternal vitamin D deficiency and offspring growth, however they did find an association between 25(OH)D deficiency and increased risk of being overweight at 1 year (620), a finding reproduced in a murine model (608). Further studies also found no association between maternal 25(OH)D status and offspring bone outcomes, however these involved only 60 Caucasian women or only accounted for differences in 25(OH)D levels between 16-20 weeks of gestation (621,622).

1.7.3 Maternal vitamin D supplementation

It could be suggested that a lack of a clear consensus on the relationship between maternal vitamin D status during pregnancy and postnatal offspring bone growth could be ameliorated by the supplementation of mothers with vitamin D during pregnancy, however supplementation trials have also shown conflicting results. A meta-analysis of 76 supplementation studies have shown some evidence for a positive association between maternal 25(OH)D status and neonatal calcium concentration, offspring birthweight and offspring bone mass (623), but there is still no unequivocal evidence to support supplementation or the role of vitamin D for a number of reasons. The measurement method of vitamin D status, the dose of vitamin D given and the consistency of study design, whether blinded or placebo controlled for example are all not yet standardised. This makes it difficult for definitive conclusions to be drawn from current studies (623,624), further suggesting the epigenetic interactions between vitamin D and bone are also not yet clear.

Studies such as the Maternal Vitamin D Osteoporosis Study Randomised Controlled Trial (MAVIDOS RCT), a multi-centre, double blind, randomised placebo-controlled trial aimed to assess whether neonates born to mothers that received vitamin D supplementation during pregnancy had a greater whole-body BMC at birth than those of mothers who had received a placebo. Whilst the study found no difference in offspring whole-body BMC, an interaction was found between treatment effect and season of birth. Specifically, when compared to offspring with un-supplemented mothers, those born in the winter months whose mothers were vitamin D supplemented were found to have a greater BMC, bone area, BMD, but no increase in birthweight or birth length. However BMC measurements were taken at 2 weeks, very early when compared to other studies (625).

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A further study which aims to elucidate the relationship between maternal supplementation, diet and development of the infant postnatally is the Southampton PRenancy Intervention for the Next Generation (SPRING) study. This study aimed to supplement a group of pregnant mothers with enhanced vitamin D₃ supplementation alongside an un-supplemented standard group with the addition of behaviour change, or dietary guidance (626,627). This will help further inform of the role of vitamin D supplementation and diet of the mother in general. In summary, despite an understanding of the role of vitamin D in bone development, the exact role of vitamin D in foetal bone development during pregnancy is not yet clear.

1.7.4 Vitamin D and the methylome

Studies examining the relationship between vitamin D status and global DNA methylation in humans have produced mixed results (628–631). A study by *Zhu et al.* (2016) found in a cross-sectional study of 454 Caucasian and African American 14-18 years olds that 25(OH)D insufficiency (<50 nmol/l) was associated with global hypomethylation in African Americans. However, in this initial study 66% of African American participants were 25(OH)D insufficient but only 2.7%, roughly seven Caucasian participants were vitamin D insufficient, suggesting the sample size was too small to ascertain whether a similar association could be seen in Caucasians. Nonetheless, the cross-sectional study showed a positive association between global methylation and 25(OH)D status. A subsequent 16 week, randomized, double-blinded, placebo-controlled trial also demonstrated that vitamin D₃ supplementation increased global methylation in African Americans with 25(OH)D insufficiency in a dose-dependent manner (629). This study indicates that supplementation may indeed influence global DNA methylation, however it also highlights vitamin D may only exert functional change in the methylome of those already vitamin D insufficient. This may be due to the fact that the rate at which vitamin D can become hydroxylated or activated in the liver may be a saturable process (632). Therefore, those already sufficient for vitamin D may not receive any benefit to supplementation and as such we may not see any functional change.

The nuclear vitamin D signalling pathway has been suggested to be regulated in part by epigenetic processes. Coactivators and corepressors recruited upon the binding of the VDR to genomic DNA have been seen to modulate the activity of chromatin remodellers, which can also be targets of the VDR itself (633). In addition, the metabolism vitamin D has been suggested to be potentially influenced by epigenetic processes. The enzymes CYP2R1, CYP27B1 and CYP24A1 all play a role in the metabolism vitamin D. All contain CpG islands, making them prone to methylation. A small study by *Zhou et al.* (2014) of vitamin D supplemented post-menopausal women found those with the smallest increase in serum 25(OH)D in response to supplementation had higher DNA methylation of CYP2R1 after supplementation. Additionally, supplementation reduced CYP24A1 methylation suggesting CYP24A1 expression increased to counteract vitamin D supplementation

and maintain homeostasis (634). A follow-up study by another group also found a higher plasma 25(OH)D concentration was associated with higher VDR methylation (351). This demonstrates the epigenetic mechanisms that contribute to the self-regulatory capability of this pathway. However these studies were conducted in peripheral blood cells (PBCs), so may not be directly comparable to the vitamin D-related activity in the liver and kidneys where most of the action of these enzymes occurs.

Animal models have also been able to provide some insight about the effects of vitamin D on the methylome. It has been suggested that Vitamin D status of a mother during pregnancy can have a persist through multiple generations, affecting the general health and epigenome of the offspring. A recent study by *Wen et al.* (2018) found a significant increase in adiposity in the offspring of pregnant rats fed a vitamin D deficient diet for four weeks. Methylation profiling revealed that when compared to a vitamin D replete group, offspring of vitamin D deficient rats showed differential methylation in 608 promoters and 204 CpG islands, involving 305 genes in DNA samples extracted from adipose tissue (608). However the exact effects of vitamin D supplementation in human mothers and the subsequent effect on the offspring epigenome has not yet reached consensus (635,636).

The MAVIDOS RCT presents a potential example of the influence of maternal vitamin D status on the methylome of the offspring, findings complemented by associations seen in the SWS study. Previous work in the SWS cohort has demonstrated perinatal differential methylation in umbilical cord blood DNA of CpG sites within the promoter region of the *RXR α* gene, a factor required for the nuclear activity of vitamin D, which were associated with both offspring bone mass at four years of age and inversely associated with maternal 25(OH)D status at one CpG site. A follow-on study in the MAVIDOS RCT also showed significant perinatal differential methylation in umbilical cord tissue DNA within the promoter region of *RXR α* induced by maternal vitamin D supplementation. As tissues genetically derived from the neonate, the use of umbilical cord tissue and blood in these studies, without needing to extract tissue from the neonate, allowed for an insight into the state of the methylome at birth and the potential effect of environmental influences on the infant methylome *in utero*.

1.8 Aims and hypothesis

Achieving an adequate peak bone mass is essential for reducing the risk for conditions such as osteoporosis later in life. Studies have shown variance in peak bone mass cannot be accounted for genotype and lifestyle factors alone (637–640), suggesting a role for the influence of environment. Both animal and human studies have demonstrated that environmental exposures in prenatal life may have the capacity to influence the trajectory of skeletal development in postnatal life through epigenetic mechanisms, consistent with the DOHaD hypothesis (524,529,530,641). Vitamin D, a factor critical to skeletal development has been proposed as an

exposure that through a period of foetal programming *in utero* may directly or indirectly influence skeletal development in postnatal life. Here, we aimed to use both umbilical cord blood and tissue, both genetically derived from the neonate, to identify epigenetic signatures potentially associated with postnatal skeletal development. Additionally, we aimed to determine whether maternal vitamin D supplementation, seen to improve bone outcomes in offspring in the MAVIDOS RCT, alters DNA methylation in genes within birth tissues that are associated with improved BMC or BMD in postnatal development. Furthermore, we intended to determine the presence of any overlap in the methylation changes within bone-related analyses which may similarly be induced by maternal vitamin D supplementation or associated with maternal vitamin D status in cord blood, tissue and additionally in UC-MSCs. This would not only allow for the potential development of prognostic biomarkers which may predict the trajectory of bone growth in postnatal life, but as tissues with a methylation profile similar to that of mesoderm-derived tissues (458), such as bone, lay the foundations for an investigation into the biological significance of potential vitamin D associated or induced changes that may enhance skeletal development. This could potentially allow us to identify individuals at risk of failing to develop an optimal peak bone mass and intervene to facilitate bone formation, ameliorating future osteoporosis risk. The schematic by which these ideas will be explored in this thesis is indicated in Figure 9.

We had access to three studies from which we were able to obtain umbilical cord tissue. The MAVIDOS RCT is a multi-centre, double blind, randomised placebo-controlled trial aimed to assess whether neonates born to mothers that received vitamin D supplementation during pregnancy had a greater whole-body BMC at birth than those of mothers who had not received supplementation (625). The SWS study was a prospective cohort study which recruited non-pregnant women aged 20-34 years in Southampton to examine how maternal and intrauterine factors interact with the genes of the offspring in the postnatal environment to influence growth and disease (642). The SPRING study aimed to assess whether healthy conversation skills or vitamin D supplementation, either individually or in combination could improving the diet quality and nutritional status of pregnant women (626). All three studies collected a wide range of data, including health, diet, physical activity, maternal and foetal anthropometry and blood throughout pregnancy into early childhood and significantly for the purposes of this study, methylation data (summarised in Table 1). This provided a valuable resource from which to examine the interaction between cord blood or tissue DNA methylation and maternal or offspring variables.

Table 1. The availability of methylation data, tissue and vitamin D supplementation for the studies used for assessing the hypothesis of this study

	Study		
	SWS	MAVIDOS RCT	SPRING
Methylation data available	Yes	Yes	No
Tissue availability	Cord blood	Pulverised cord	Cells and whole cord
Vitamin D supplementation	No	Yes	Yes

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The hypothesis of this study is that vitamin D supplementation during pregnancy may induce changes in DNA methylation in foetal tissues which influence later bone development. This can be addressed in two-parts:

- i) DNA methylation in birth tissues is associated with BMC or BMD in postnatal development.
- ii) Maternal vitamin D supplementation alters DNA methylation in genes within birth tissues which may be associated with improved BMC or BMD in postnatal development.

Aims:

1. Determine whether there are DNA methylation signatures in umbilical cord blood DNA associated with offspring BMC or BMD at birth, four, six or eight years of age in the SWS cohort.
2. Determine whether maternal vitamin D supplementation induces differential methylation changes in DNA extracted from MAVIDOS RCT umbilical cord tissue and whether associations exist between umbilical cord blood DNA methylation and maternal late-stage pregnancy vitamin D status in the SWS cohort.
3. Identify whether there are DNA methylation signatures in MAVIDOS RCT umbilical cord DNA associated with postnatal BMC or BMD at birth and at four years of age.
4. Determine whether short or long-term supplementation of UC-MSCs with vitamin D alters the differentiation potential and DNA methylation of UC-MSCs *in vitro*.
5. Assess the effects of both maternal vitamin D supplementation in the MAVIDOS RCT, maternal late-stage pregnancy vitamin D status in the SWS cohort and postnatal measures of BMC or BMD in both cohorts on the acceleration or deceleration of the Horvath and Knight methylation clocks.

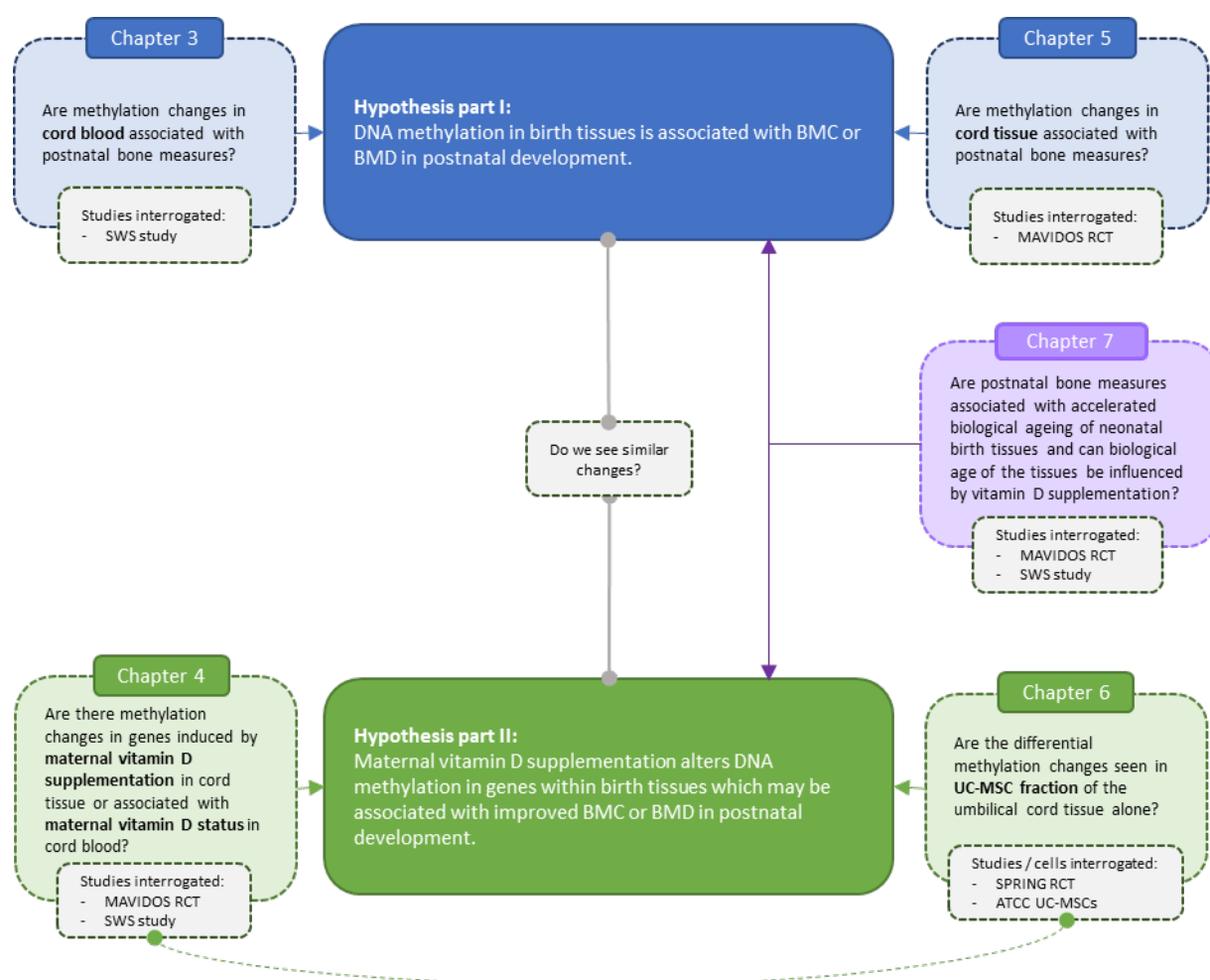


Figure 9. Thesis schematic flow diagram

A thesis diagram to address the manner in which chapters will seek to address the hypotheses proposed within this thesis.

2 Chapter 2 - Methods

2.1 Materials

Chemical / reagent	Company	Catalogue / ref. number
Cell culture		
Antibiotic-Antimycotic (100X)	ThermoFisher	15240062
American Type Culture Collection® Primary Umbilical Cord-Derived Mesenchymal Stem Cells	LGC Standards	PCS-500-010
DMEM/F-12	ThermoFisher	11320074
DMEM/F-12, no phenol red	ThermoFisher	21041025
FastRead Counting Slides	Immune systems	BVS100
Foetal bovine serum (FBS), Qualified, HI	ThermoFisher	10500-064
2% Gelatin solution	Sigma-Aldrich	G1393
Human Osteosarcoma SaOS-2 Cell Line	Cell Lines Service	36025
L-Glutamine	Sigma-Aldrich	G7513
DMEM	ThermoFisher	41966-029
Penicillin-Streptomycin	Sigma-Aldrich	P4333
Phosphate Buffered Saline (PBS) Tablets	Fisher Scientific	12821680
Human osteosarcoma SaOS-2 cell line	Cell lines service	36025
Trypsin-EDTA 0.25%	ThermoFisher	25200-056
Cell sorting		
CD90 microbeads, human	BD Biosciences	130-096-253
MS sorting columns	BD Biosciences	130-042-201
Flow cytometry		
Compensation beads	BD Biosciences	552843
FITC Mouse Anti-Human CD44	BD Biosciences	560977
FITC Mouse IgG2b κ Isotype Control	BD Biosciences	555742
PE Mouse Anti-Human CD29	BD Biosciences	561795
PE Mouse IgG1, κ Isotype Control	BD Biosciences	555749
Propidium iodide	Sigma-Aldrich	P4170
General		
1α,25-dihydroxyvitamin D ₃	Enzo Life Sciences	BML-DM200-0050
CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)	Promega UK	G3582
Chloroform	Fisher Scientific	BP1145-1
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E5134
Ethanol 99%+	Fisher Scientific	E/0600/05
GeneElute™ Mammalian Genomic DNA Miniprep kit	Sigma	G1N350-1KT
Isopropanol	Fisher Scientific	BP2618-1
DNase and RNase (nuclease) free water, DEPC treated	Fisher Scientific	BP561-1
QIAamp DNA blood mini kit	Qiagen	51104
Sodium acetate buffer solution	Sigma-Aldrich	S7899
Tris-Acetate-EDTA buffer, 50X Solution (TAE buffer)	Fisher Scientific	BP1332-1
UltraPure™ Phenol:Chloroform:Isoamly AlcoholPhenol-chloroform	Invitrogen	15593-049
Zymo EZ-96 DNA Methylation Gold Kit	Zymo research	D5008
RT-qPCR		

Deoxynucleotide Triphosphates (dNTPs)	Promega	U1420
DNase I Amplification Grade	Sigma-Aldrich	AMPD1-1KT
GeNorm 12 Kit (Human)	Primer Design	ge-SY-12
M-MLV Reverse Transcriptase kit	Promega	M170B
QuantiFast SYBR Green PCR Kit	Qaigen	204054
Quantitect primers	Qaigen	249900
Random nonomers	Sigma-Aldrich	R7647
RNeasy mini kit	Qaigen	74104
TRI Reagent	Sigma-Aldrich	T9424
Vector		
Antarctic Phosphatase	New England Biolabs	M0289S
Carbenicillin disodium salt	Sigma-Aldrich	C3416
Cell-culture grade water	Sigma	W3500
Dual-Luciferase® Reporter Assay System	Promega	E1910
FastDigest SacI	ThermoFisher	FD0524
FastDigest KpnI	ThermoFisher	FD1133
FuGENE 6 transfection reagent	Promega	E2691
GeneJET Plasmid Midiprep Kit	ThermoFisher	K0481
Monarch® PCR & DNA Cleanup Kit	New England Biolabs	T1030S
Opti-MEM® Reduced Serum Medium	Invitrogen	31985062
pGL4.23 [luc2/minP] Vector	Promega	E8411
QIAprep® spin miniprep kit	Qaigen	27104
Quick Ligation™ Kit	New England Biolabs	M2200S
Single-Use JM109 Competent Cells	Promega	L2005
Super optimal broth with catabolite repression (SOC) media	Sigma-Aldrich	S1797
Zymoclean Gel DNA recovery kit	Cambridge Bioscience	D4001

2.2 Cohort details

2.2.1 Southampton Women's Survey study

All patient-facing activities described within this section were conducted by Southampton Women's Survey (SWS) study research nurses and ancillary staff.

2.2.1.1 Cohort details

The SWS study is a prospective cohort study composed of 12,583 non-pregnant women aged 20 to 34 years of age recruited into the study between April 1998 and October 2002 as residents of the city of Southampton, UK. The recruitment activities for the SWS study (Figure 10) were conducted by the 20-member multidisciplinary SWS research team without the involvement of the author of this dissertation. The SWS study was approved by the Southampton and South West Hampshire local ethics research committee (307/97, 153/99w, 005/03/t and 06/Q1702/104) and all the study participants gave written informed consent to be included and by a parent or guardian with parental responsibility on behalf of their children.

2.2.1.2 Maternal assessment

An assessment of lifestyle and anthropometry was conducted at entry into the study and for those who became pregnant, during early pregnancy (11 weeks) and late pregnancy (34 weeks) where details of maternal smoking, ethnicity and body mass index (BMI) were recorded.

2.2.1.2.1 Assessment of 25(OH)D status

Maternal 25-hydroxyvitamin D3 (25(OH)D) status was ascertained at 11- and 34-weeks gestation, where a venous sample of blood was obtained and an aliquot of maternal serum was frozen at -80°C. Serum concentrations of both 25(OH)D₂ and 25(OH)D₃ were measured by radioimmunoassay (DiaSorin, Minnesota, USA). The assay was based upon the dissociation of 25(OH)D from its binding protein and subsequent binding to vitamin D-specific antibodies, coated on to magnetic microparticles. Once bound, isotope-linked vitamin D was added to compete for any unoccupied vitamin D-specific antibody binding sites and any unbound material removed in a wash cycle. The samples were measured for 1 minute with a gamma counter to determine the quantity of bound isotope, a measurement which was inversely proportional to the concentration of 25(OH)D in the sample. Sample total 25(OH)D concentrations could then be determined by interpolation from a calibration standard curve.

All samples were analysed in a single batch at the end of the study at Medical Research Council Human Nutrition Research, Cambridge, UK. The assay met the requirements of the UK National Vitamin D External Quality Assurance Scheme (DEQAS) and showed intra-assay and inter-assay coefficient of variation (CV) values of <10%.

2.2.1.3 *Offspring anthropometrics and DXA*

At birth, gestational age was recorded, birthweight determined using calibrated digital scales (Seca, UK) and crown to heel length determined measured using a neonatometer (CMS Ltd, UK). Within two weeks of birth and with maternal consent, total body dual-energy X-ray absorptiometry (DXA) scans were conducted with a Lunar DPX-L scanner (GE Corporation, Madison, WA, USA) using paediatric software (paediatric small scan mode v4.7c). Neonates were swaddled to reduce the incidence of movement artefacts in the scan. Measurements of bone mineral content (BMC), bone mineral density (BMD) and bone area (BA) for whole-body including the head were obtained. The instrument underwent daily quality assessment and was calibrated against a water phantom weekly.

A subset of children aged four years were recruited sequentially from the SWS cohort to attend the Osteoporosis centre at Southampton General Hospital where an assessment of bone mass and body composition was conducted. At this visit, written consent for DXA was obtained from the mother or father/guardian. The child's height was measured using a Leicester height measurer (Seca Ltd, UK) and weight in underpants only, using calibrated digital scales (Seca Ltd, UK). A whole-body scan was obtained using a Hologic Discovery instrument (Hologic Inc., Bedford, MA, USA) in paediatric scan mode. BMC, BMD and BA were recorded for whole-body less head to exclude any scan artefacts introduced by head movement and the potential of such movement artefacts to compromise downstream analysis. Scans with an unacceptable level of movement artefacts were excluded. The manufacturer's coefficient of variation for the instrument was 0.75% for whole-body bone mineral density. DXA scan assessments were further repeated at 6 and 8 years of age.

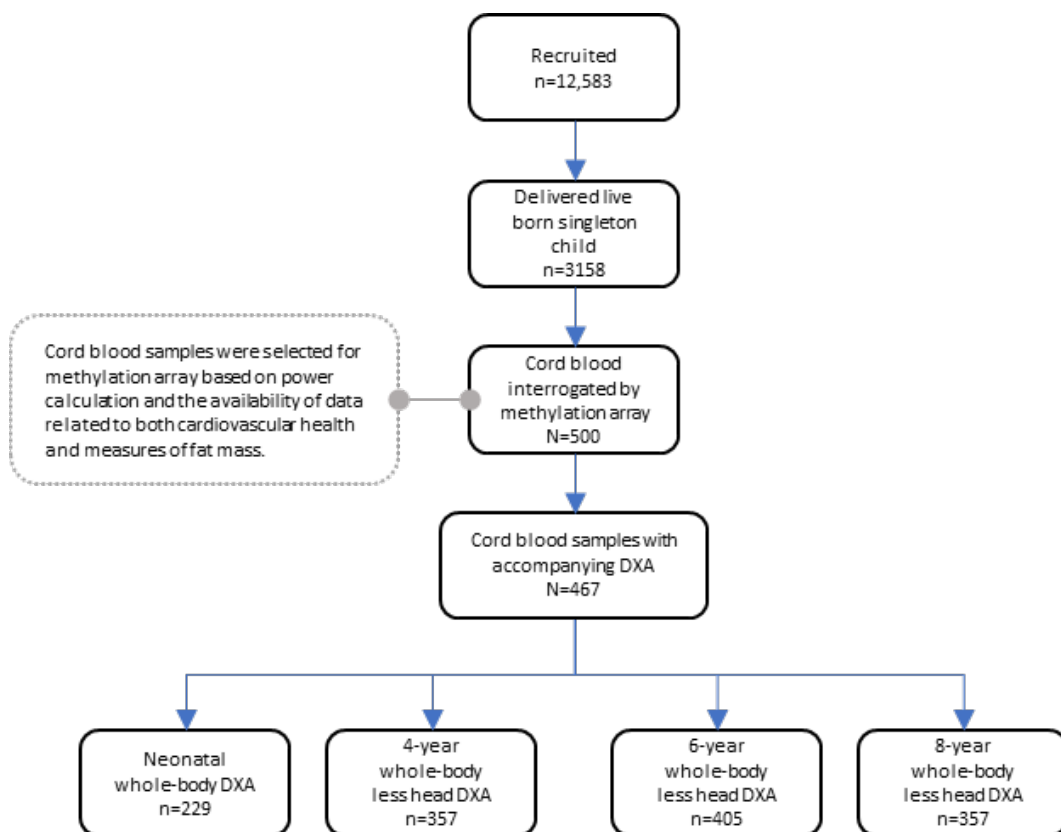


Figure 10. Flow chart depicting the recruitment of participants for the SWS study

Non-pregnant women aged 20-34 years were recruited to the study ($n=12,583$) between April 1998 and October 2002. A total of 3158 women subsequently became pregnant and delivered a liveborn singleton infant between December 1998 and December 2007. Women who delivered a live born singleton child had phenotypic characteristics recorded in detail during pregnancy and their offspring followed up into childhood. Mothers registered with specific GP practices were invited to take part in the bone sub-study component of the SWS study. GP practices were selected to avoid participants participating in more than one sub-study and were representative of the population of Southampton as a whole. Cord blood samples were selected for methylation array analysis ($n=500$) based on the availability of companion phenotypic measures of cardiovascular health and fat mass, alongside a power calculation which determined the number of samples required for detection of an appropriate effect size. Post-QC DNA methylation data and companion phenotypic data was available for 467 participants, of which DXA data was available for 229 at birth, 357 at four years of age, 405 at 6 years of age and 357 at eight years of age. Recruitment activities requiring patient contact or interaction were conducted by the multidisciplinary, 20-member SWS research team. No recruitment activities depicted in the above flow chart were conducted by the author of this dissertation. Differential methylation analyses were conducted with respect to umbilical cord blood DNA methylation and DXA measures of total BMC (kg), total BMD (g/cm^2) at birth, 4, 6 and 8 years of age. A comparison of the top 100 dmCpGs when ranked by FDR was made between all age groups within BMC or BMD related analysis groups. Comparisons between BMC and BMD related analyses were made between measures taken at the same age, where dmCpGs were produced in both analyses with an $\text{FDR} < 0.25$.

2.2.2 *Maternal Vitamin D Osteoporosis Study randomised controlled trial*

All patient-facing activities described within this section were conducted by Maternal Vitamin D Osteoporosis Study (MAVIDOS) randomised controlled trial (RCT) research nurses and ancillary staff.

2.2.2.1 *Study details*

The MAVIDOS RCT is a multi-centre, double blind, randomized placebo trial of vitamin D supplementation in the UK. Recruitment activities for the MAVIDOS RCT were conducted by the 15-member, multidisciplinary MAVIDOS RCT research team. Recruitment activities are shown in Figure 11, where contributions to the recruitment process by the author of this dissertation are highlighted. The study was conducted in accordance with guidelines laid down in the Declaration of Helsinki and was approved by the Southampton and South West Hampshire Research Ethics Committee. MAVIDOS was registered prospectively (ISRCTN:82927713; EUDRACT:2007-001716-23); full approval from UK Medicines and Healthcare products Regulatory Agency was granted and written, informed consent was obtained from all participants.

2.2.2.2 *Maternal assessment*

All participants were given standard antenatal care and able to self-administer antenatal vitamins containing up to 400 international units (IU)/day vitamin D. At 14 weeks, prior to commencing study medication a detailed assessment of diet (including calcium and vitamin D intake), smoking, alcohol consumption, health, physical activity, medications and supplements was ascertained using interviewer-led questionnaires. Ethnicity was reported by the participant as either white or non-white. Maternal anthropometry including height, weight, skin fold thickness (triceps, biceps, subscapular and suprailiac) and grip strength are measured. Assessments were repeated at 34-weeks' gestation.

2.2.2.2.1 *Assessment of 25(OH)D status*

A venous non-fasted blood sample was obtained at 14 weeks gestation at which medication began to be dispensed and at 34 weeks gestation. Serum was stored at -80°C until assay. 25(OH)D was then assessed via chemiluminescent assay (liaison automated platform; DiaSorin, Stillwater, MN, USA). The assay was based upon the dissociation of 25(OH)D from its binding protein and subsequent binding to vitamin D-specific antibodies, coated on to magnetic microparticles. Once bound, vitamin D linked to an isoluminol derivative was added to compete for any unoccupied vitamin D-specific antibody binding sites and any unbound material removed in a wash cycle. The light signal was measured by a photomultiplier as relative light units which was inversely proportional to the concentration of 25(OH)D in the sample. Sample total 25(OH)D concentrations could then be determined by interpolation from a calibration standard curve.

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All samples were analysed in a single batch at the end of the study at Medical Research Council Human Nutrition Research, Cambridge, UK. Assay performance and quality control through participation in DEQAS, US National Institute of Standards and Technology and the UK National External Quality Assessment Service are given elsewhere (643,644).

2.2.2.3 *Offspring anthropometrics and DXA*

At birth a research nurse measured the baby's anthropometric indices, including length, weight, skinfolds, head and abdominal circumference. Within two weeks of birth all neonates underwent a DXA scan assessment of the lumbar spine and whole-body BMC, BMD and BA using a Hologic Discovery instrument (Hologic Inc., Bedford, MA, USA) or Lunar DPX-L scanner (GE Corporation, Madison, WA, USA) using neonatal software. To maximise scan quality neonates were swaddled to reduce the incidence of movement artefacts in the scan. All DXA images were reviewed for quality and movement artefacts by two operators who were blinded to treatment allocation.

At four years of age, children of Southampton participants were invited to attend the osteoporosis centre at Southampton General Hospital for assessment of both body composition and bone mass (March 2013 to October 2018). At this visit, informed consent was obtained from the parent/guardian and health, diet and lifestyle information gathered through an interview-led questionnaire. The child's height, without shoes, was measured three times using a portable stadiometer (Leicester height measurer, Seca Ltd, UK) to the nearest 0.1 cm and a mean calculated. Weight was also measured in light clothing using calibrated electronic scales (Seca Ltd, UK) to the nearest 0.1kg. DXA scans were obtained for whole-body, lumbar spine and left hip BMC, BMA and BA using a Hologic Discovery instrument (Hologic Inc., Bedford, MA) in paediatric scan mode. The analyses at four, six and eight years of age within this thesis use the whole-body minus head measurements to exclude any scan artefacts introduced by head movement and their potential to compromise downstream analysis. DXA scans were reviewed by two operators, both blinded to treatment allocation. Scans with movement artefacts affecting the whole body, both arms or both legs were removed from the analysis. If movement artefacts affected only one limb, the region of interest in the unaffected limb was transposed into that of the limb with the movement artefact.

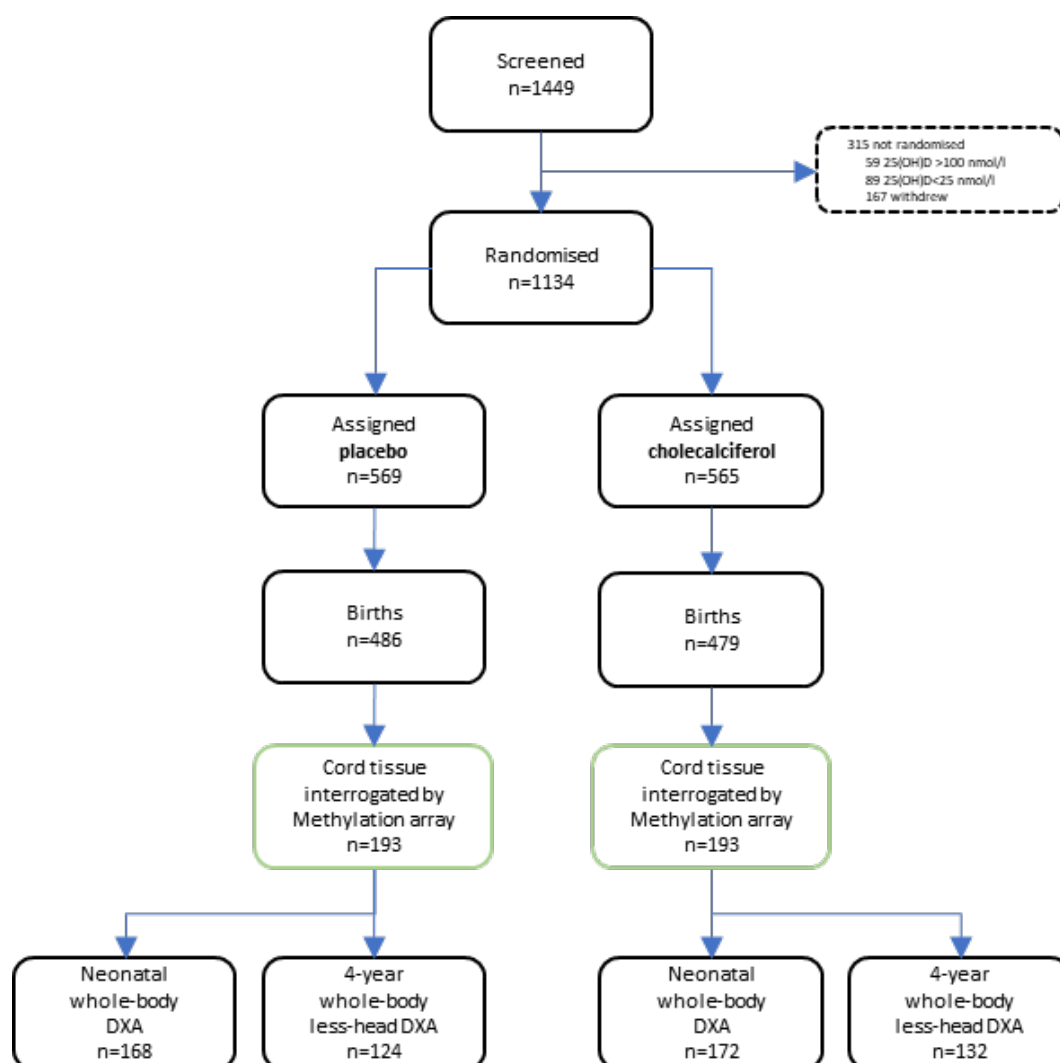


Figure 11. Flow chart depicting the recruitment of participants for the MAVIDOS RCT

Women attending one of three UK hospitals (University Hospital Southampton NHS Foundation Trust, Southampton; Oxford University Hospitals NHS Foundation Trust, Oxford; Sheffield Hospitals NHS Trust, University of Sheffield, Sheffield) for early pregnancy ultrasound screening (11 to 14 weeks' gestation) between October 6th 2007 and February 11th 2014 were asked to participate in the study. Women were screened (n=1449) based on inclusion criteria including being over 18 years of age, singleton pregnancy and gestation less than 17 weeks based on last menstrual period and ultrasound. Women were excluded based on known metabolic bone disease, renal stones, hyperparathyroidism, hypercalciuria, those taking medication known to interfere with fetal growth, fetal anomalies on ultrasonography, and women already using >400 IU/day vitamin D supplementation. Women with 25(OH)D between 25 nmol and 100 nmol/l and a serum calcium of <2.75 mmol/l were eligible to enrol in the study. Eligible participants (n=1134) were randomized to receive either cholecalciferol 1000 IU/day (n=565) or matched placebo (n=569). There were 486 live births in the control group and 479 in the cholecalciferol group. A total of 193 samples from each treatment arm underwent methylation array analysis. Within the placebo arm whole-body DXA data was available for 168 participants at birth and 124 at four years of age. Within the cholecalciferol arm whole-body DXA data was available for 172 participants at birth and 132 at four years of age. Recruitment activities requiring patient contact or interaction were conducted by the multidisciplinary, 15-member MAVIDOS research team. Activities in the above flow chart conducted by the author of this dissertation are highlighted with a green border. Differential methylation analyses were conducted with respect to umbilical cord tissue DNA methylation and DXA measures of total BMC (kg), total BMD (g/cm²) at birth and four years of age. A comparison of the top 100 dmCpGs when ranked by FDR was made between both age groups within BMC or BMD related analysis groups. Comparisons between BMC and BMD related analyses were made between measures taken at the same age, where dmCpGs were produced in both analyses with an FDR<0.25. In addition, differential methylation analyses were conducted with respect to umbilical cord tissue DNA methylation and maternal cholecalciferol supplementation in both the whole study group and the mothers insufficient for 25(OH)D in early pregnancy (n=224). Comparisons were made between both cholecalciferol supplementation analyses and between BMC, BMD at birth and four years and cholecalciferol supplementation analyses.

2.3 DNA extraction

2.3.1 SWS

2.3.1.1 DNA extraction from SWS cord blood samples

At birth, the umbilical cord was swabbed with a 70% alcohol wipe, venous umbilical cord blood aspirated (25 ml) by a SWS research nurse and stored at 4°C overnight. The whole blood was centrifuged (3500 rpm, 10 minutes) and the buffy coat layer carefully extracted with a sterile pastette. Genomic DNA was extracted from the buffy coat of umbilical cord blood samples using the QIAamp DNA blood mini kit (Qiagen). The kit allowed for the isolation of DNA from potential contaminants of downstream processes through the adherence of the DNA to a silica spin column membrane, allowing contaminants to be washed away and the DNA eluted separately. Briefly, buffy coat (200 µl) was added to a microcentrifuge tube containing QIAGEN Protease (20 µl) and buffer AL added (200 µl). The sample was pulse vortexed (15 seconds) and incubated at 56°C (10 minutes). The microcentrifuge tube was pulse centrifuged to remove sample from the walls of the tube, 100% ethanol (Fisher Scientific) (200 µl) added, the sample pulse vortexed (15 seconds) and pulse centrifuged once more to remove extraneous sample from the walls of the tube. The mixture was aliquoted into a QIAamp mini spin column within a collection tube, centrifuged (8000 rpm, 1 minute) and the spin column placed into a new, clean collection tube. Buffer AW1 was added (500 µl), the spin column centrifuged (8000 rpm, 1 minute) and placed in a new collection tube. Buffer AW2 was added (500 µl), the spin column centrifuged (14000 rpm, 3 minutes), placed in a new collection tube and centrifuged with the same parameters for 1 further minute. The spin column was transferred to a microcentrifuge tube, nuclease-free water added and allowed to incubate at room temperature (RT) (1 minute). Finally, the spin column was centrifuged to elute the DNA. The quality of genomic DNA was assessed by agarose gel electrophoresis and both quality and quantity assessed on the NanoDrop ND-1000 spectrophotometer (NanoDrop technologies) as in section 2.3.4. DNA was stored at -80°C. This work was conducted by Nikki Graham at the University of Southampton.

2.3.1.2 Quality assessment and Infinium MethylationEPIC BeadChip array analysis

Prior to dispatch, 500 DNA samples were randomised to positions on EPIC array eight-sample chip plans by Robert Murray at the University of Southampton. Samples were then dispatched to Julie McIsaac of the Kobor lab, Centre for Molecular Medicine and Therapeutics, University of British Columbia, Canada where the DNA samples were bisulfite converted (section 2.3.1.2.1), quality checked and run on an Illumina Infinium MethylationEPIC BeadChip (EPIC) array (Illumina Inc, San Diego, CA) (Figure 12). Once the EPIC array was completed, intensity data (IDAT) files were provided for download to the author of this dissertation for differential methylation analyses to be conducted.

2.3.1.2.1 *Bisulfite conversion*

The Zymo EZ-96 DNA Methylation Gold Kit (Zymo Research) was used for the bisulfite conversion of genomic DNA prior EPIC array interrogation. Incubation of target DNA with sodium bisulfite catalysed the conversion of unmethylated cytosine residues in genomic DNA to uracil, whereas methylated cytosines in cytosine-phosphate-guanine (CpG) dinucleotides remained unaltered. This allowed for a distinction to be made between methylated and unmethylated DNA sequences in any subsequent downstream methylation analysis. Briefly, DNA (1 µg) was made up to a final volume of 20 µl with nuclease-free water in a 96-well conversion plate, CT reagent added (120 µl) and the solution mixed by pipetting. The plate was sealed with strip caps and a foil seal then incubated at 98°C for 10 minutes, 64°C for 2.5 hours and finally 4°C for up to 20 hours in an Applied Biosystems™ Veriti™ 96-well thermal cycler (ThermoFisher). The DNA solution was transferred to a Silicon-A 96-well binding plate containing M-binding buffer (400 µl), mixed via pipetting, centrifuged (3000 g, 10 minutes) and the flow-through discarded. M-wash (400 µl) was added, the plate centrifuged (3000 g, 5 minutes) and flow-through discarded. M-desulphonation (200 µl) buffer was added and incubated at RT (20 minutes), after which the plate was centrifuged (3000g, 5 minutes) and the flow-through discarded. M-wash buffer was added (400 µl), the plate centrifuged (3000g, 5 minutes) and the flow-through discarded. M-wash (400 µl) was added once more, the plate centrifuged and flow-through discarded. The Silicon-A binding plate was positioned on an elution plate, nuclease-free water added (30 µl) ensuring the membrane was saturated and the plate incubated at RT (5 minutes). The plate stack was centrifuged and eluted DNA stored at -80°C until required. This work was conducted by Julie McIsaac of the Kobor lab, Centre for Molecular Medicine and Therapeutics, University of British Columbia, Canada.

2.3.2 *MAVIDOS RCT*

2.3.2.1 *DNA extraction from MAVIDOS RCT cord tissue samples*

DNA isolation was performed using a phenol-chloroform extraction method. In brief, hyaluronidase solution 10-11 U/ml (500 µl) was added to crushed umbilical cord (500 mg ±10%), the samples pulse-spun and incubated at 37°C (30 minutes, shaking at 150 rpm). An equal volume of TNES (Tris 50 mM, NaCl 400mM, ethylenediaminetetraacetic acid (EDTA) 100mM, Sodium dodecyl sulphate 0.5%) solution was added and samples were then homogenised using the Xiril Dispomix Homogeniser using the programme in Table 2. Next, proteinase K 20 mg/ml (5 µl) was added and the samples, mixed and incubated overnight at 55°C. Samples were transferred to 2.2 µl Eppendorf tubes, 2.6M NaCl (500 µl) added, the samples mixed and centrifuged (20 minutes, 13,000 rpm, RT). The supernatant was then split between two new Eppendorf tubes, an equal volume of ice-cold 100% ethanol added and the samples mixed. The precipitated DNA was spooled into RNase-free water (500 µl) containing RNase A 10 mg/ml (5 µl) and incubated at 37°C (30 minutes). Phenol/chloroform (500 µl) was added to each sample, mixed and centrifuged

(12,000 rpm, 5 minutes). The top aqueous layer was removed to a new tube and the phenol/chloroform wash repeated. $1/10^{\text{th}}$ the volume of 3M pH 5.2 NaAc and two volumes (900 μl) of ice cold 100% ethanol were added, mixed and precipitated DNA was spooled into nuclease-free water (100-500 μl). If no substantially sized clump of DNA appeared, the sample was centrifuged (12,000 rpm, 10 minutes) and the supernatant removed. The pellet was resuspended in nuclease-free water (250 μl). Duplicate DNAs were then combined, allowed 24 hours to resuspend into solution at 4°C before quantity and quality was assessed by Nanodrop ND-1000 spectrophotometer (NanoDrop technologies) as in section 2.3.4. DNA was stored at -80°C. This work was conducted by the author of this dissertation.

2.3.2.2 *Quality assessment and Infinium MethylationEPIC BeadChip array analysis*

Samples were selected for array analysis based on the availability of DXA information at the ages of birth and four years and the quality of sample (appendix 9.5.1). The umbilical cord tissue DNA samples, including inter-chip and intra-chip replicates were then randomised and arranged to positions on EPIC array 8-sample chips such that placebo and vitamin D supplementation samples alternated across all chips by the author of this dissertation. The samples were dispatched to by Julie McIsaac of the Kobor lab, Centre for Molecular Medicine and Therapeutics, University of British Columbia where they were bisulphite converted (section 2.3.1.2.1), quality checked and run on an EPIC array (Figure 12). Once completed, IDAT files were provided for download to the author of this dissertation for the subsequent analysis of differential methylation in the samples.

2.3.3 *American type culture collections umbilical cord mesenchymal stromal cells (ATCC UC-MSCs)*

2.3.3.1 *DNA extraction from short-term treated 1α , 25-dihydroxyvitamin D₃ ($1,25(\text{OH})_2\text{D}$) ATCC UC-MSCs*

ATCC UC-MSC cells were grown at 6×10^5 cells per flask in T175 flasks and treated with either 50nm $1,25(\text{OH})_2\text{D}$ (n=6) or vehicle (n=6) for 24 hours. Cells were trypsinised from flasks as described in section 2.5.2, pelleted, and all excess media removed. DNA was extracted from cell pellets using the Sigma GeneElute™ Mammalian Genomic DNA Miniprep Kit. This kit allowed for the extraction of cell DNA through the denaturation of cells in a chaotropic salt-containing solution and the addition of ethanol, meaning when transferred to and centrifuged in a spin column DNA to bound to the spin column silica membrane, whereas unwanted lysate was washed away. To do so, pellets were allowed to thaw and resuspended in resuspension solution (200 μl). RNase A was then added and allowed to incubate at RT (2 minutes). Cells were then through the addition of Proteinase K solution (20 μl), followed by lysis solution C (200 μl), vortexed until homogenous and incubated at 70°C (10 minutes). GenElute Miniprep binding columns were prepared through the addition of column preparation solution (500 μl), centrifuged (12000 g, 1 minute) and flow-through discarded. Ethanol (100%) (200 μl) was added to the cell lysate,

vortexed until homogenous and transferred to the binding columns which were centrifuged (6500 g, 1 minute) and the flow-through discarded. Columns were placed in new collection tubes, wash solution added (500 µl) and the columns centrifuged (6500 g, 1 minute). Columns were transferred to a new collection tube, wash solution added (500 µl) and centrifuged (16000 g, 4 minutes) to dry the column. To elute DNA, columns were transferred to a new collection tube, nuclease-free water added (50 µl) incubated for 5 minutes at RT and columns centrifuged (6500 g, 1 minute). This step was repeated two more times for total of three sequential elutions of DNA. Quality and quantity of the extracted DNA was assessed using a NanoDrop ND-1000 (NanoDrop technologies) and agarose gel electrophoresis (section 2.3.4). DNA was stored at -80°C. This work was completed by the author of this dissertation.

2.3.3.2 Quality assessment and Infinium MethylationEPIC BeadChip array analysis

Prior to dispatch, ATCC UC-MSC DNA samples, including inter-chip and intra-chip replicates were randomised and arranged to positions on EPIC array eight-sample chip plans such that vehicle and 1,25(OH)₂D treated samples alternated across all chips by the author of this dissertation. The samples were dispatched to Julie McIsaac of the Kobor lab, Centre for Molecular Medicine and Therapeutics, University of British Columbia, Canada where samples were bisulfite converted (section 2.3.1.2.1), quality checked and run on an EPIC array (Figure 12). Once the EPIC array was completed, IDAT files were provided for download to the author of this dissertation for differential methylation analyses to be conducted.

2.3.4 DNA quality assessment

2.3.4.1 NanoDrop spectrophotometer

The quality and quantity of genomic DNA was assessed by NanoDrop ND-1000 spectrophotometer (NanoDrop technologies). Briefly, 1.5 µl was loaded on to the NanoDrop ND-1000 spectrophotometer and concentration. The 260/280 ratio, which may provide an indication of potential protein or residual phenol contamination and the 260/230 ratio, a potential indication of residual phenol, guanidine or glycogen contamination were both recorded. A 260/280 ratio of ≥ 1.6 confirmed an acceptable quality of a DNA sample and a 260/280 ratio of ≥ 1.8 confirmed an acceptable quality of an RNA sample.

2.3.4.2 Agarose gel electrophoresis

The quality of genomic DNA samples, including the presence of any contamination or degradation was determined using agarose gel electrophoresis on a 0.8% agarose gel. All samples were prepared with an appropriate quantity of DNA or RNA, DNA Gel loading dye (2 µl) (ThermoFisher) and made to a final volume (10 µl) with nuclease-free water. All gels were imaged using the Syngene Ingenius gel imager and associated GeneSnap software (Syngene).

Table 2. The Xiril Dispomix Homogeniser programme used for the homogenisation of crushed MAVIDOS RCT umbilical cord tissue

Time / seconds	Activity
5	+4000 rpm
3	Off
5	-4000 rpm
3	Off
5	+4000 rpm
3	Off
5	-4000 rpm

2.4 Methylation array bioinformatics analysis

All methylation array and subsequent bioinformatics analysis described within this section was conducted by the author of this dissertation.

2.4.1 QC and pre-processing

The EPIC array incorporates two generations of probe assay to detect methylation at a CpG site, as shown in Figure 12 (304). The probe assays produce either a methylated or un-methylated signal on red or green fluorescence channels depending on the probe type. The raw signal intensities generated from each channel were stored in IDAT files. IDAT files were then processed in R using the minfi package (v.1.30.0), the IlluminaHumanMethylationEPICanno.ilm10b4.hg19 and IlluminaHumanMethylationEPICmanifest datasets. To generate the beta values IDAT files were read into a minfi RGChannelSet object named an 'RGset' that stored the raw intensity data. Raw signal intensities were then used to calculate the ratio between the methylated and un-methylated signals, the result of which was used to generate a beta value between 0 and 1. Prior to differential methylation analysis taking place the beta values underwent a series of quality control (QC) steps (Figure 13). Initially, normalised beta values were generated for all samples through the beta-mixture quantile (BMIQ) normalisation method using the champ.norm function of the chip analysis methylation pipeline (ChAMP) package (v2.14.0). Principal component analysis (PCA) plots were generated to determine sources of variance in the dataset. Hierarchical clustering was then conducted based on Euclidian distance, to determine whether inter-chip and inter-chip samples cluster based on beta values. Median absolute deviation score (MADscore) a robust z-score measurement method was used to determine outliers. Samples and any associated replicates with a MADscore <-5 were omitted from any further analysis. Post-removal of outliers and replicated, raw data was re-normalised before any subsequent analysis. Filtering of probes was then conducted using the ChAMP package (v.2.14.0). CpG probes with a detection *p*-value of greater than 0.01, or with a bead count <3 were set to NA and samples with NA in >5% of samples were also excluded from any further analysis. Probes with a single-nucleotide polymorphism (SNP) within a CpG site, or at the single nucleotide extension were removed, along with probes previously indicated to be cross-reactive (645–647). As the samples originated from both male and female donors, CpG probes located on sex chromosomes were also removed.

Estimated cell-type compositions for the respective tissue types were estimated for each sample using the meffil package (v1.1.0). To estimate the effects of cell-type composition on methylation for each sample, meffil used as a reference previous work by *Gervin et al.* (2016) for umbilical cord blood-based differential methylation analyses and *Lin et al.* (2018) for umbilical cord tissue-based differential methylation analyses (648,649). Estimated cell-type composition for each sample was integrated into any models using that sample data. Additionally, ComBat was used to

adjust beta values for batch/chip effect as indicated by singular value decomposition (SVD) plots post-normalisation.

2.4.2 Differential methylation analysis

Differential methylation analysis was carried out using limma (v3.40.6) in R. Covariates for each regression model were initially based on biological relevance and a correlation matrix was generated to identify any other significant correlation between the phenotypic characteristics of the respective study and the 100,000 CpGs demonstrating the greatest variance in methylation from the EPIC array. Differentially methylated CpGs with a false discovery rate (FDR) of less than 0.25 still provide some functional or biologically relevant insight into the most significant methylation changes within this analysis, so the top 100 differentially methylated cytosine-phosphate-guanine dinucleotides (dmCpGs) from these analyses were included in further analysis. The quality of each model was assessed by its genomic inflation factor (λ), where >1.2 indicated an unacceptable level of inflation. Selected models with a $\lambda > 1.2$ were adjusted for their inflation and bias with the bacon package (v.1.12.0) (650). The bacon package produced inflation and bias adjusted p -values which were then subsequently corrected for multiple testing using the Benjamini-Hochberg method. Differentially methylated regions were determined by DMRcate (651).

2.4.3 Pathway analysis

2.4.3.1 Cytoscape

Pathway analysis was conducted with the top 100 dmCpGs in a selected differential methylation analysis. CpGs were included if they were present in the illumina EPIC manifest file and any duplicate genes were removed. Initially, enriched gene ontology (GO) terms were determined using the missMethyl (v.1.20.4) (652) package in R. Protein-protein interaction networks were also generated using GeneMANIA (v.3.5.2) (653) in Cytoscape (v.3.8.2) (654), wherein the networks were clustered using the MCODE (v2.0.0) (655) algorithm and enriched GO terms determined with Biological Networks Ontology tool (BiNGO) (v.3.0.4) (656).

2.4.3.2 Ingenuity pathway analysis

Pathway analysis was conducted with the top 100 dmCpGs in a selected differential methylation analysis. CpGs were included if they were present in the illumina EPIC manifest file and any duplicate genes were removed. Gene identifiers for each dmCpG were uploaded into the Ingenuity Pathway Analysis (IPA) software (Qiagen). Each gene identifier was mapped to a corresponding gene in the Ingenuity Pathway Knowledge Base and the core analysis function included in the software was used to determine associations in biological processes canonical pathways, upstream transcriptional regulators and gene networks.

2.4.4 *Histone mark and chromatin state analysis*

To determine the enrichment of dmCpGs among chromatin enhancer states and histone modifications we obtained chromatin immunoprecipitation sequencing (ChIP-seq) peak regions in the broadPeak format for processed and consolidated ChIP-seq experiments conducted by the National Institute of Health Roadmap Epigenomics Mapping Consortium (657,658) in human umbilical vein endothelial cells (HUVEC) as a surrogate for umbilical cord blood and tissue from the Roadmap Epigenomics project data portal (https://egg2.wustl.edu/roadmap/web_portal/). The expanded 18-state chromatin state model for HUVECs was also obtained from the Roadmap Epigenomics project. We assessed the enrichment of dmCpGs among the chromatin states and histone modifications using Fisher exact tests, with all Infinium MethylationEPIC BeadChip array CpGs as background.

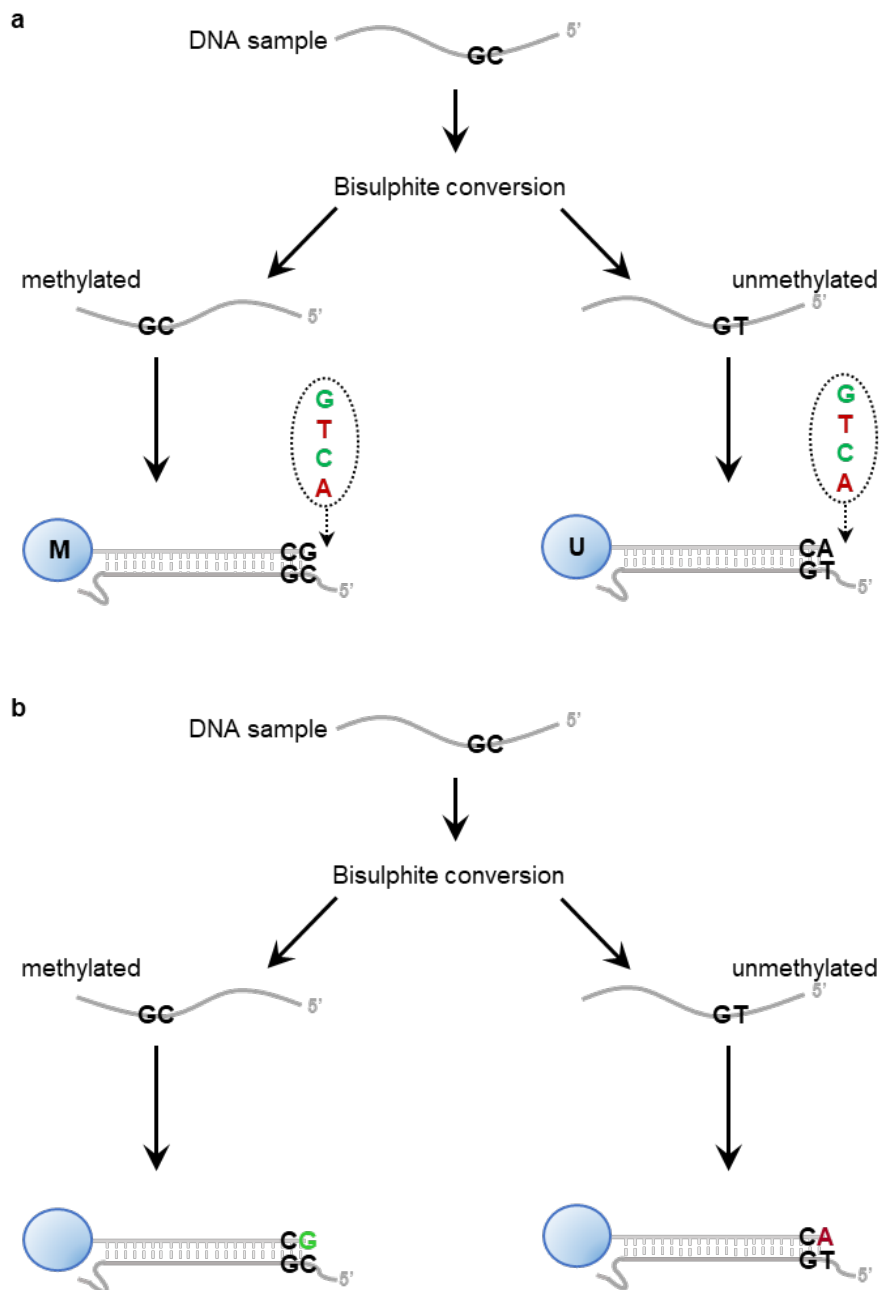


Figure 12. An illustration of the Infinium MethylationEPIC BeadChip array assay probe types a) I and b) II

Bisulfite converted DNA is hybridised to an Illumina Infinium MethylationEPIC BeadChip which consists of two types of Infinium probe, Infinium probes I and II. Infinium probe type a) I probes use two bead types per CpG locus, one for each of the methylated and unmethylated states. Both the bead types incorporate the same labelled nucleotide for the same target CpG and so therefore producing the same fluorescence. However, the nucleotide added is determined by the base downstream of the 'C' of the target CpG. The methylation state of this locus is then calculated by comparing the intensities from the two different probes in the same colour. The Infinium type b) II probes use a single bead type and the methylation state is determined at the single base extension step after hybridisation. Methylation state is detected by single base extension at the 'C' of the target CpG which will always result in the addition of a labelled 'G' or 'A' nucleotide complementary to a methylated 'C' or an unmethylated 'T' respectively. As each locus is detected in two colours, the methylation state can be determined by comparing the two colours from one position. Adapted from Maksimovic *et al.* (2012) (678).

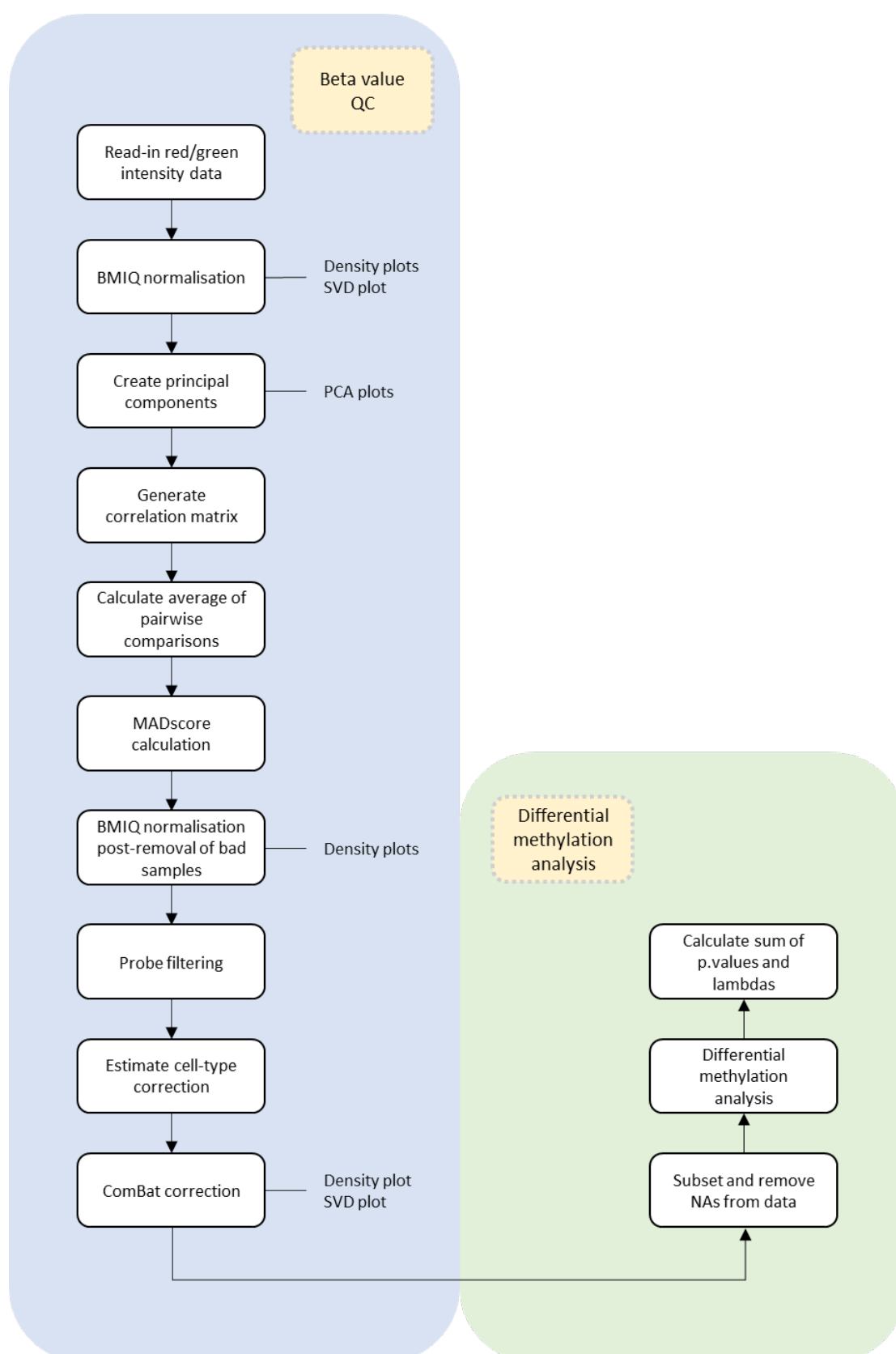


Figure 13. A flow chart diagram of the EPIC array BMIQ differential methylation analysis pipeline

The beta value QC and differential methylation analysis components required for the analysis of data produced from the interrogation of DNA on the EPIC array.

2.4.5 Transcription factor enrichment

The R package RcisTarget (v1.6.0) (659) was used to identify the transcription factor (TF) binding site motifs enrichment in a list of genes associated with the top 100 dmCpGs produced from a differential methylation analysis. Briefly, RcisTarget identified transcription factor binding site motifs (TFBS motifs) in the 500bp upstream of the of transcription start site (TSS) in the provided gene set by reference to a database of TFBS motifs aggregated from several publicly available datasets (660–666). This database also contained genome wide rankings of all human genes for each motif based on the presence of the motif within a genes putative regulatory regions, determined from the interrogation of deoxyribonuclease I (DNase I) hypersensitivity, TF and histone ChIP-seq, DNA sequencing, formaldehyde-assisted isolation of regulatory elements sequencing and assay for transposase-accessible chromatin (ATAC) sequencing experimental data (667). These rankings and the input gene set were used along with an area under the recovery curve (AUC) method to determine whether each TFBS motif was significantly enriched in the gene-set. Raw AUCs for each motif were then normalised to generate a normalised enrichment score (NES), where $NES = (AUC - x) / y$, x referring to the mean of all AUC scores across all features in the motif database and y referring to the standard deviation of all the AUC scores. A NES score of ≥ 4.0 indicated significant enrichment of a TFBS motif within the gene set (659,667,668).

2.4.6 Methylation clocks

2.4.6.1 Horvath methylation clock epigenetic age and age acceleration

Epigenetic age was calculated using the method as described in *Horvath* (2013) (669). The Horvath clock utilises 353 CpGs present on the Infinium HumanMethylation450 BeadChip (450K) (Illumina Inc, San Diego, CA) methylation array to estimate epigenetic age, however 19 of these are specific to this platform and as such are not present on the EPIC array. Consequently, 19 CpGs were removed from the predictor, however studies by both *McEwan et al.* (2018) and *Dhingra et al.* (2019) established this has no effect on the fidelity of the estimations when conducted on the EPIC array (670,671). Epigenetic age acceleration was calculated of the residuals of the regression of epigenetic age against observed gestational age, calculated from the last menstrual period and ultrasound measurements for the MAVIDOS RCT and ultrasound for the SWS study.

2.4.6.2 Knight methylation clock epigenetic gestational age and gestational age acceleration

Epigenetic gestational age was calculated using the method as described by *Knight et al.* (2016) (672). This clock uses 148 CpGs for the estimation of epigenetic gestational age, however 5 CpGs used are specific to the array on which it was developed, the 450K methylation array and not present on the EPIC array. Consequently, five CpGs were removed from the predictor, although this has been previously shown not to adversely affect the accuracy of the epigenetic gestational age calculation. Gestational age acceleration was calculated as the residuals from the regression

of epigenetic gestational age against observed gestational age, which was calculated from last menstrual period and ultrasound measurements for the MAVIDOS RCT and ultrasound for the SWS study.

2.4.6.3 *Statistical analysis*

Gestational acceleration or age acceleration was regressed against phenotypic characteristics using a multivariate regression model consisting of the estimated cell-type composition for the respective tissue, position on beadchip, sex, smoking during pregnancy, respective DXA age (if a bone-related variable of interest). Regression diagnostics, including quantile-quantile, heteroscedasticity, Cook's distance and residuals plots were used to assess the regression model. Phenotypic data was transformed prior to regression using either log or square root transformations where necessary to meet the assumptions of linear regression. Potential outlier samples were assessed by inclusion or exclusion of the sample in a sensitivity analysis to ascertain whether they drive the associations seen.

2.5 **Cell culture**

All cell-culture or cell-culture based experimentation conducted within this dissertation was conducted by the author of this dissertation.

2.5.1 *Cell culture medium*

2.5.1.1 *Primary Umbilical Cord Mesenchymal Stem Cell culture medium*

Primary human UC-MSCs obtained from American type culture collections were cultured in UC-MSC medium (Dulbecco's modified eagle medium (DMEM) containing 15% foetal bovine serum (FBS) and 1% antimycotic-antibiotic (ThermoFisher)).

2.5.1.2 *Osteosarcoma SaOS-2 cell line culture medium*

SaOS-2 cells were cultured in SaOS-2 complete media (Nutrient Mixture F-12 (DMEM/F12) containing 5% FBS, 1% L-glutamine and 1% penicillin-streptomycin).

2.5.2 *Culture conditions of ATCC UC-MSC and SaOS-2 cells*

ATCC UC-MSCs and SaOS-2 osteosarcoma cells were maintained in T175 tissue culture flasks. All mesenchymal stromal cells isolated from umbilical cord were maintained in 100 mm culture dishes on a surface of 0.1% gelatin. All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ and were passaged at 70-80% confluence whereby 0.25% trypsin-EDTA (ThermoFisher) was added to cells and the culture incubated for a minimum of three minutes. Once cells were detached, four volumes of UC-MSC medium were added, the cells transferred to a 50ml centrifuge tube and centrifuged (1000 g, 5 minutes). Supernatant was then removed and cells resuspended in a known quantity of UC-MSC medium and quantified using FastRead slides.

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Cells were re-suspended and re-seeded in T175 flasks at a density of 50,000 cells/cm² and 10,000 cells/cm² for the SaOS-2 cell line and UC-MSCs respectively in an appropriate medium.

2.5.3 *In-vitro cell culture 1,25(OH)₂D treatment protocol*

Stock 1,25(OH)₂D (Enzo Life Sciences) powder was reconstituted in ethanol to form a stock solution at a concentration of 100 µm, which was stored at -20°C. This stock solution was then diluted on the day of treatment to 100x final in-well concentration in either UC-MSC or DMEM/F12 media (500µl) according to the cell type being treated, such that ethanol concentration per well did not rise above 0.1% (Table 3). Treatments below 1µm were formed by serial dilution. Treatments were then aliquoted into the appropriate wells such that they were diluted 100x. Cells were treated for 24 hours and the media was either replaced with fresh medium, or the cells assayed as required.

Table 3. 1,25(OH)₂D concentration per well and associated ethanol concentration when treating cells *in vitro*

1,25(OH) ₂ D concentration per well/nm	Ethanol per well/%
100	0.1
50	0.05
10	0.01

2.6 Assessment of UC-MSC response to vitamin D stimulation

All work described within this section was conducted by the author of this dissertation.

2.6.1 Vector-vitamin D responsive element (VDRE) constructs

2.6.1.1 Generating vector-VDRE construct

2.6.1.1.1 Oligonucleotide annealing

Oligonucleotides (oligos) for both forward and reverse complementary strands were designed to contain a 3x repeated hexameric VDRE consensus sequence characterised from the osteocalcin (OC) promoter by *Ozono et al.* (673), or the CYP24A1 (CYP) promoter by *Chen et al.* (674) (Figure 14). Oligos were designed with KpnI and SacI restriction sites to allow ligation as an insert into the multiple cloning region of the Promega pGL4.23 luc2/minP vector plasmid (Figure 15). The polyacrylamide gel electrophoresis (PAGE) purified oligos, synthesised by Sigma-Aldrich were reconstituted to a final concentration of 200 μ M using nuclease-free water. Oligos were then diluted to a final working concentration of 2 μ M in annealing buffer (Tris 10 mM, EDTA 1 mM, NaCl 50 mM) and placed in a $\sim$ 100°C water bath and allowed to cool overnight. Oligos were stored at 4°C.

2.6.1.1.2 Restriction digest and purification of oligonucleotides and vector

Both the oligos (3 μ g) and vector (3 μ g) were digested with KpnI (5 units) and SacI (5 units) in a total volume of 100 μ l and incubated at 37°C overnight.

2.6.1.1.2.1 Purification of oligonucleotides

Restricted oligonucleotide DNA (1 μ g) was purified using a PCR clean-up kit (New England Biolabs). The kit allowed for the binding of restricted oligonucleotide to the silica membrane within each spin column, allowing for any downstream contaminants to be washed away and the elution of purified DNA. Unless stated otherwise, all centrifugation steps were carried out at 16000 g. Briefly, restricted oligonucleotide was made up to the suggested volume (50 μ l) with nuclease-free water. DNA clean-up binding buffer (100 μ l) and ethanol (95%) (300 μ l) were added to the restricted oligonucleotide and the solution mixed by pipetting. The sample was transferred to a spin column in a collection tube, centrifuged (1 minute) and the flow-through discarded. DNA wash buffer was added to the column, centrifuged, the flow-through discarded and the step repeated once more. To elute the DNA the column was transferred to a microcentrifuge tube, nuclease-free water added (6 μ l), incubated at RT (1 minute) and centrifuged (1 minute). DNA was checked for integrity or to assess the digestion by agarose gel electrophoresis as in section 2.3.4.2 according to the conditions in Table 4. DNA was stored at 4°C.

2.6.1.1.2.2 Gel purification of vector

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The vector (3 µg) was run on an agarose gel as described in section 2.3.4.2 according to conditions in Table 4. The vector DNA bands of the appropriate size were visualised using a ultraviolet (UV) light box, cut from the gels and DNA extracted from the gel sections using the Zymoclean Gel DNA recovery kit (Zymo Research). This kit allowed for the extraction of purified DNA from agarose gel by dissolving the buffer in an ethanol containing agarose dissolving buffer, centrifugation in a silica membrane-containing spin column to which DNA would bind and could be washed, after which purified DNA was eluted separately from potential downstream contaminants. Unless stated otherwise, all centrifugation steps were carried out at 16000 g. Briefly the gel section was transferred to a microcentrifuge tube and three volumes of agarose dissolving buffer added to the tube. Samples were allowed incubate at 55°C (10 minutes) until completely dissolved, the melted solution transferred to a Zymo-Spin™ column in a collection tube upon which and the column centrifuged (1 minute). Flow-through was discarded and DNA wash buffer added (200 µl), after which samples were centrifuged (30 seconds) and the step repeated once more. To elute the DNA, the columns were transferred to a microcentrifuge tube, nuclease-free water (6 µl) added to the column matrix and the column centrifuged (1 minute). The linearised vector plasmid were stored at 4°C.



Figure 14. The annealed oligonucleotide sequences containing 3x repeats of the a) hexameric osteocalcin-derived VDRE sequence and b) hexameric CYP24A1-derived VDRE sequence, accompanying restriction sites and spacing bases
 The oligonucleotide sequence containing the repeated hexameric VDRE sequences (blue), restriction sites (green and purple) and additional bases to allow binding of cellular machinery (yellow). The arrows indicate cleavage sites of restriction enzymes which generate sticky ends, allowing ligation into the multiple cloning region of the pGL4.23 enzyme which has been similarly restricted.

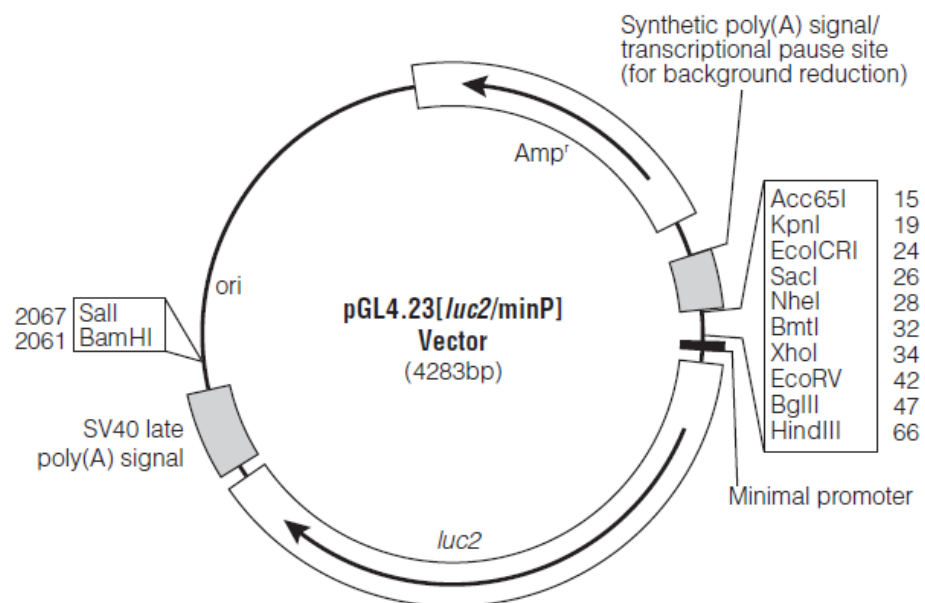


Figure 15. The plasmid map for the pGL4.23 [luc2/minP] vector

Table 4. Conditions for Agarose Gel Electrophoresis

	Percentage agarose gel / %	Run voltage / V	Run time / min
RNA	2	150	15
DNA (Vector)	0.8	120	20
DNA (Oligonucleotides)	3	120	20

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2.6.1.1.3 *Antarctic phosphatase treatment*

A reaction (10 µl) containing linearised vector (500 ng), antarctic phosphatase (AnP) (5 units) and 10x antarctic phosphatase rxn buffer (1 µl) was incubated at 37°C for 60 minutes, then heat inactivated at 80° for 2 minutes. Post-AnP treatment the linearised vector plasmid were stored at 4°C.

2.6.1.1.4 *Ligation*

AnP-treated linearised vector (200 ng) and a 3-fold molar excess of VDRE-containing oligonucleotide (insert) (3 ng) were made up to a final volume of 10 µl with nuclease-free water. Next, 2x ligation reaction buffer was added (10 µl) and mixed. Quick T4 DNA ligase (2,000 units) was then added, mixed and incubated at RT for 5 minutes. The solution was chilled on ice until transformation.

2.6.1.2 *Transformation of vector-VDRE construct*

2.6.1.2.1 *Transformation media*

2.6.1.2.1.1 *Luria Broth agar*

To make media for agar plates, Luria Broth (LB) powder (10 g) and agar powder (7.5 g) were made to a total volume of 500 ml using ultra-pure water. The bottle was autoclaved at 121°C for 15 minutes using the Boxer 200/100L autoclave, and 100ug/ml of carbenicillin (500 µl) added and mixed when cool enough to touch. LB agar plates were prepared by pouring 25 ml of cooled autoclaved LB agar into 90mm Petri dishes and allowed to set.

2.6.1.2.1.2 *Luria Broth media*

LB powder (10 g) was made to a total volume of 500 ml, mixed and autoclaved at 121°C for 15 minutes using a Boxer 200/100L autoclave. Once cooled, Carbenicillin at 100 µg/ml (500 µl) was added to the mixture and mixed thoroughly. The media was stored at 4°C until use.

2.6.1.2.2 *Transformation into JM109 cells*

JM109 *Escherichia coli* (*E. coli*) bacterial cells (Promega) were thawed on ice. Ligated DNA (see section 2.6.1.1.4) (25 ng) was added to a vial of JM109 cells ($>1 \times 10^8$ cfu/µg). Positive control (circular plasmid) and negative control (restricted, AnP-treated plasmid) transformation reactions were included as controls. The tubes were vortexed and incubated on ice for 10 minutes. Once incubated, the cells were transferred to a pre-heated water bath (42°C) for exactly 45 seconds and then returned to ice for 2 minutes. Super optimal broth with catabolite repression media (SOC) (100 µl) (Sigma-Aldrich), a media the composition of which is optimised for the growth and maximal transformation efficiency of *E. coli* was then added to the cells. Cells were next incubated for 60 minutes at 37°C in a shaking incubator at 225 rpm. Post-incubation the cells were aliquoted and spread using sterile culture loops on LB agar plates then left overnight at 37°C.

2.6.1.2.3 *Quality check and creating stock of plasmid constructs*

2.6.1.2.3.1 Growth and purification of plasmid vector

Single JM109 colonies were used to inoculate LB media (5 ml) and incubated at 37°C in a shaking incubator at 225 rpm overnight. Plasmid DNA was extracted using the QIAprep spin miniprep kit (Qiagen). The kit allowed for the isolation of plasmid DNA by the alkaline lysis of bacterial cells and the adsorption of the DNA on to a silica membrane in a high salt environment. Lysate could then be cleared by centrifugation, leaving only purified plasmid DNA. Briefly, bacterial cultures were decanted into 15 ml centrifuge tubes, centrifuged at 4°C (5400 g, 10 minutes) and the supernatant removed. The pelleted bacterial cells were resuspended in buffer P1 (250 µl) and transferred to a microcentrifuge tube, after which buffer P2 was added (250 µl) and the contents of the tube mixed by inversion. Buffer N3 was added, the tube mixed by inversion and centrifuged (17900 g, 10 minutes). The supernatant (800 µl) was transferred to a QIAprep 2.0 spin column in a 5ml collection tube and centrifuged (3000 g, 1 minute). Columns were then washed by the addition of buffer PB (500 µl) and centrifugation (3000 g, 1 minute), followed by the addition of buffer PE (750 µl) and further centrifugation (3000 g, 1 minute). Columns were transferred to a microcentrifuge tube and centrifuged (17900 g, 1 minute) to remove residual buffer. Finally, to elute plasmid DNA, the spin column was placed in a clean, 1.5ml microcentrifuge tube, nuclease-free water added (50 µl) to the centre of each column, allowed to incubate at RT (1 minute) and centrifuged (17,900 g, 1 minute). Once eluted, plasmid DNA quantity and quality were assessed using a nanodrop spectrophotometer as in section 2.3.4.1 and stored at 4°C.

2.6.1.2.3.2 Size of plasmid vector

To ascertain whether the plasmid DNA was of the correct size, 500 ng DNA was digested with KpnI restriction enzyme as stated in 2.6.1.1.2 to produce a linearised vector. Once digested, the linearised vector was run on a 0.8% agarose gel as in section 2.3.4.2 according to the conditions in Table 4 to assess the integrity of the samples and determine which samples contained plasmid with insert for sequencing.

2.6.1.2.3.3 Sequence of plasmid vector

Purified plasmid DNA was diluted in nuclease-free water (1:8) post-miniprep to a final volume of 20 µl. All transformation reactions excluding controls were sequenced along with the stock pGL4.23 plasmid. Plasmids were sequenced by GATC Biotech using pGL3-RV3 and pGL2 primers for forward and reverse directions. Sequences were aligned using clustal omega to check for similarity to either the predicted pGL4.23-ostecalcin VDRE (pGL4.23-OC VDRE) or pGL4.23-CYP24A1 (pGL4.23-CYP VDRE) VDRE sequence.

2.6.1.2.3.4 Preparation of glycerol stock

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To create glycerol stocks to allow for the future culture and propagation of plasmid DNA, the transformed bacterial culture (350 µl) was added to sterile 30% glycerol (350 µl), vortexed thoroughly and snap frozen on dry ice. The aliquots were then stored at -80°C.

2.6.1.2.4 *Preparing vector constructs for transfection*

2.6.1.2.4.1 Midiprep

A 5 µl sterile loop was used to inoculate an LB agar plate with DNA glycerol stock and incubated at 37° overnight. Single bacterial colonies were then sub-cultured into 100ml fresh LB broth and incubated overnight in a shaking incubator (37°C at 225 rpm). Bacterial cultures were harvested by centrifugation at 4°C (5000 g, 10 minutes), the supernatant discarded and plasmid DNA isolated and purified using the GeneJET Plasmid Midiprep Kit (Thermo Fisher). The kit allowed for the isolation of plasmid DNA through the alkaline lysis of bacterial cells to liberate plasmid DNA. Unwanted lysate was precipitated out via neutralisation and upon addition of the total lysate to a spin column purified plasmid DNA adhered to the silica membrane, whereas unwanted lysate was washed away leaving the purified plasmid DNA to be eluted separately. Briefly, resuspension solution was added to the pellet (2 ml) and the samples vortexed or pipetted until homogenous. Lysis solution (2 ml) was then added and mixed by inversion until viscous and slightly clear, after which the sample was incubated at RT (3 minutes). Neutralising solution was added (2 ml), mixed by inversion, endotoxin binding reagent added (0.5 ml), the sample further mixed by inversion and incubated at RT (3 minutes). Once incubated, 96% ethanol was added, mixed by inversion and centrifuged (5000 g, 40 minutes). Supernatant was removed from the pelleted white precipitate, 96% ethanol added (3 ml) and mixed by inversion. A volume of supernatant was transferred to a spin column in a collection tube as to not overfill, centrifuged (2000 g, 3 minutes) and the process repeated until all lysate had been passed through a spin column. Wash solution I was then added (4 ml), the column centrifuged (3000 g, 2 minutes) and the flow-through discarded. The wash step was then repeated twice with wash solution II. Columns were centrifuged to remove residual wash solution and transferred to a new 15 ml collection tube wherein elution buffer was added (0.35 ml) to the centre of the column membrane, incubated at RT (2 minutes) and centrifuged. Once eluted plasmid DNA quantity and quality was assessed using a nanodrop spectrophotometer and agarose gel electrophoresis according to section 2.3.4. Purified plasmid was stored at 4°C.

2.6.1.2.4.2 Sterilising plasmid DNA for cell culture

Plasmid DNA (50 µg) was made up to 300 µl with nuclease-free water, then 1/10th the volume of sodium acetate (pH5.2) and two volumes of ice cold 100% ethanol were added and incubated overnight at -20°C. Plasmid DNA was then centrifuged (13,000 rpm, 15 minutes) and all further steps were carried out in a class II safety cabinet. The supernatant was removed and the DNA resuspended in 70% ethanol (1 ml). The samples were centrifuged as above and the supernatant

aspirated and pellets air-dried for 5 minutes. The DNA pellet was resuspended in cell-culture grade water (200 µl), the quantity and quality of plasmid DNA determined using the nanodrop spectrophotometer as in section 2.3.4.1.

2.6.1.3 *Transfection of vector-VDRE construct*

2.6.1.3.1 *Transfection of SaOS-2 osteosarcoma and ATCC UC-MSCs*

SaOS-2 cells were seeded at 1×10^5 cells per well of a 24-well plate in DMEM/Ham's F-12 media (1 ml) and incubated overnight. ATCC UC-MSC cells were seeded at 2.5×10^4 cells per well of a 24-well plate in UC-MSC medium (1 ml) and incubated overnight.

2.6.1.3.2 *Transfection of plasmid DNA*

Cells were seeded according to section 2.5.2. After 24 hours cells were transfected with plasmid DNA using the FuGENE 6 transfection reagent (Promega). FuGENE 6 is a cationic non-liposomal transfection reagent which forms a positively charged complex with plasmid DNA. This allows the complex to bind to the negatively charged cell membrane and enter the cell via the endosomal pathway. To transfect the cells, briefly, Opti-MEM media and the FuGENE 6 reagent were both allowed to reach RT and FuGENE 6 vortexed immediately before use. The volume of Opti-MEM required to make a final transfection mixture volume of 25 µl/well was added to an Eppendorf and FuGENE 6 (3 µl) was added directly to the medium. The mixture was then vortexed, pulse spun and incubated for 5 minutes at RT. Once incubated, the reporter plasmid (492.5 ng/well) and Renilla luciferase control plasmid (4.5 ng/well) were added to the appropriate reagent mixtures, pulse spun and left to incubate at RT for 15 minutes. Final transfection mix (25 µl/well) was added to cells in the appropriate wells and carefully pipetted up and down to mix the medium. After six hours the appropriate wells were treated with $1,25(\text{OH})_2\text{D}$ as described in section 2.5.3. After 24 and 48 hours the appropriate wells were prepared for the luciferase reporter assay as described in section 2.6.1.4.1.

2.6.1.4 *Luciferase reporter assay*

2.6.1.4.1 *Assessing luciferase activity*

Media was removed and cells rinsed with phosphate buffered saline (PBS). All liquid was aspirated and 1x passive lysis buffer (Promega) added to each well (150 µl). Plates were stored at -80°C until use. To assay, plates were thawed at RT on a rocking platform for 15 minutes until all cells were lysed. If lysis was incomplete, cells were mixed with a pipette or further incubated or repeatedly freeze-thawed until complete. Lysed cell material (30 µl) was transferred to a white opaque 96 well plate for the assessment of luciferase activity.

Luminescence was determined using the Promega GloMax® Explorer System luminometer, using the pre-set 'Dual luciferase reporter assay system' program. Prior to assay the injectors were

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flushed with deionised water, 70% ethanol, deionised water and finally air. Once flushed, injectors were primed with either Luciferase Assay Reagent II (500 µl) or Stop and Glo reagent (500 µl). The luciferase assay reagent II was added to each well (100 µl), five seconds elapsed and the subsequent firefly luciferase luminescence read in each well. Stop and Glo reagent was then added (100 µl), five seconds elapsed and the subsequent *Renilla* luciferase luminescence read in each well.

2.6.1.4.2 Statistical analysis

Firefly luciferase values were normalised to the *Renilla* luciferase expression. Statistical analysis was conducted using GraphPad Prism 7. A three-way analysis of variance (ANOVA) analysis was conducted between treatment time, vector with insert or without and vehicle or 1,25(OH)₂D (50 nm) treated cells.

2.6.2 RT-qPCR

2.6.2.1 Isolation of total RNA

2.6.2.1.1 TRI Reagent

TRI Reagent (TRIzol) was used for the isolation of SaOS-2 and UC-MSC RNA directly from 6-well culture plate wells. TRIzol (500 µl) was added to each well and incubated at RT (5 minutes), after which samples were transferred to microcentrifuge tubes and centrifuged at 4°C (12000 rpm, 10 minutes). The upper phase was transferred to a clean tube, chloroform added (500 µl), briefly vortexed to mix and left to incubate at RT (15 minutes). Once incubated, samples were centrifuged at 4°C (12000 rpm, 15 minutes) and the now distinct clear top layer transferred to a new microcentrifuge tube. To the new tube isopropyl alcohol was added (75%), mixed by inversion and incubated at -20°C to precipitate overnight. Post-incubation RNA was pelleted by centrifugation at 4°C (12000 rpm, 20 minutes) and the supernatant removed. The remaining pellet was washed by the addition of ethanol (75%) (1000 µl), mixed and centrifuged at 4°C (7500 rpm, 5 minutes). The previous washing step was repeated once more and the pellet allowed to dry at RT (5-10 minutes). The RNA pellet was resuspended in nuclease-free water (30 µl) and the quantity and quality of extracted RNA was analysed using a Nanodrop spectrophotometer as in section 2.3.4.1. A NanoDrop 260/280 ratio of ≥1.8 confirmed the quality of the samples.

2.6.2.1.2 Qaigen RNeasy kit

The Qaigen RNeasy kit was used for the isolation of SaOS-2 cell and UC-MSC RNA from cells seeded and cultured in 6-well cell culture plates. Cells were trypsinised as described in section 2.5.2, the cells pelleted by centrifugation (1000 g, 5 minutes) and media removed completely. Cell pellets were lysed with the addition of buffer RLT (350 µl) and vortexed (1 minute) to homogenise the cell lysate. Ethanol (70%) (350 µl) was added to promote selective binding of RNA to the RNeasy mini spin column membrane, mixed by pipetting and the sample (700 µl) transferred to an

RNeasy Mini spin column in a collection tube. The column was then centrifuged (8000 g, 15 seconds) and the flow-through discarded. To wash away potential contaminants of the final extracted RNA product, buffer RW1 was added (700 μ l), the column centrifuged (8000 g, 15 seconds) and the flow-through discarded. Buffer RPE (500 μ l) was then added to the column, the previous centrifugation step repeated and the flow-through discarded. An additional volume of buffer RPE (500 μ l) was also added and the column centrifuged (8000 g, 2 minutes) to dry the spin column membrane. Finally, to elute the RNA the column was transferred to a microcentrifuge tube, nuclease-free water applied to the spin column membrane and then centrifuged (8000 g, 1 minute). The quantity and quality of extracted RNA was analysed using a Nanodrop spectrophotometer as in section 2.3.4.1. The quality of the RNA samples was confirmed by a NanoDrop 260/280 ratio of ≥ 1.8 .

2.6.2.2 *cDNA conversion*

Unless stated otherwise, all incubation steps above RT were conducted in an Applied Biosystems™ Veriti™ 96-well thermal cycler (ThermoFisher).

The RNA was diluted to 125ng/ μ l in nuclease-free water and 1 μ g (8 μ l) added to each reaction. Amplification grade DNase I (1 unit) and 10x reaction buffer (1 μ l) (Sigma-Aldrich) and were added to each reaction, briefly centrifuged and incubated at RT for 15 minutes. Stop solution was added (1 μ l) (Sigma-Aldrich), heated at 70°C for 10 minutes and maintained at 4°C. Next, 1 μ l of deoxynucleotide triphosphate (dNTP) mix (10 nM) (Promega) and 1 μ l of random nonamers (50 μ M) (Sigma-Aldrich) were added to the DNase treated RNA. The RNA was then centrifuged briefly and incubated as above. To generate the cDNA, 10x M-MLV reverse transcriptase buffer (4 μ l) (Promega), M-MLV reverse transcriptase (200 units) (Promega) and nuclease-free water (5 μ l) (Promega) were added to each reaction. RNA was incubated at RT for 10 minutes, 37°C for 50 minutes, 90°C for 10 minutes and maintained at 4°C. The final cDNA concentration was at 50 ng/ μ l and diluted to 15 ng/ μ l for RT-qPCR using nuclease-free water.

2.6.2.3 *Real time quantitative polymerase chain reaction (RT-qPCR)*

A total of 30 ng of cDNA was amplified in a 10 μ l reaction mixture containing primer (1 μ l), Quantifast SYBR green (5 μ l) and nuclease-free water. RT-PCR was carried out using the Roche LightCycler® 480 Instrument II (Roche). The program entailed 40 amplification cycles. Each cycle was 95°C for 10 seconds and 60°C for 30 seconds at which time a fluorescence reading was acquired. Melt curve acquisition was performed to assess each sample for the presence of any primer-dimer or contaminating DNA. The program consisted of 95°C for 15 seconds, 60°C for 1 minute and an increase of temperature to 95°C during which continuous fluorescence readings were acquired.

2.6.2.3.1 *GeNorm assay*

The GeNorm assay kit (PrimerDesign) allows for the selection of appropriate reference genes for RT-qPCR analysis. Briefly, a RT-qPCR assay for six reference genes commonly used for UC-MSCs were run on three samples for each of a set of 100 nm, 50 nm, 10 nm, 1 nm, 0.1 nm and 0.01 nm 1,25(OH)₂D treated and un-treated ATCC UC-MSCs. Reference genes analysed were Beta-2-microglobulin, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), 60S ribosomal protein L13a, Ubiquitin C, Cytochrome C and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each 10 µl reaction consisted of 1 µl primer (working concentration 300 nm), SYBR green master mix (5 µl), RNase-free water (1 µl) and cDNA (3 µl). Melt curves were also run to determine the presence of any primer-dimer or genomic DNA contamination. Results were interpreted using qbase+ qPCR analysis software.

2.6.2.3.2 *Analysis*

Target gene cycle threshold (CT) values and melt curve analysis were generated by Roche LightCycler® 480 SW 1.5.1 software. CT values were normalised to the mean CT of YWHAZ and GAPDH, established as appropriate reference genes for UC-MSCs using GeNorm analysis (see section 2.6.2.3.1). In order to quantify the relative expression of target genes, the $2^{-\Delta\Delta Ct}$ method was used and plotted as fold-change in expression. Multiple t-tests, one per row were used to assess the statistical significance of results using GraphPad 7 software.

2.6.3 *Primary UC-MSC isolation*

2.6.3.1 *Isolation of mesenchymal stromal cells from Nipper and SPRING umbilical cord*

Umbilical cords (UC) were collected post-processing and stored in Hanks balanced salt solution for an average of 1 hour at 4°C until processing. To extract mesenchymal stromal cells (MSCs) from the Wharton's jelly, UCs were cut to 4 cm lengths and excess blood removed. One section of cord was snap frozen and stored at -80°C for future analysis. The remaining sections were cut longitudinally to expose the Wharton's jelly and then digested face-down in enzymatic solution (5 ml pre-warmed UC-MSC medium containing final concentrations of Collagenase (I) (2 mg/ml), collagenase (IV) (2 mg/ml) and hyaluronidase (100 IU/ml)), ensuring only the Wharton's jelly came into contact with the enzymatic solution. UC were then incubated at 37°C for 45 minutes.

Following incubation, the UC sections were transferred to a petri dish containing equilibrated UC-MSC medium and the digested Wharton's jelly was teased into the UC-MSC medium. Medium was then transferred to a 15ml centrifuge tube, resuspended and centrifuged at 300g for 5 minutes at RT. The pellet was then resuspended in UC-MSC medium and the wash step repeated. Cells were then plated onto two 100mm culture dishes pre-coated with 0.1% gelatin and incubated until 70-80% confluent. Media was changed every two days.

2.6.3.2 *Freezing for storage and future processing*

Primary MSCs isolated from umbilical cord tissue were frozen for later resuscitation, DNA and RNA extraction according to the culture schedule in Figure 16.

To freeze for later resuscitation, cells were trypsinised as in section 2.5.2 and resuspended in UC-MSC medium. Cells were quantified with FastRead slides and half of the cells reseeded at a minimum of 350 cells/cm². Remaining cells were centrifuged at 1000g for 5 minutes at RT, supernatant removed and freezing media (8 ml) (Dimethyl sulphoxide 1x, FBS 10x) added to cells which were then chilled on wet ice. Cryovials were frozen in a Mr. Frosty tub at -80°C for 24 hours before transferring to liquid nitrogen storage.

To freeze for future DNA and RNA extraction, cells were trypsinised as in section 2.5.2 and centrifuged at 1000g for 5 minutes at RT. Supernatant was removed and the pellet resuspended in UC-MSC medium (4 ml) which was evenly distributed between four 1.5 ml Eppendorf tubes. Cells were centrifuged once more at 1000g for 5 minutes at RT, supernatant discarded and the cells pulse centrifuged to remove any remaining medium before snap freezing on dry ice and transferring to -80°C.

2.6.3.3 *Resuscitating cells for culture*

Cells were semi-immersed in a water bath at 37°C until defrosted. A minimum of 500,000 cells were added to each T175 tissue culture flask.

2.6.4 *Primary UC-MSC enrichment*

Umbilical cord tissue is a heterogenous mixture of endothelial, epithelial, smooth muscle and stromal cells. Therefore, to exclude the potential for the proliferation of contaminating cell types introduced during the isolation process (as detailed in section 2.6.3), a series of steps were taken to enrich our UC-MSC cultures.

2.6.4.1 *Preparation and staining of cells for flow cytometry*

Cells were trypsinised, washed twice with PBS. A minimum of 200,000 cells were made up to a final volume of 100 µl in with PBS and labelled with the cluster of differentiation 29-phycoerythrin (CD29-PE) (20 µl) or cluster of differentiation 44-Fluorescein isothiocyanate (CD44-FITC) (20 µl) antibodies, vortexed and incubated in the dark for 20 minutes at 4°C or with propidium iodide (PI) (5 µl) for 10 minutes. Isotype-matched control antibodies (20 µl) were used as controls. The sample was washed twice with staining wash (PBS 98%, FBS 2%) (4°C, 1 ml) vortexed and centrifuged for 5 minutes at ~1000g. The supernatant was poured off and the wash step repeated. The sample were resuspended in PBS (200 µl) and passed through a FACSCalibur flow cytometer using CellQuest software to capture 10,000 events for each FACS tube.

2.6.4.2 *Flow cytometry analysis*

Data was analysed using FlowJo10 software. Unless stated otherwise 10,000 events were captured for each assay and the proportion of stained cells in a population assessed alongside an overlaid unstained, control population. The gating strategy for flow cytometry assays in this study dictates that $\geq 99\%$ of the unstained control population is to be held in the lower left-hand quadrant gate, to which the staining of the rest of the population can be determined.

2.6.4.3 *Validation of anti-CD29 PE and anti-CD44 FITC markers antibodies*

A vial of isolated UC-MSCs was resuscitated at P3 and grown until 70-80% confluent. The cells were prepared for flow cytometry as described in section 2.6.4.1. The FACSCalibur flow cytometer was calibrated with compensation beads stained with the selected antibodies and voltages adjusted appropriately for PE and FITC fluorescence channels. Once compensated, cells were either unstained or stained with CD29, CD44 or both CD29 and CD44 antibodies as described above. A total of six assays were conducted in duplicate, gated according to section 2.6.4.2 and all results overlaid to demonstrate the fraction of cells staining positively for their respective assay antibody.

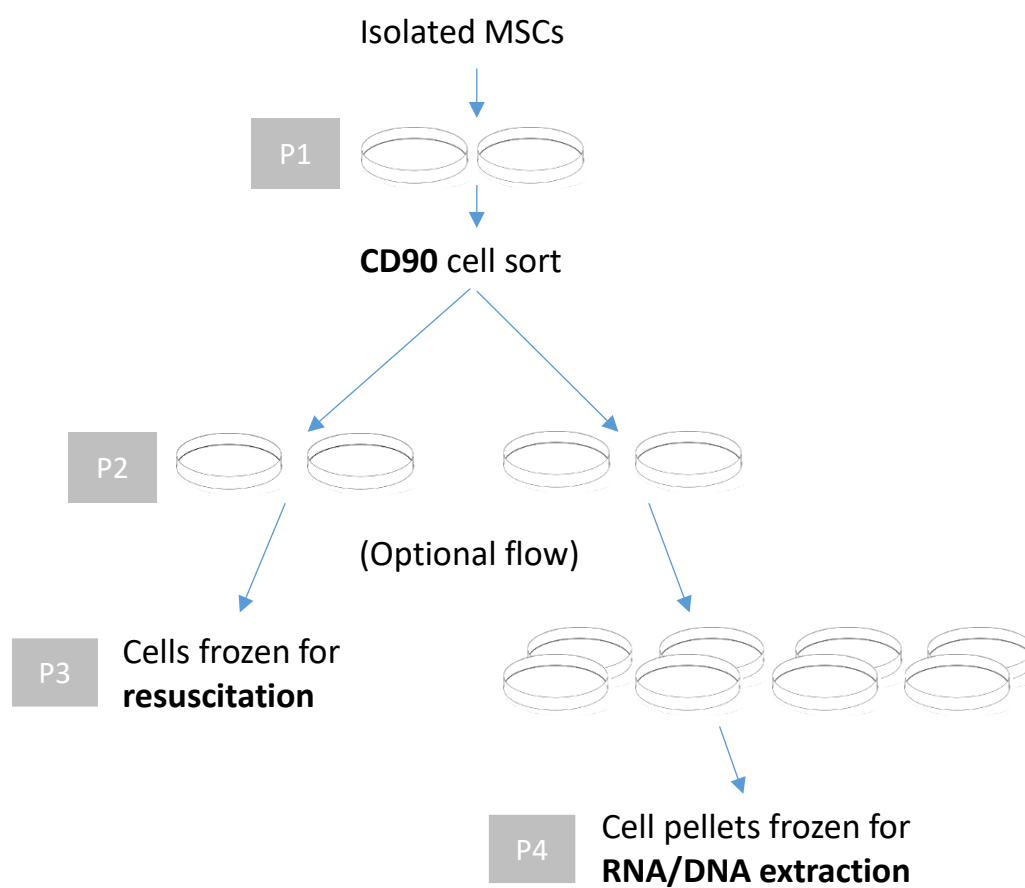


Figure 16. The culture schedule for MSCs isolated from umbilical cord from isolation to freezing

2.6.4.4 *Cell sorting*

Unless stated otherwise, all centrifugation steps and reagents were chilled to 4°C prior to use.

UC-MSC cultures were positively sorted according to the presence of the CD90 antigen, one of three markers defined by the International Society for Cell and gene Therapy (ISCT) as expressed by mesenchymal stromal cells. CD90 positive cells were isolated from the culture using the Miltenyi Biotech MiniMACS kit and CD90 microbeads. A collection column from this kit, when placed in a magnetic field allowed for the retention of CD90 microbead labelled cells from a culture passed through the column. Unlabelled cells, or the CD90 negative fraction of the culture passed through the column unencumbered. Once removed from the magnetic field, magnetically retained CD90 microbead labelled cells were eluted as the positively sorted cell fraction. Briefly, cells were grown until 70-80% confluent at passage 1 (P1), trypsinised as described in section 2.5.2 and passed through a 40 µM cell-strainer. Cells were centrifuged (1000 g, 3 minutes), the cell suspension removed by pipetting and the cell pellet resuspended in buffer (80 µl) (PBS, 0.5% bovine serum albumin, 2 mM EDTA). CD90 microbeads (20 µl) were added, mixed well by inversion and the mixture incubated at 4°C (15 minutes). Once incubated, additional buffer was added (2 ml) and the mixture centrifuged (300 g, 10 minutes, 4°C). Post-centrifuging, the supernatant was removed by pipetting and the cell and bead mixture resuspended in buffer (500 µl). Isolation columns were prepared by placing in the magnetic field of a MACS separator above a 15 ml collection tube, buffer added (500 µl) and the flow-through discarded. The cell suspension was applied to the column and allowed to pass through, after which three volumes of buffer (500 µl) were added and the flow-through collected as the CD90-negative fraction of cells. The column was removed from the magnetic field and placed on a new collection tube, buffer added (1 ml) to the column and the CD90-positive fraction of cells immediately flushed through by the firm application of the plunger supplied with the column. The eluted, positively sorted fraction was washed twice in UC-MSC medium (section 2.5.1.1) and seeded onto 0.1% gelatin coated 100 mm culture dishes in UC-MSC medium (10 ml).

2.6.4.4.1 *Cell sorting analysis*

Cultures were grown until 70-80% confluent and then trypsinised as in section 2.5.2. The culture was cell sorted as in section 2.6.4.4 whereby unsorted, CD90 negative and positive fractions were isolated from the process. Each fraction of cells was split into four treatment groups, un-stained, CD29+ CD44+, stained with an isotype control antibody, PI stained and processed for flow cytometry as in section 2.6.4.1.

2.6.4.4.2 *Isolated UC-MSC culture on plastic and gelatin*

Cultures were grown until 70-80% confluent and then trypsinised as in section 2.5.2. Cells were then and seeded onto 100 mm, uncoated culture dishes or culture dishes coated with 0.1%

gelatin. Cells were seeded, grown until 70-80% confluent and processed for flow cytometry as mentioned in section 2.6.4.1.

2.7 Statistical analysis

Unless stated otherwise, all experiments were performed at least twice in duplicate wells. All bioinformatical analysis was carried out in R (v3.5.1). Graphs were created using the ggplot2 package in R or GraphPad Prism 7, unless otherwise specified. An FDR <0.05 or a p -value <0.05 was classed as statistically significant.

3 Chapter 3 – DNA methylation at birth is associated with BMC and BMD in infancy and childhood in the SWS cohort

3.1 Introduction

Peak bone mass is the amount of skeletal tissue accrued at the end of skeletal maturation, a stage of growth which typically occurs between 25 and 30 years of age and will significantly, in-part influence both bone mineral content (BMC) and bone mineral density (BMD) into adulthood (675). After reaching peak bone mass the skeleton is predominantly remodelled, therefore the amount of bone tissue accrued in early life and the rate of bone loss in later life will both contribute significantly to the risk of conditions characterised by a paucity of bone, such as osteoporosis. The variance in peak bone mass within a population has contributions from both genetics and environment, where both the pre and postnatal environment may account for around 20–40% of variance in peak bone mass (9,510,512). The effect of environment on phenotype may be in part mediated by epigenetic processes, one of the most commonly studied of which is DNA methylation (534,676). Previous studies in the SWS cohort focusing on methylation of the retinoid X receptor- α (*RXR α*) gene, a factor required for nuclear $1\alpha,25$ -dihydroxyvitamin D3 ($1,25(\text{OH})_2\text{D}$) activity found the perinatal methylation of four promoter cytosine-phosphate-guanine (CpG) sites of the *RXR α* gene in umbilical cord blood DNA was inversely associated with childhood BMC at four years of age (2,3). This indicates it may be possible to identify epigenetic marks at birth which may be useful predictive biomarkers of later bone outcomes and individuals potentially at risk for bone disease. We therefore aimed to further this work by interrogating epigenome-wide DNA methylation of Southampton Women's Survey (SWS) umbilical cord blood DNA using the Illumina Infinium MethylationEPIC BeadChip (EPIC) array which allows for the measurement of DNA methylation across 850,000 CpG sites, permitting an in-depth analysis of the methylation changes across the genome associated with BMC or BMD at the ages of birth, four, six or eight years of age.

3.1.1 Aims

The aim of this chapter was to identify DNA methylation signatures in umbilical cord blood DNA associated with offspring BMC or BMD at birth, four, six or eight years of age in the SWS cohort.

3.2 Results

To identify and compare methylation changes associated with BMC and BMD at birth, four, six and eight years of age, the 467 DNA samples were extracted from the umbilical cord blood of neonates within the SWS cohort and analysed using the EPIC array. The EPIC array interrogates 850,000 CpG sites in the genome with CpG sites focused within gene promoters, enhancers and

key regulatory sequences (677). Differential methylation analyses and comparisons made between analyses are detailed in (Figure 17).

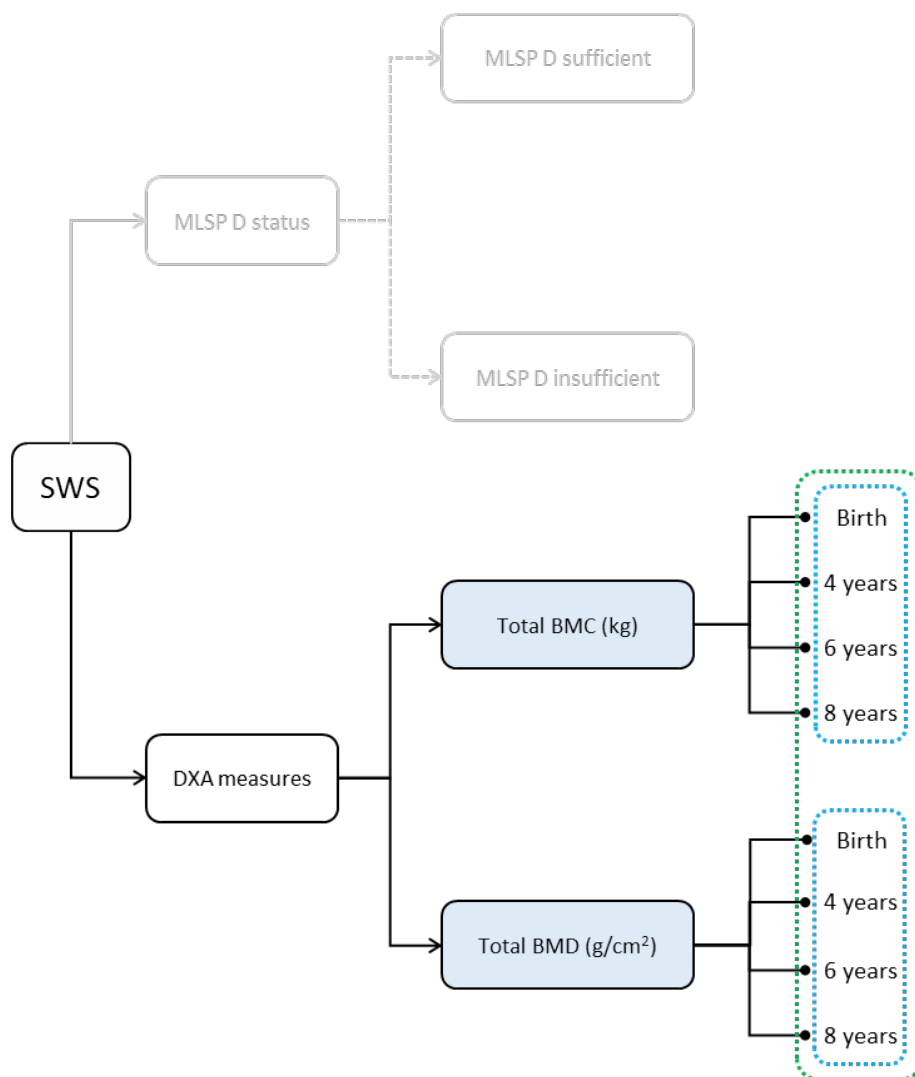


Figure 17. Differential methylation analyses and comparisons made between analyses with respect to DXA bone measures of both total body BMC and BMD at birth, four, six and eight years of age in the SWS cohort

Within the SWS cohort, differential methylation analyses were conducted with respect to umbilical cord blood DNA methylation and DXA measures of total BMC (kg), total BMD (g/cm²) at birth, 4, 6 and 8 years of age. A comparison of the top 100 dmCpGs when ranked by FDR was made between all age groups within BMC or BMD related analysis groups, indicated by light blue boxes. Comparisons between BMC and BMD related analyses were made between measures taken at the same age, where dmCpGs were produced in both analyses with an FDR<0.25 (green boxes).

3.2.1 Cohort characteristics

In the SWS cohort there were 467 individuals for which both umbilical cord blood EPIC array methylation data and usable dual energy X-ray absorptiometry (DXA) data at either: birth, four, six or eight years of age was available. Two samples were removed during quality control (QC), and after exclusion of individuals born prematurely (<37 weeks) $n=444$ individuals remained in the final analysis cohort. Median maternal age at birth was 31.49 years, and 13.1% smoked during pregnancy. The median maternal serum 25-hydroxyvitamin D3 (25(OH)D) concentration increased slightly from 63.5 nmol/l in early pregnancy (11 weeks) to 66 nmol/l in late pregnancy (34 weeks) (Table 5).

The neonates were all Caucasian, a reflection of the predominant ethnicity of the population within the immediate geographical area of the city of Southampton. Individuals had a median gestational age of 40.14 weeks and were 51.4% male. With respect to season, previously seen to be a potential mediating factor for the influence of maternal vitamin D supplementation on postnatal bone growth (625), 28.8% of the offspring were born in the autumn, 27% in the summer, 23.4% in the winter and 20.7% in the spring. Table 6 shows the median offspring age at DXA and accompanying median BMC and BMD measures at the ages of birth, four six and eight years of age.

3.2.2 SWS QC

3.2.2.1 Beta values QC

The EPIC array incorporates two generations of probe assay to detect methylation at a CpG site, as shown in Figure 12 (678). The excitation of integrated fluorophore-tagged nucleotides generates fluorescence intensity values which are imported into R as intensity data (IDAT) files to generate beta values for each probe site.

Prior to differential methylation analysis taking place the beta values underwent a series of QC steps (Figure 13). Beta values were used to generate principal component analysis (PCA) and singular value decomposition (SVD) plots to indicate any clustering of samples and the contribution of each phenotypic characteristic to variance within the dataset. A PCA plot of the first two principal components indicated strong clustering based on sex (Figure 18a), but no clustering or batch effect based on beadchip (Figure 18b). However, in contrast, a post-normalisation SVD plot demonstrated a significant influence from beadchip (Sentrix ID) (Figure 19) suggesting the influence of a batch effect and so a need for batch correction. Beta values were therefore adjusted using the ComBat function of the surrogate variable analysis (SVA) package (v3.20.0) to obtain batch-corrected beta values that could be used for further investigation.

The final step of QC was the calculation of the median absolute deviation score (MADscore), used to identify any samples which may be outliers in the dataset, where a score of < -5 was classified

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as an outlier. Two samples were found to be outliers and excluded from further analysis, leaving 465 samples in the dataset.

Table 5. Maternal cohort characteristics for the SWS cohort

Values are n (%), or median (5th, 95th percentile). A sufficient early or late pregnancy serum 25(OH)D equates to a blood serum 25(OH)D concentration of ≥ 50 nmol/l, insufficient ≥ 25 nmol/l and < 50 nmol/l and deficient < 25 nmol/l. Group/number refers to the subgroup of a phenotypic characteristic group, or the number within the phenotypic characteristic group if no subgroups exist.

Phenotypic characteristic	Group/number	N (%) or median (5th, 95th percentile)
<i>Ethnicity</i>	White	440 (100%)
	Other	0 (0%)
<i>Maternal age at birth</i>	444	31.49 (24.59, 36.39)
<i>Smoking during pregnancy</i>	No	386 (86.9)
	Yes	58 (13.1)
<i>Maternal BMI (kg/m²)</i>	444	24.07 (19.64, 35.11)
<i>Early pregnancy: Serum 25(OH)D at 11 weeks (nmol/l)</i>	351	63.5 (25.5, 109)
<i>Early pregnancy: Serum 25(OH)D at 11 weeks categories (nmol/l)</i>	Sufficient	233 (66.4)
	Insufficient	105 (29.9)
	Deficient	13 (3.7)
<i>Late pregnancy: Serum 25(OH)D at 34 weeks (nmol/l)</i>	422	66 (28.95, 124.95)
<i>Late pregnancy: Serum 25(OH)D at 34 weeks categories (nmol/l)</i>	Sufficient	282 (66.8)
	Insufficient	129 (30.6)
	Deficient	11 (2.6)

Table 6. Offspring cohort characteristics for the SWS cohort

Group/number refers to the subgroup of a phenotypic characteristic group, or the number within the phenotypic characteristic group if no subgroups exist.

Phenotypic characteristic	Group/number	N (%) or median (5th, 95th percentile)
<i>Gestational age (weeks)</i>	444	40.14 (37.86, 41.88)
<i>Sex of offspring</i>	Female	216 (48.6)
	Male	228 (51.4)
<i>Season of birth</i>	Spring	92 (20.7)
	Summer	120 (27)
	Autumn	128 (28.8)
	Winter	104 (23.4)
<i>Baby DXA: Age (days)</i>	219	6 (1, 14)
<i>Baby DXA: Total BMC (kg)</i>	219	0.06 (0.04, 0.09)
<i>Baby DXA: Total BMD (g/cm²)</i>	219	0.52 (0.49, 0.58)
<i>4-year DXA: Age (years)</i>	346	4.11 (4.03, 4.22)
<i>4-year DXA: Total BMC (kg)</i>	339	0.37 (0.3, 0.46)
<i>4-year DXA: Total BMD (g/cm²)</i>	339	0.49 (0.43, 0.56)
<i>6-year DXA: Age (years)</i>	398	6.7 (6.33, 7.35)
<i>6-year DXA: Total BMC (kg)</i>	384	0.53 (0.43, 0.67)
<i>6-year DXA: Total BMD (g/cm²)</i>	384	0.59 (0.52, 0.69)
<i>8-year DXA: Age (years)</i>	391	9.21 (8.92, 9.63)
<i>8-year DXA: Total BMC (kg)</i>	388	0.71 (0.56, 0.93)
<i>8-year DXA: Total BMD (g/cm²)</i>	388	0.66 (0.57, 0.74)

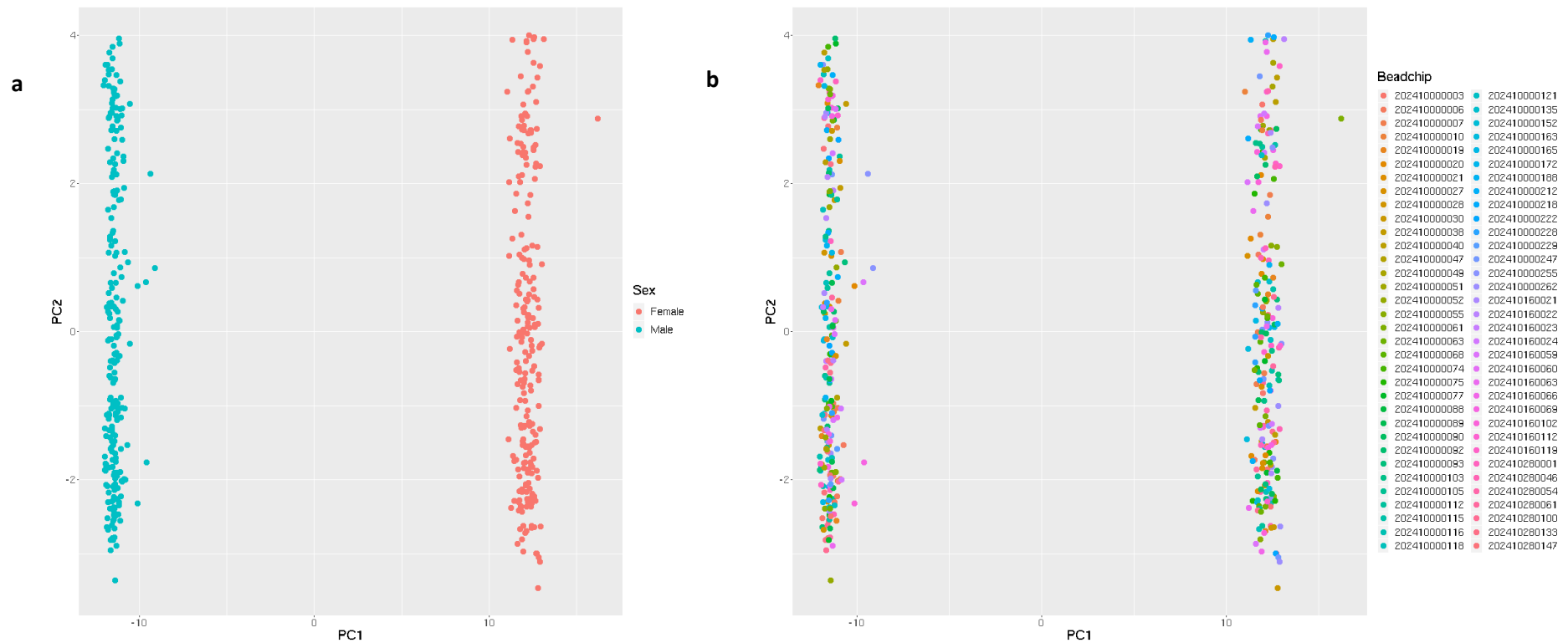


Figure 18. Scatter plots of the first two principal components generated from the principal component analysis of SWS UCB DNA sample methylation data coloured by a) offspring sex and b) beadchip

Scatter plots of the first two principal component values generated for each sample by the principal component analysis of methylation data acquired from the EPIC array interrogation of SWS cohort umbilical cord blood DNA, coloured by a) offspring sex and b) EPIC array beadchip. Plots of the first two principal components showed a) strong clustering of samples based on sex indicating a strong influence of sex on methylation within the dataset and no samples with a misclassified sex. In contrast b) no noticeable clustering of samples was seen with respect to beadchip, suggesting no batch effect was present in the data.

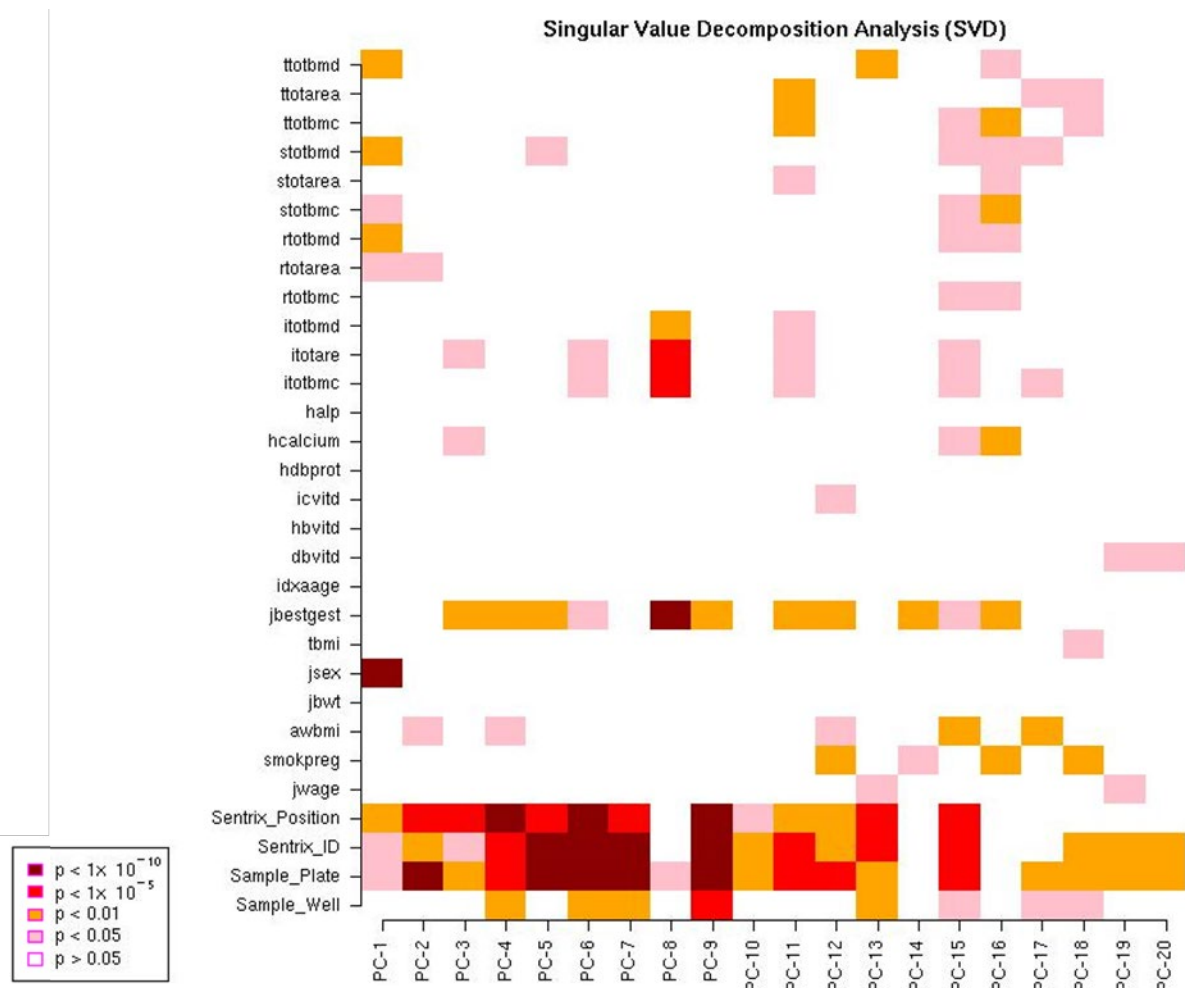


Figure 19. Singular value decomposition plot of post-normalisation methylation data generated from the EPIC array interrogation of UCB DNA from the SWS cohort

The EPIC array interrogation of SWS umbilical cord blood DNA generated methylation data for each sample which post-QC was subjected to a singular value decomposition (SVD) analysis. SVD analysis allowed for the assessment of each phenotypic characteristic as a potentially significant source of variance in the methylation dataset. A post-normalisation SVD plot of SWS UCB DNA methylation data indicated Sample plate, beadchip (indicated by Sentrinx_ID), position on beadchip (indicated by Sentrinx_position), sex (jsex), maternal BMI (awbmi), bone area and bone mineral density at four years of age (rtotarea, rtotbmd), bone mineral content and bone mineral density at six years of age (stotbmc, stotbmd) and BMD at eight years of age as significant (p -value < 0.05) sources of variance in the methylation dataset when accounting for the first two principal components.

3.2.2.2 Probes QC

The EPIC array uses a detection p -value to determine whether the fluorescence signal received from each probe is above background levels by reference to a set of negative control probes. Within this study CpG probes with a detection value greater than 0.01 or a bead count <3 were set to NA. If NA in more than 5% of samples the probes were excluded from further analysis. In this dataset 11977 probes were removed based on a detection p -value <0.01 and 733 probes removed based on a bead count of <3 . Since the EPIC assay was introduced CpG probes have been found to contain single-nucleotide polymorphisms (SNPs) and to cross-hybridise to multiple regions in the genome, identified in *Zhou W et al.* (2017), *Pidsley et al.* (2016) and *Nordlund et al.* (2013) (645–647). To prevent the probes confounding further analysis 94772 probes with SNPs at the CpG site or in the single-nucleotide extension were removed according to *Zhou W et al.* (2017) and 691 according to *Pidsley et al.* (2016). Probes were also removed on the basis that they cross-hybridize, leading to the removal of 3072 probes according to *Pidsley et al.* (2016) and *Nordlund et al.* (2013). Additional filtering was conducted using the `champ.filter` function of the chip analysis methylation pipeline (ChAMP) package (v2.14.0) leading to the removal of 2843 probes as non-CpG related probes and 16458 probes removed as they were located on sex chromosomes. Post-filtering 735545 probes remained for 465 samples.

3.2.2.3 Establishing model covariates

The differential methylation analyses of both BMC or BMD at ages of birth, four, six and eight years of age in the SWS cohort required a set of covariates to be established for each regression model. Covariates were initially selected based on their biological relevance. Smoking during pregnancy was selected as a covariate for its potential to induce perinatal methylation changes suggested to persist into adulthood and for its potential relationship with birthweight (679–681). Both sex and gestational age influence anthropometry at birth and have also been linked to differential methylation at a number of CpG sites (614,682–684). To account for the age at which DXA measurements were taken and the potential influence that may have on measures of BMC or BMD the model was also adjusted for DXA age. Furthermore, the model was also adjusted for estimated cell counts using *Gervin et al.* (2016) to ensure any methylation differences between samples were not driven by cell type composition (648). Finally, a correlation matrix was generated to identify any other significant correlation between the phenotypic characteristics of the SWS cohort and the 100,000 CpGs demonstrating the greatest variance in methylation from the EPIC array (appendix 9.2.1). The position of the sample on the beadchip (Sentrix position) indicated a significant correlation (p -value = $1.72e^{-8}$) with the CpGs demonstrating the greatest variance in methylation and so was included in the final set of covariates (Table 7).

3.2.2.4 *Model QC*

Post-differential methylation analysis each model was assessed by its inflation factor (λ), a quantile-quantile plot (qqplot) and p -value distribution histogram. Models with a $\lambda > 1.2$ were considered inflated and adjusted for their inflation and bias with the bacon package (v.1.12.0) and the newly generated p -values corrected for multiple testing (650). The models showing a predominantly normal qqplot and a p -value distribution histogram with a sloping curve were taken forward for analysis.

Table 7. The covariates adjusted for in each regression model supplied to limma for differential methylation analyses in the SWS cohort

SWS cohort term	Definition
Sentrix_position	Position on chip
smokpreg	Smoking during pregnancy
jsex	Sex
jbestgest	Gestational age
(x)dxage	Respective DXA age
Bcell	Estimated B cell composition
CD14	Estimated CD14 cell composition
CD4T	Estimated CD4T cell composition
CD8T	Estimated CD8T cell composition
Gran	Estimated granulocyte cell composition
NK	Estimated natural killer cell composition

3.2.3 Differential methylation analysis in relation to BMC

3.2.3.1 Differentially methylated CpGs

Differential methylation analysis to investigate the association between DNA methylation and BMC at birth, four, six and eight years was carried out using the limma package (v3.40.6). The *p*-values produced from the analysis were adjusted for multiple testing using the Benjamini-Hochberg method and assessed for inflation. Where differentially methylated cytosine-phosphate-guanine dinucleotides (dmCpGs) were associated with a known gene, the gene name was used for further analysis.

A differential methylation analysis assessing the relationship between DNA methylation in umbilical cord blood and BMC at birth found no dmCpGs with a false discovery rate (FDR)<0.25. In contrast, there were 17 dmCpGs associated with BMC at four years of age with an FDR<0.25, but no dmCpGs with an FDR<0.05. The analysis of the association between umbilical cord blood DNA methylation and BMC at six years of age was over-inflated and remained over-inflated post-adjustment with the bacon algorithm and therefore was not included in this analysis. Finally, no dmCpGs with an FDR<0.25 were found to be associated with BMC at eight years of age. The top dmCpGs associated with BMC at four years of age are listed in Table 8 and the top 100 dmCpGs in appendix 9.4.1.1.1.

3.2.3.2 Genomic locations of dmCpGs associated with BMC at four years of age

The locations of the top 100 dmCpGs whose methylation status was associated with BMC at four years of age was determined relative to CpG islands and genomic structure.

The BMC associated dmCpGs were most commonly located within open sea regions, which contained 51% of the top 100 dmCpGs, 16% were located within CpG islands and 33% in CpG shores or shelves. Relative to genomic structure, 61% of BMC associated dmCpGs were located in the gene body, 13% in the five prime untranslated region (5'UTR) and the first exon, 23% were located within 1500 bases upstream of the transcriptional start site (TSS) and 3% located in a three prime untranslated region (3'UTR). The distribution of the top 100 dmCpGs in the genome relative to both CpG islands and genomic structure are shown for the analyses of BMC at four years of age in Figure 20.

Table 8. The results of differential methylation analysis investigating the association between umbilical cord blood DNA methylation and measures of total body BMC at four years of age for offspring within the SWS cohort

Methylation data produced from the EPIC array interrogation of SWS umbilical cord blood DNA was used to conduct a differential methylation analysis investigating the association between umbilical cord blood (UCB) DNA methylation and DXA measurements of total body BMC at four years of age for offspring within the SWS cohort. The regression model for the analysis is detailed in table 7. This analysis found three dmCpGs with an FDR of <0.10 ($n=339$). No notable inflation was observed $\lambda=1.02$. DmCpGs associated with a known gene are represented by their respective associated gene symbol.

Model	Sample number	Lambda	FDR					Top 10 dmCpGs (FDR <0.25)
			<0.25	<0.20	<0.15	<0.10	<0.05	
4 years	339	1.02	17	9	3	3	0	IGF-1R, POU2F1, cg06957851, cg13649309, PLXNA4, cg02634733, cg04164351, ZNF669, SSH3, TBCD

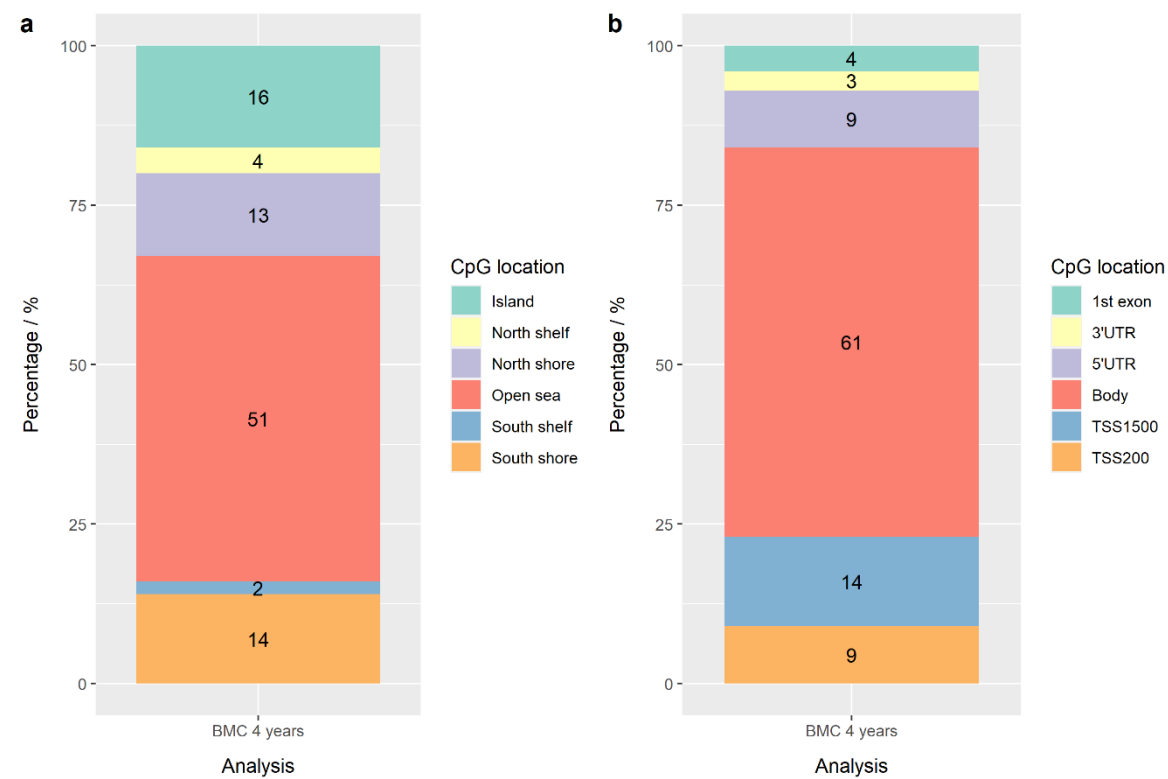


Figure 20. The location of the top 100 dmCpGs associated with BMC at four years of age relative to a) CpG islands and b) genomic structure in the genome
The top 100 dmCpGs associated with BMC at four years of age, relative to CpG islands were found largely within open sea regions and relative to genomic structure, most commonly within gene bodies. CpG shores, shelves and open sea regions are defined in appendix 9.3.

3.2.3.3 *Transcription factor enrichment analysis of dmCpGs associated with BMC at four years of age*

The RcisTarget package (v3.12) is a tool which examines dmCpGs associated with a known gene for the enrichment of specific transcription factor binding site motifs in a 500bp region upstream of the TSS (659). Significance was determined by a normalised enrichment score (NES) ≥ 4 .

Transcription factor motif enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age showed the significant enrichment (NES ≥ 4) of nine transcription factor binding site motifs. Enriched transcription factors included the Transcription Factor 7 (TCF7) motif (NES = 4.74) in 19 genes, the Chromodomain Helicase DNA Binding Protein 1 (CHD1) motif (NES = 4.52) in 14 genes and the Heat Shock Factor 1 transcription factor motif (NES = 4.35) in 22 genes (Table 9). Full list of motif references, sequence logo graphs and enriched genes in appendix 9.4.1.1.2.

3.2.3.4 *Differentially methylated regions associated with BMC at four years of age*

Differentially methylated CpGs work in concert to regulate gene expression, therefore the presence of any regions of differential methylation (DMRs) is often significant in determining the functional significance of methylation changes. DMRcate (v.2.2.3) is a package which determines DMRs by combining the *p*-values of all individual CpG probes in an area using Stouffer's method (685). This produces a Stouffer's value (SV) which is used as a significance threshold to determine what may be biologically relevant to the study. The dmCpGs associated with the analysis of BMC at four years of age were assessed for regions of differential methylation, however no significant DMRs (SV < 0.05) were identified.

3.2.3.5 *Pathway enrichment analysis of dmCpGs associated with BMC at four years of age*

To determine the functional significance of the methylation changes associated with BMC at four years of age, a protein-protein interaction network was generated based on predicted, experimental and literature-derived evidence using Cytoscape (653). Cytoscape is software that allows for the visualisation of molecular interaction networks and integration with gene expression profiles and other state data. The MCODE algorithm was then used within Cytoscape to detect densely connected regions or groups of genes in the protein-protein interaction network which may represent molecular complexes. These groups of genes or clusters were assessed for gene ontology (GO) term enrichment, where initially genes were referenced against the Gene Ontology resource (686,687), a repository containing all currently known functional attributes of each gene and their involvement in a biological process or molecular function and the cellular compartment in which they may be active, each denoted by a unique GO term. GO terms significantly over-represented in each cluster when compared to the rest of the genome was then determined, where GO terms with an FDR < 0.05 were classed as significantly enriched.

3.2.3.5.1 *Cytoscape pathway enrichment analysis*

Initial clustering of the top 100 dmCpGs associated with BMC at four years of age produced three clusters (Table 10). Clusters one and three indicated the significant enrichment ($FDR < 0.05$) of 3 and 1 pathways respectively, however cluster 2 showed no significant pathway enrichment. GO term enrichment analysis indicated enrichment for a pathway involved in cellular processes (GO: 9987, $FDR = 2.26e^{-2}$) and protein tyrosine kinase activity (GO: 4713, $FDR = 4.26e^{-2}$). Full ontology results are available in appendix 9.4.1.1.3.

3.2.3.6 *Enriched histone mark and chromatin states associated with BMC at four years of age*

Post-translational histone modifications influence gene expression through dynamic regulation of the chromatin structure and play a role in DNA replication and repair, chromatin compaction and cell-cycle control. The Encyclopaedia of DNA Elements (ENCODE) project tool chromHMM identifies combinations of histone modifications to annotate the genome into predicted regulatory regions including promoters, enhancers, active poised or repressed chromatin and a number of other chromatin states (688). To explore the chromatin landscape surrounding our dmCpGs, we obtained chromatin immunoprecipitation sequencing (ChIP-seq) peak regions for selected histone marks in the broadPeak format and a 15-state chromatin state model generated from human umbilical vein endothelial cells from the Roadmap Epigenomics project (657). Initially, overlap of the top 100 dmCpGs with either histone marks or chromatin states was determined and then a Fisher's exact test used to determine whether there was a significant enrichment of dmCpGs overlapping these regions.

Histone mark and chromatin state enrichment for the top 100 dmCpGs associated with BMC at four years of age showed a significant under-representation of dmCpGs overlapping H3K27me3 modifications ($FDR = 0.02$).

Table 9. The transcription factor binding site motifs enriched within the top 100 dmCpGs associated with BMC at four years of age in the SWS cohort

The R package RcisTarget (v1.6.0) was used to identify the transcription factor (TF) binding site motifs enrichment in a list of genes associated with the top 100 dmCpGs produced from the differential methylation analysis assessing the relationship between UCB DNA methylation and BMC at four years of age. The analysis found the dmCpGs significantly enriched ($NES \geq 4$) for 8 transcription factor motifs.

Analysis	Rank	TF	Normalised enrichment score	Motifs	Annotation	Multiple motifs enrichment range		Genes enriched
						Maximum NES	Minimum NES	
<i>BMC at 4 years</i>								
	1	TCF7	4.74	1	Inferred	NA	NA	19
	2	CHD1	4.52	1	Direct	NA	NA	14
	3	HSF1	4.35	1	Inferred	NA	NA	22
	4	IRX6	4.29	1	Inferred	NA	NA	23
	5	SP100	4.13	1	Inferred	NA	NA	37
	6	TCF7L1	4.08	1	Inferred	NA	NA	20
	7	ZKSCAN1	4.06	1	Inferred	NA	NA	21
	8	MAFK	4.04	1	Inferred	NA	NA	11

Table 10. Gene clusters produced during the pathway analysis of the top 100 dmCpGs associated with the differential methylation analyses for BMC at four years of age in the SWS cohort

Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age produced three distinct clusters of dmCpGs. However only clusters 1 and 3 saw significant enrichment of pathways with the involvement of three or more dmCpGs. Nodes represent individual genes in each cluster and edges the total number of connections between all genes in each cluster.

Model	Cluster	Genes	Nodes	Edges	Score	Pathways FDR <0.05	Gene count ≥3
4 years	1/3	OCA2, TCF7L1, COPA, WWC1, DYRK1A, TBCD, DDX43, IGF-2R, IGF-1R, NCOR2, PAN3, PAPP, HECW1, RAB3GAP2, JARID2	15	38	4.375	3	1
	2/3	FBXO18, PRKCZ, PEA15, EPM2AIP1, CNKSR2, UBE4A, IARS2, PDCD6, BTBD3, SEC11A, ELP2, ZNF608, TIAM2, ADD1, ABR, SP100, COL8A1, SRBD1	18	39	4.000	0	NA
	3/3	SMARCE1, EPHA5, HDAC4, DYNC1H1, ZCCHC7, POU2F1, TRIO, SRD5A1, KCNH7, MYT1L, RPA1, DMP1, STK4, SSH3, CENPE, DPYSL2, UST, ARFIP1, ALDH18A1, HARS, SPG20	21	43	3.636	1	1

3.2.4 Differential methylation analysis in relation to BMD

3.2.4.1 Differentially methylated CpGs

The association between umbilical cord blood DNA methylation and BMD at birth, 4, 6 and 8 years of age was examined. Analysis of the dataset found 1, 0, 0 and 3 dmCpGs in cord blood DNA significantly associated with BMD at birth, four, six and eight years of age respectively ($FDR < 0.05$). Whilst there were no dmCpGs at an $FDR < 0.05$ associated with BMD at four or six years of age, there were 16 and 4 dmCpGs associated with BMD at four and six years of age with an $FDR < 0.25$ respectively.

The most significant dmCpG associated with BMD at birth was cg04104541, located in the region 1500 bases upstream of the TSS of the Transmembrane Serine Protease 11F (*TMPRSS11F*) gene ($FDR = 0.01412$) and its methylation was negatively associated with BMD at birth (Figure 21). GO annotations for *TMPRSS11F* suggest the gene plays a role in serine-type endopeptidase activity. The top dmCpG associated with BMD at four years of age was cg02548571, an intergenic CpG ($FDR = 0.205$), whereas the top dmCpG associated with BMD at six years of age was cg22950153, positioned in body of the Immunoglobulin Superfamily DCC Subclass Member 3 (*IGDCC3*) gene ($FDR = 0.1118$). At eight years of age the most significant dmCpG associated with BMD was cg04611479, located in the 5' UTR of the Membrane Palmitoylated Protein 3 (*MPP3*) gene ($FDR = 0.04005$) the methylation of which was positively associated with BMD at eight years of age (Figure 22). MPP3 is a member of the membrane-associated guanylate homologs (MAGUKs) protein family which interact with the cytoskeleton and regulate signalling pathways, cell proliferation and intercellular junctions. The top dmCpGs associated with BMD at birth, four, six and eight years of age ($FDR < 0.25$) are listed in Table 11 and the top 100 dmCpGs for each analysis in appendix 9.4.2.

The overlap between the methylation signatures associated with BMD at birth, four, six and eight years of age was determined using the online venn diagram generator, Venny (689). CpG probe names of the top 100 dmCpGs from each analysis were inputted. A comparison between BMD at birth and four years of age found an overlap of 2 dmCpGs, between four and six years there were 6 dmCpGs in common, between six and eight years there was an overlap of 8 dmCpGs and between birth and six years 1 dmCpG. In addition, differential methylation analyses of BMD at four and eight years of age found 8 dmCpGs in common and an overlap of 26 dmCpGs between BMD at birth, four and six years of age. Whereas between BMD at four, six and eight years of age there was only a single dmCpG in common (Figure 23).

3.2.4.2 Genomic locations of dmCpGs

The locations of the top 100 dmCpGs associated with BMD at birth, four, six and eight years of age were determined relative to CpG islands and genomic structure.

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Collectively the dmCpGs were positioned most commonly in open sea regions, CpG islands, or northern shelves and shores and the fewest dmCpGs in southern shelves or shores. Relative to genomic structure these dmCpGs were primarily within the gene body or upstream of the TSS, within the 5'UTR and least frequently in the first exon or 3'UTR. The percentage values for each group and the distribution of the top 100 dmCpGs in the genome relative to both CpG islands and gene structure for analyses of BMD at birth, four, six and eight years of age are shown in Figure 24.

Table 11. Results of differential methylation analysis investigating the association between UCB DNA methylation and measures of BMD at birth, four, six and eight years of age for offspring within the SWS cohort

Methylation data produced from the EPIC array interrogation of SWS umbilical cord blood DNA was used to conduct differential methylation analyses to investigate the association between UCB DNA methylation and DXA measurements of total body BMD at the isolated time points of birth, four, six and eight years of age for offspring within the SWS cohort. The differential methylation analysis investigating the association between UCB DNA methylation with BMD at birth found one significant dmCpG (FDR<0.05) (n=219). 16 dmCpGs were found to be weakly associated (FDR<0.25) with BMD at four years of age (n=339), 2 weakly associated with BMD at 6 years of age (n=384) and 3 significantly associated with BMD at 8 years of age (n=388). DmCpGs associated with a known gene are represented by their respective associated gene symbol.

Model	Sample number	Lambda	FDR					Top 10 dmCpGs (FDR <0.25)
			<0.25	<0.20	<0.15	<0.10	<0.05	
Birth	219	1.05	5	5	5	1	1	TMPRSS11F, cg02270765, HIRA, HEBP2, NAV2
4 years	339	1.02	16	0	0	0	0	cg02548571, IGF-2R, ZNF669, C17orf75, IGF-1R, NHP2L1, cg13649309, POU2F1, CRADD, cg23376847
6 years	384	1.02	4	2	2	0	0	IGDCC3, cg14662769, cg00062285, cg04692555
8 years	388	1.17	16	14	5	5	3	MPP3, cg07666211, CKB, cg15416279, SLC15A1, CAMK2D, ABR, COCH, KANSL2, LRRC8D

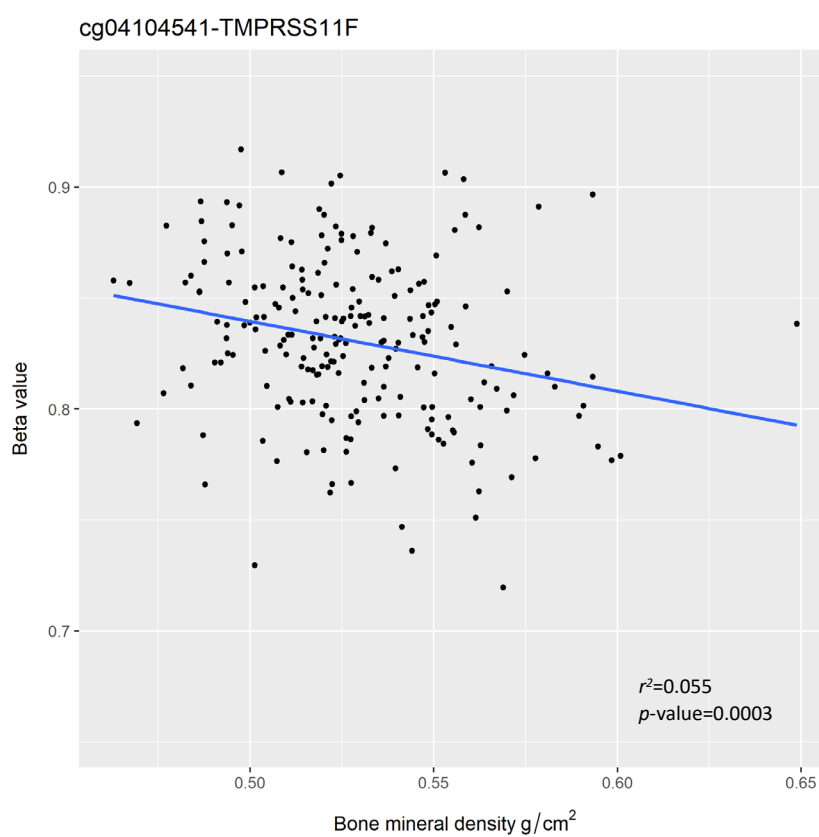


Figure 21. The association between cg04104541 methylation in UCB DNA and BMD at birth in the SWS cohort
The differential methylation analysis assessing the association between the methylation of 219 samples of UCB DNA and BMD at birth in the SWS cohort found significant differential methylation of CpG cg04104541, mapped to the TMPRSS11F gene. The methylation of the dmCpG showed an inverse association with BMD at birth.

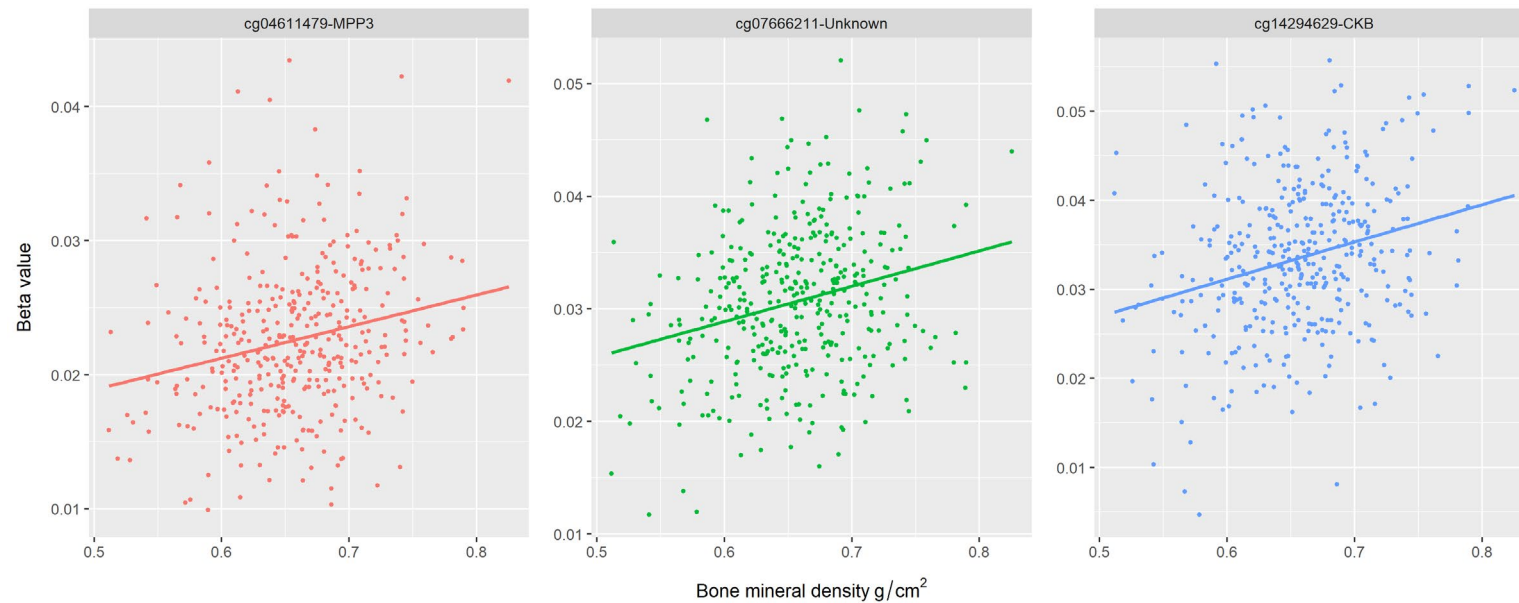


Figure 22. The association between methylation and BMD for the three dmCpGs significantly (FDR <0.05) associated with BMD at eight years of age in the SWS cohort

The differential methylation analysis assessing the association between the methylation of 388 samples of UCB DNA and BMD at eight years of age in the SWS cohort found significant (FDR <0.05) differential methylation of CpGs cg04611479, cg07666211 and cg14294629 which were mapped to the MPP3 gene, an intergenic region and the CKB gene respectively. The methylation of all three dmCpGs showed a positive association with BMD at eight years of age.

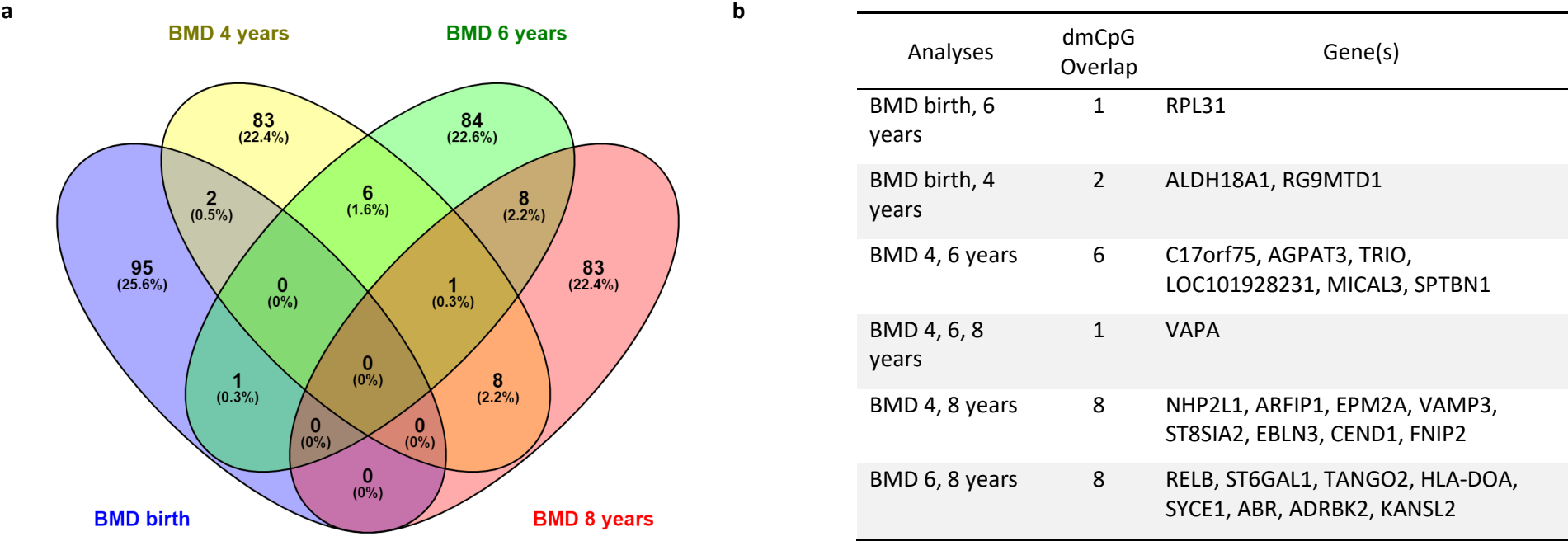


Figure 23. The a) venn-diagram indicating the overlap between the top 100 dmCpGs for each differential methylation analysis investigating the relationship between umbilical cord blood DNA methylation and BMD at of birth, four, six and eight years of age and the b) gene associated with each overlapping dmCpG

A comparison of the top 100 dmCpGs when ranked by FDR was compared between differential methylation analyses investigating the relationship between umbilical cord blood DNA methylation and measures of BMD at birth, four, six and eight years of age in the SWS cohort. A a) venn diagram depicts the overlap between dmCpGs taken from each analysis, and b) the overlapping dmCpG probe name, or the gene name if located within a known gene.

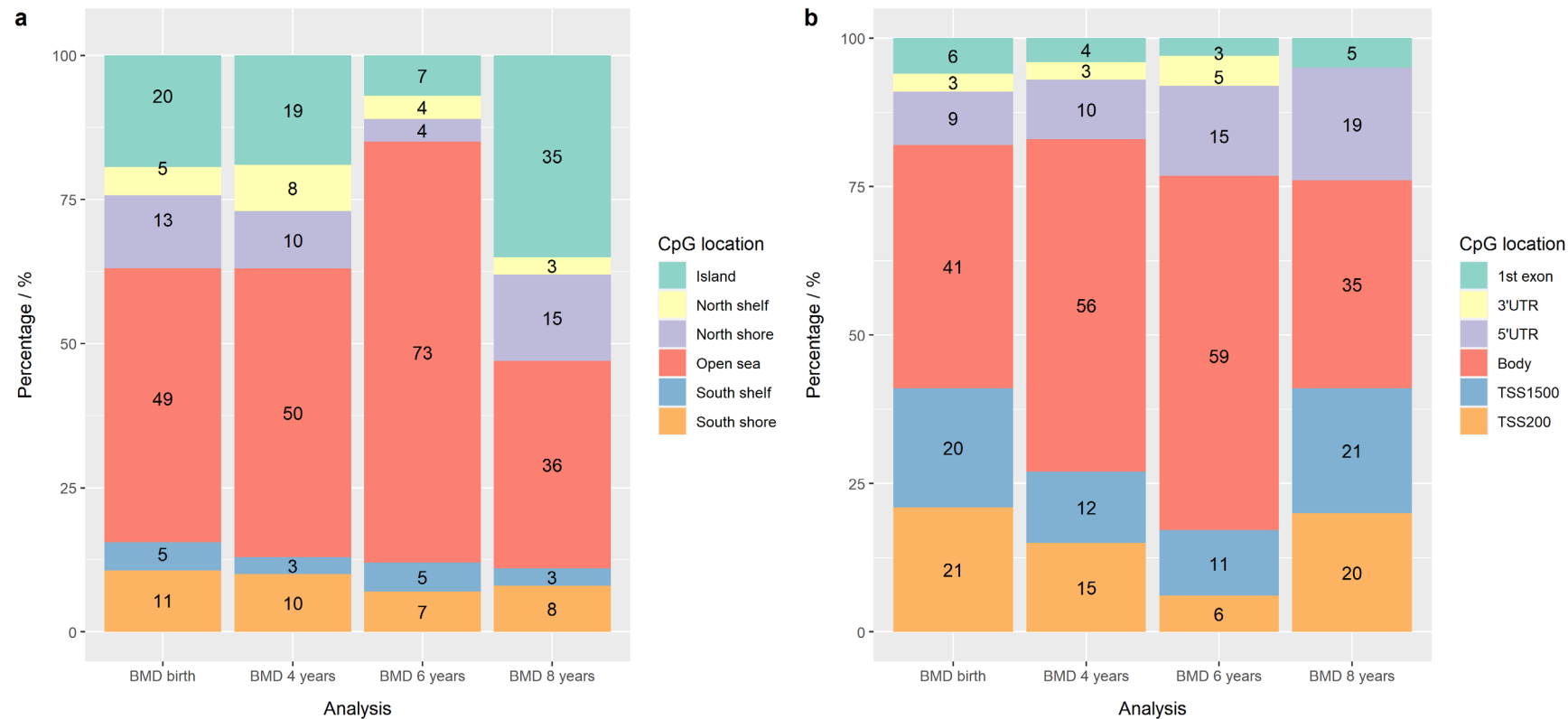


Figure 24. The location of the top 100 dmCpGs associated with BMD at birth, four, six and eight years of age relative to a) CpG islands in the genome b) genomic structure

The top 100 dmCpGs associated with the differential methylation analysis investigating the association between UCB DNA methylation and BMD at birth in the SWS cohort were shown to be largely in an open sea region and predominantly within the body of the gene, or 1500 bases upstream of the TSS. The dmCpGs associated with BMD at four and six years of age were largely within an open sea gene body region, whereas the dmCpGs associated with the analysis interrogating association between UCB methylation and BMD at eight years of age were more commonly found in a CpG island, north shore or shelf and up to 1500 bases upstream of a TSS. CpG shores, shelves and open sea regions are defined in appendix 9.3.

3.2.4.3 Transcription factor enrichment analysis

Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at birth, four, six and eight years found significant enrichment ($NES > 4$) for 1, 7 and 4 transcription factor binding site motifs respectively (Table 12). The dmCpGs associated with BMD at birth indicated enrichment for the Enhancer Of Zeste Homolog 2 (EZH2) transcription factor motif ($NES = 4.22$) in 43 genes. The dmCpGs associated with BMD at four years of age showed enrichment for seven transcription factor binding site motifs including the Odd-Skipped Related Transcription Factor 1 (OSR1) motif ($NES = 4.67$) in 13 genes, multiple RNA Polymerase II Subunit A (POLR2A) motifs ($NES = 4.65-3.01$) in 60 genes and the DNA Polymerase Iota (POLI) motif ($NES = 4.38$) in 12 genes. At six years of age, associated dmCpGs were enriched for four POU domain containing transcription factors, the POU Class 5 Homeobox 1 (POU5F1) transcription factor motif ($NES = 4.23$) in 10 genes, POU Class 3 Homeobox 1 (POU3F1) transcription factor motif ($NES = 4.17$) in 9 genes, POU Class 2 Homeobox 1 (POU2F1) transcription factor motif ($NES = 4.09$) in 12 genes and POU Class 3 Homeobox 2 (POU3F2) transcription factor motif ($NES = 4.03$) in 10 genes. Full list of motif references, sequence logo graphs and enriched genes in appendix 9.4.2.

3.2.4.4 Differentially methylated regions

Differential methylation analyses for BMD at birth, four, six and eight years were assessed for regions of differential methylation, but no regions of significant differential methylation ($SV < 0.05$) were found.

3.2.4.5 Pathway enrichment analysis

3.2.4.5.1 Cytoscape pathway analysis

Clustering of the top 100 dmCpGs associated with BMD at birth, four, six and eight years of age produced 2, 2, 0 and 1 distinct clusters of genes (Table 13) respectively. GO term enrichment analysis showed dmCpGs associated with BMD at birth were enriched for the integrin-mediated signalling pathway (GO: 7229, $FDR = 1.91e^{-2}$), multicellular organismal homeostasis (GO:48871, $FDR = 2.11e^{-2}$) and cell-matrix adhesion (GO:7160, $FDR = 2.11e^{-2}$). Furthermore, dmCpGs associated with BMD at four years indicated enrichment for induction of apoptosis (GO:6917, $FDR = 6.11e^{-4}$), induction of programmed cell death (GO:12502, $FDR = 6.11e^{-4}$), induction of apoptosis by extracellular signals (GO:8624, $FDR = 1.05e^{-3}$). However at eight years of age only a single pathway was enriched for activity in the cytosol (GO:5829, $FDR = 4.00e^{-2}$) (Figure 25). Full ontology results for each differential methylation analysis are available in appendix 9.4.2.

3.2.4.6 Histone mark and chromatin state analysis

There was a significant under-representation of the top 100 dmCpGs associated with BMD at four years of age overlapping H3K27me3 histone modifications ($FDR = 0.0441$). At six years of age, we saw a significant under-representation of dmCpGs overlapping H3K4me1 ($FDR = 0.0223$), H3K4me3

(FDR=0.0318) and H3K9ac (FDR=0.0361) histone marks. However, at eight years of age there was significant enrichment for dmCpGs overlapping the three histone marks H3K4me1 (FDR=0.0002), H3K4me3 (FDR=0.0024) and H3K27ac (FDR=0.0468) (Figure 26a). Additionally, significant enrichment of the dmCpGs in active TSS regions of the genome (FDR= 0.0401) and under-representation in quiescent/low areas (FDR= 0.015) was shown at eight years of age (Figure 26b).

Table 12. The transcription factor binding motifs enriched within the top 100 dmCpGs associated with BMD at birth, four and six years of age in the SWS cohort

Transcription factor enrichment analysis of the top 100 dmCpG associated with BMD at birth, four six and eight years of age found the enrichment of 1, 7, 4 and 0 transcription factor motifs respectively with a normalised enrichment score (NES) >4.

Analysis	Enriched TFs	Rank	TF	Normalised enrichment score	Motifs	Annotation	Multiple motifs enrichment range		Genes enriched
							Maximum NES	Minimum NES	
<i>BMD at birth</i>									
	1	1	EZH2	4.22	1	Direct	NA	NA	43
<i>BMD at 4 years</i>									
	7	1	OSR1	4.67	1	Inferred	NA	NA	13
		2	POLR2A	NA	8	Direct	4.65	3.01	60
		3	POLI	4.38	1	Direct	NA	NA	12
		4	CLOCK	4.29	1	Direct	NA	NA	13
		5	TET1	4.19	1	Direct	NA	NA	38
		6	ZBTB14	4.19	1	Direct	NA	NA	32
		7	CCDC169-SOHLH2	4.1	1	Inferred	NA	NA	11
<i>BMD at 6 years</i>									
	4	1	POU5F1	4.23	1	Direct	NA	NA	10
		2	POU3F1	4.17	1	Inferred	NA	NA	9
		3	POU2F1	4.09	1	Inferred	NA	NA	12
		4	POU3F2	4.03	1	Direct	NA	NA	10

Table 13. Gene clusters produced during the pathway analysis of the top 100 dmCpGs associated with the differential methylation analyses for BMD at birth, four and six years of age in the SWS cohort
 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMD at birth, four, six and eight years of age produced 2, 2, 0 and 1 distinct clusters of dmCpGs respectively. However only one cluster associated with BMD at birth, one associated with BMD at four years of age and the cluster associated with BMD at eight years saw significant enrichment of pathways with the involvement of three or more dmCpGs.

Model	Cluster	Genes	Nodes	Edges	Score	Pathways FDR <0.05	Gene count ≥3
Birth	1/2	PHF2, CDV3, LAMA5, OLIG3, ITGB4, NFYC, ITGA2CADPS, STAT3, TSHZ2, KIAA0040, ACACA, POLD3, CDH4, SNX19, MYB, TSPAN17, FBXO3, TRIM37, PTF1A	20	52	4.476	20	10
	2/2	ATP5G3, ZHX2, RTTN, ALDH9A1, SIN3A, PTPN3, BIRC5, ITGA3, INTU, FAM3C, NAV2, HEBP2, ABHD5, JAKMIP1, LSAMP, NIFK, ITGB1, ADAMTS2, CCDC68, LONRF1, CEBPB, ALLC, DUSP6	23	57	4.333	0	NA
4 years	1/2	FDPS, CASP8, CRADD, PAPP A, FAS, HTT, CHD1L	7	20	3.500	158	29
	2/2	MAEA, POU2F1, EPM2AIP1, UBE4A, SH3BP4, MEGF11, TRAPPC10, AP1G1	8	15	3.111	0	NA
8 years	1/1	ZCCHC7, ABR, CAMK2D, C11ORF70, XRCC4, KCND2, ZNF180, CKB, SH3D19, EREG	10	22	3.636	3	1



Figure 25. GO terms significantly enriched (adjusted p -value <0.05) for the top 100 dmCpGs associated with BMD at birth, four and eight years of age in the SWS cohort

Cytoscape was used to generate PPI networks from the the top 100 dmCpGs associated with BMD at birth, four and eight years of age in the SWS cohort and the MCODE algorithm used to cluster densely connected regions which may denote molecular complexes. Each gene in a gene cluster was referenced against the Gene Ontology Resource tool repository for their involvement in a biological process or molecular function and the cellular compartment in which they may be active. Over-representation of GO-terms was determined for each gene cluster using the genome as a background. Significant enrichment was shown for the dmCpG clusters produced from the clustering of the top 100 dmCpGs associated with BMD at birth, four and eight years of age. The dmCpGs associated with BMD at six years of age produced no clusters and so no significant gene ontology pathway enrichment was identified. A larger point size and $-\log_{10}$ adjusted p -value indicates a greater significance and number of dmCpGs enriched for the pathway.

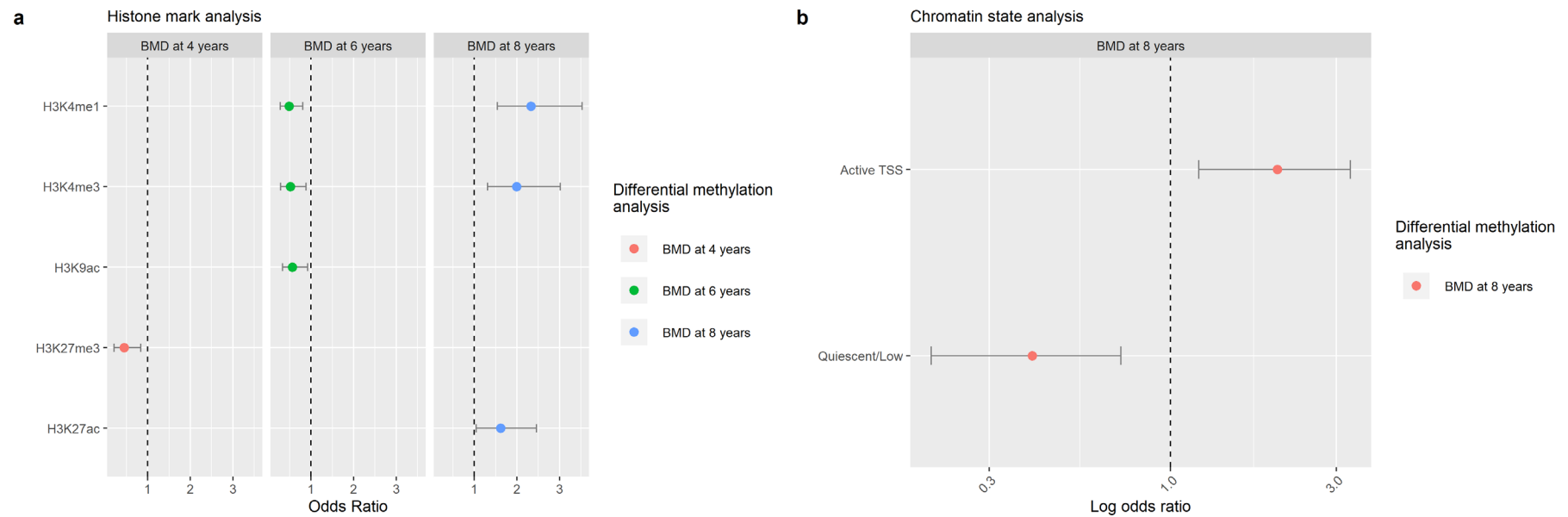


Figure 26. Odds ratio graphs indicting over-representation (odds ratio >1) or under-representation (odds ratio <1) of the top 100 dmCpGs associated with BMD at four, six and eight years of age overlapping either a) histone marks or b) chromatin states in the genome

The a) histone mark enrichment analysis of the top 100 dmCpGs associated with the differential methylation analyses of BMD at birth, four, six and eight years of age showed no significant (FDR <0.05) over or under-representation of histone marks associated with BMD at birth. However at four years of age there was a significant under-representation of H3K27me3 and at six years of age under-representation of H3K4me1, H3K4me3 and H3K9ac. At eight years there was over representation of H3K4me1, H3K4me3 and H3K27ac marks. The b) chromatin state analysis showed no significant over or under enrichment at birth, four or six years of age, but over representation of dmCpGs within an active TSS and under representation of dmCpGs within a quiescent area of the genome at eight years of age.

3.2.5 *Overlap between dmCpGs associated with BMC and BMD in the SWS cohort*

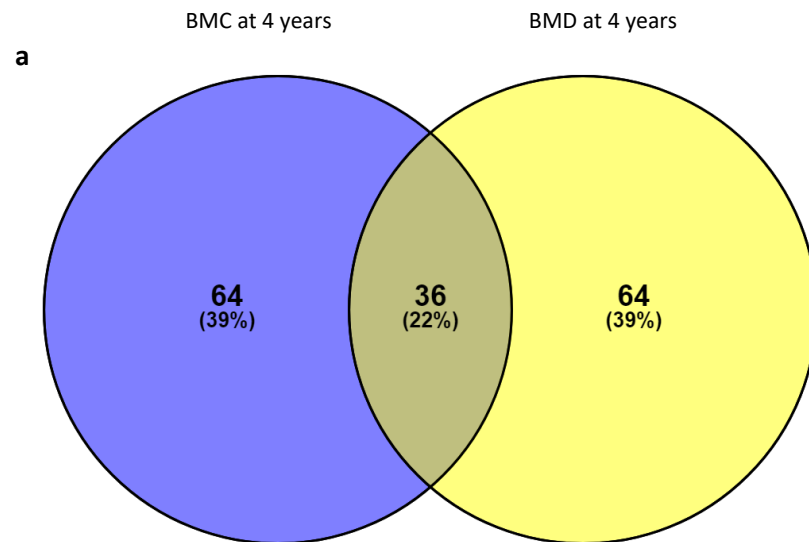
3.2.5.1 *dmCpG overlap*

To determine whether there may be common methylation changes associated with both BMC and BMD within the SWS cohort, a comparison was made between the top 100 dmCpGs associated with BMC and BMD within each age group. As the only BMC related differential methylation analysis to produce a methylation signal, BMC at four years of age was compared with BMD at four years of age from which an overlap of 36 out of a combined total of 200 dmCpGs was identified (Figure 27).

3.2.6 *Does maternal late-stage pregnancy vitamin D status potentially influence the methylation changes associated with offspring postnatal bone measures?*

Mineralisation of the foetal skeleton occurs primarily in the late second and third trimesters where demand for calcium can be up to 250 mg/day (690,691). Given the dependence of calcium homeostasis on vitamin D and its metabolites, we aimed to determine whether maternal late-stage pregnancy vitamin D (MLSP D) status may influence the methylation signatures associated with bone outcomes through the adjustment of our differential methylation analysis regression models for MLSP vitamin D status.

Differential methylation analyses were repeated for BMC at four years of age and BMD at the ages of birth, four, six and eight years of age with adjustment for MLSP vitamin D status (full results in Table 14). A comparison of the top 100 dmCpGs produced from the differential methylation analyses of BMC at four years of age adjusted and unadjusted for MLSP vitamin D status found 69 of a combined total of 199 dmCpGs from both analyses in common. Similarly, a comparison of the top 100 dmCpGs produced from the adjusted and un-adjusted differential methylation analyses for BMD at birth found 46 of 198 dmCpGs in common, at four years of age 77 of 200 dmCpGs were found to be in common, at six years of age 71 of 200 dmCpGs were found to be shared between analyses and between the adjusted and un-adjusted differential methylation analyses at eight years of age 74 of 200 dmCpGs were found in common.

**b**

Analyses	dmCpG Overlap	Gene(s)
BMC 4 years, BMD 4 years	36	IGF1R, POU2F1, cg06957851, cg13649309, cg04164351, ZNF669, SSH3, TBCD, AGPAT3, UBE4A, cg07601720, TNFRSF10A, ALDH18A1, EPM2AIP1, RPA1, EPHA5, LOC101928231, PTPRO, TRIO, KCNH7, cg01022012, cg02519452, NUBP1, ARFIP1, cg06139720, NHP2L1, REST, NAA50, MIR183, IGF2R, TRAPPC10, PAPP, cg01902998, cg02548571, TRDMT1, CASC18

Figure 27. The a) venn-diagram overlap between the top 100 dmCpGs from differential methylation analyses interrogating the relationship between SWS cohort umbilical cord blood DNA methylation, BMC at four years of age and BMD at four years age and the b) associated identity of each overlapping dmCpG

A comparison of the top 100 dmCpGs when ranked by FDR was compared between differential methylation analyses investigating the relationship between umbilical cord blood DNA methylation and measures of BMC and BMD at four years of age in the SWS cohort. A a) venn diagram depicts the overlap between dmCpGs taken from each analysis, and b) the overlapping dmCpG probe name, or the gene name if located within a known gene.

Table 14. Results of differential methylation analyses investigating the association between cord blood DNA methylation and BMC at four years, BMD at birth, four, six and eight years of age adjusted and non-adjusted for maternal vitamin D status in late-stage (34 weeks) pregnancy

Differential methylation analyses were used to assess the association between methylation data collected from the EPIC array interrogation of SWS umbilical cord blood DNA, and measures of BMC and BMD at birth, four six and eight years of age. Analyses where dmCpGs were produced with $FDR < 0.25$ were repeated using regression models adjusted for maternal vitamin D status in late-stage pregnancy (34 weeks) to determine whether maternal late-stage pregnancy vitamin D (MLSP D) status may influence the methylation signatures associated with bone outcomes. Cgs associated with a known gene were represented by their respective associated gene symbol.

Bone measure	Model	Adjusted for MLSP vitamin D	Sample number	Lambda	FDR					Top 10 dmCpGs (FDR <0.25)
					<0.25	<0.20	<0.15	<0.10	<0.05	
BMC	4 years	✗	339	1.02	17	9	3	3	0	IGF-1R, POU2F1, PLXNA4, ZNF669, SSH3, TBCD, AGPAT3, DMP1, UBE4A, LINC00939
		✓	326	1.02	11	7	6	0	0	cg06957851, IGF-1R, cg13649309, PLXNA4, POU2F1, SSH3, cg02634733, ALDH18A1, ZNF669, EPM2AIP1
BMD	Birth	✗	219	1.05	5	5	5	1	1	TMPRSS11F, HIRA, HEBP2, NAV2, ZNF512B, OLIG3, ESRRA, SPTB, SIN3A, WDR60
		✓	207	1.04	54	45	18	8	0	cg09651845, NAV2, GDE1, TMPRSS11F, SIN3A, INSR, cg02270765, NFYC, GNAI1, HIRA
	4 years	✗	339	1.02	16	0	0	0	0	IGF-2R, ZNF669, C17orf75, IGF-1R, NHP2L1, POU2F1, CRADD, RPA1, TBCD, ARFIP1
		✓	326	1.02	0	0	0	0	0	NA
	6 years	✗	384	1.02	4	2	2	0	0	IGDCC3, IFT140, PRPF8, PUM1
		✓	367	1.02	2	2	0	0	0	cg14662769, IGDCC3
	8 years	✗	388	1.17	16	14	5	5	3	MPP3, CKB, SLC15A1, CAMK2D, ABR, COCH, KANSL2, LRRC8D, GJB2, ASB1
		✓	372	1.13	44	7	4	2	0	MPP3, CKB, cg07666211, CAMK2D, SLC15A1, KANSL2, LINC01132, UBE2Z, SIGIRR, GJB2

3.3 Discussion

3.3.1 Summary

Previous work in the SWS study investigating umbilical cord blood DNA methylation identified differential methylation changes in both *RXR α* and Cyclin-dependant kinase N2A (*CDKN2A*), genes both linked to skeletal development which were predictive of postnatal measures of bone growth (2,3,692). Whilst these CpGs were not present on the EPIC array and so could not be validated in the context of this study, here, we expanded upon this work by conducting a genome wide scan of SWS umbilical cord blood DNA for epigenetic signatures and found further significant differential methylation changes associated with BMD at birth and eight years of age. The dmCpGs associated with BMD at eight years of age could act as potential prospective biomarkers of BMD from birth, however extensive validation would be required in further cohorts. These dmCpGs are also most likely specific to umbilical cord blood due to the generally cell-type specific nature of DNA methylation (693).

Interestingly, there was a lack of overlap in the top dmCpGs associated with each analysis between age groups. This lack of overlap may be due to the nature of early life being a period of dynamic change in physiology and rapid growth. Children become ambulatory between the ages of birth and four years of age, increasing mechanical loading, a major regulator of skeletal bone mass and geometry (694). Furthermore, puberty can begin as early as eight years of age, a period where an increase in sex hormones facilitates rapid bone growth (695,696). This progression in development may therefore instigate significant changes in biological processes such as skeletal development which may lead to changes in methylation unique to that age group. In contrast, we did see common dmCpGs associated with both BMC at four years of age, which overall showed weaker associations ($FDR < 0.25$) and BMD at four years of age. However, this may not be surprising as BMD is a metric of BMC per unit area. The analyses of BMC at birth, six and eight years of age showed no dmCpGs associated with umbilical cord blood DNA whatsoever. This may be potentially due to the confounding effect differences in height can have on measures of BMC for a given age and so perhaps made for a weaker regression model. This may also be an indication of a lack of power in the study, or the associations may also simply not exist. This suggests the need for replication, which if being done for the sake of biomarker validation could take place in further umbilical cord blood DNA samples from a number of other cohorts, however if it is to ascertain the biological relevance of the change, the replication would need to occur in the cells which ultimately become osteoblasts *in vivo*, bone marrow mesenchymal stromal cells (BM-MSCs).

Further analyses of the dmCpGs associated with both BMC and BMD produced some functional insights into the potential effects of these differential methylation changes. Pathway enrichment analysis of the more weakly significant dmCpGs associated with BMC at four years of age found

enrichment for both cellular processes and tyrosine kinase activity, indicating methylation changes may be collectively contributing to changes in cellular signalling. Additionally, significant enrichment was found for eight transcription factor binding site motifs. There were no regions of differential methylation identified.

Alongside the significant ($FDR < 0.05$) differential methylation changes in umbilical cord blood DNA associated with BMD at birth and eight years of age, weaker associations were found with both BMD at four and six years of age. Pathway analysis showed enrichment for the integrin-mediated signalling pathway and transcription factor activity associated with BMD at birth, the positive regulation of cellular and biological processes associated with BMD at six years of age and activity in the cytosol associated with BMD at eight years of age. There was also some overlap between the dmCpGs associated with BMD at birth, four, six and eight years. The analyses associated with BMD at birth, four, six and eight years of age again indicated no regions of differential methylation.

3.3.2 Top hits

Whilst there were no significant associations between umbilical cord tissue DNA methylation and BMC at four years of age, there were significant associations with BMD at both birth and eight years of age. *TMPRSS11F*, associated with BMD at birth and *MPP3*, associated with BMD at eight years of age have not been reported to be linked to skeletal development and therefore further work may be required to understand the significance of the differential methylation changes seen within these genes. However, creatine kinase brain-type (*CKB*) ($FDR = 0.04005$) a significant differentially methylated CpG associated with BMD at eight years of age has potential functional relevance to this study. Creatine kinase is an enzyme responsible for maintaining energy homeostasis in a number of cell types with high energy demands including skeletal, cardiac, smooth muscle, brain and neuronal cells and a number of others. Four isoforms of creatine kinase are currently understood to exist in mammalian cells, two mitochondrial and two cytosolic which are *CKB* and creatine kinase muscle-type (*CKM*). These cytosolic creatine kinase enzymes can form the homodimers *CKMM* and *CKBB* and the heterodimer *CKMB*, only one of which is typically expressed in a given tissue. The *CKBB* homodimer is expressed in a range of tissues, including osteoclasts, the precursor cell of which are present in umbilical cord blood (697–699). Osteoclasts exhibit a high citric acid cycle activity and rate of mitochondrial respiration, both required to generate high levels of adenosine triphosphate (ATP) to maintain ATPase activity and facilitate bone resorption, making expression of *CKBB* crucial to maintain the high-energy conditions needed for normal osteoclast function (700). Consequently, knockout of *CKBB* in mice has demonstrated that *in vivo* mice were protected against an induction of bone loss, whereas *in vitro* osteoclasts derived from the *CKBB* null mice showed reduced bone-resorbing activity. In humans, clinical cases of osteogenesis imperfecta, typically treated with nitrogen-containing

bisphosphonates (NBPs) have reported both osteopetrosis and increased levels of serum CKBB (701). It was found NBPs act to suppress osteoclast activity or lead to an increase in osteoclast apoptosis-related events and the release of CKBB, indicating the similar role of CKBB in human bone remodelling (702). Within the context of this study, we saw a significant increase in the methylation of cg14294629 within the promoter region of the *CKB* gene which was positively associated with BMD (Figure 21). An increase in methylation within the promoter region of a gene is typically associated with a suppression of transcriptional activity, however any potential effect of methylation on the transcriptional activity of this gene would have to be further validated. If validated this could indicate a methylation change potentially associated with a decreased osteoclastic resorptive activity, favouring bone formation.

3.3.3 *Transcription factor enrichment*

Transcription factor enrichment analysis found significant enrichment of the top 100 dmCpGs associated with BMC at four years, BMD at birth, four and six years of age.

Enriched within the dmCpGs associated with BMC at four years of age was the TCF7 transcription factor binding site motif (NES=4.74). TCF7 belongs to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, a group of transcription factors which act as the major end point to Wnt/ β -catenin signalling. In the nucleus TCF7 binds β -catenin as a coactivator to influence transcriptional changes, altering cellular behaviours such as movement, proliferation, differentiation, survival and self-renewal (703). The Wnt signalling pathway is integral to skeletal development, such that inactivation of β -catenin has been seen to block osteoblastic differentiation and delay chondrocyte maturation, both of which subsequently impair endochondral ossification (260,261). Indeed, *Mikasa et al.* (2011) showed dominant-negative *TCF7* transgenic mice exhibited dwarfism, retarded mineralisation in the limbs, ribs and vertebrae along with an inhibition of endochondral ossification due to a reduction in chondrocyte proliferation (704). Furthermore, *Mikasa et al.* (2011) observed *RUNX2*-induced expression of TCF7 in a pre-osteoblastic cell line and a paucity of TCF7 expression in *RUNX2*-null suggesting that *RUNX2* mediated induction of TCF7 expression may also be important for osteoblastic development (704). However, it should be noted that TCF7 is also expressed in a number of other cell types including T-cells, in which the family of TCF/LEF transcription factors were discovered. TCF7 also plays a critical role in the development of both natural killer cells and innate lymphoid cell development (705–707). The nature of our tissue subjected to interrogation by epigenome-wide association study (EWAS), umbilical cord blood, means T-cell, NK, MSC and other innate lymphoid cell types are most likely present in this tissue making it difficult to interpret the TCF7 transcription factor activity. Both determination of possible upstream regulation and an examination of the dmCpGs enriched by this transcription factor may therefore be pertinent to determine its functional relevance to this study.

Transcription factor enrichment analysis of the dmCpGs associated with BMD at birth found enrichment for EZH2 (NES=4.22). EZH2 is a catalytic subunit of the polycomb repressive complex 2 (PRC2) which catalyses the trimethylation of histone 3 at lysine residue 27 (H3K27me3), a histone mark associated with transcriptional silencing. EZH2 is one of a number of epigenetic regulators that plays a critical role during mesenchymal lineage commitment and osteoblast differentiation. A genome-wide screen to identify EZH2-target genes in human mesenchymal stromal cells (MSCs) found pre- to post-osteogenic differentiation the number of genes with bound EZH2 reduced from over 4000 to less than 30. *In vitro*, loss or inhibition of EZH2 has been shown to enhance osteogenic and inhibit adipogenic differentiation of human adipose mesenchymal stromal cells (AD-MSCs), human BM-MSCs and murine pre-osteoblastic cells (708–710). However, a study from *Dudakovic et al.* (2018) showed the role of EZH2 during skeletal development *in vivo* may be dependent on the stage of development. The conditional knock-out (cKO) of EZH2 in early mesenchymal lineage cells caused skeletal patterning defects. Conversely, cKO of EZH2 in committed pre-osteoblastic cells lead to normal development of the skull, clavicles and long bones albeit with increased bone marrow adiposity, reduced trabecular bone mass and male body weight. This suggests the role of EZH2 and its effects on bone formation are stage-dependent and it can both suppresses lineage commitment and facilitates proliferation of osteoprogenitors (2). This study has demonstrated an enrichment of EZH2 in the genes associated with the top 100 dmCpGs associated with BMD at birth. This enrichment may be due to EZH2 enrichment in the promoters of anti-osteogenic or anti-proliferative genes, leading to their transcriptional silencing through H3K27 trimethylation. However, the lack of histone mark enrichment data for the genes associated with the top 100 dmCpGs makes this assumption difficult to confirm. Alternatively, the enrichment for EZH2 may be a reflection of the necessity for EZH2 in skeletal development in the early mesenchymal lineage as described in *Dudakovic et al.* (2018) (711).

BMD at four years of age was enriched for 7 transcription factor binding site motifs (Table 12). Whilst the function of both the RNA Polymerase II Subunit A (POL2RA) and DNA Polymerase Iota (POLI) transcription factors are transcriptionally related, the significance of enrichment for both Odd-Skipped Related Transcription Factor 1 (OSR1) and Clock Circadian Regulator (CLOCK) to this study is not yet clear. However the enrichment of the Ten-eleven translocation methylcytosine dioxygenase 1 (TET1) (NES=4.19) transcription factor motif is of particular significance to this study. DNA methylation is considered a long-term epigenetic mark that is stable and maintained through cell division (541,712). However, demethylation does occur both passively and actively. Passive demethylation occurs during successive rounds of replication in the absence of functional DNA methylation maintenance machinery, whereas active demethylation occurs where TET enzymes sequentially oxidise the CpG methylation mark, 5-methylcytosine (5mC) to form the intermediates 5-formylcytosine and 5-carboxylcytosine. Demethylation is then completed by replication dependant dilution or by thymine-DNA glycosylase-mediated base excision repair of

these intermediates (713–716). Functionally, demethylation has been linked to the control of pluripotency, maintaining cell identity, nuclear reprogramming and cellular differentiation (717). There is also significant evidence of DNA methylation as an inhibitor of osteogenic and adipogenic differentiation of mesenchymal stem cells *in-vitro*, where differential methylation in the promoter region of osteogenic genes such as runt-related transcription factor 2 (*RUNX2*), osteopontin (*OPN*), alkaline phosphatase (*ALP*) and osteocalcin (*OC*) has been seen to associate with, or partly regulate differentiation (718–722). A study by *Zhang et al.* (2011) showed that upon inhibition of demethylation processes during the osteogenic differentiation of adipose-derived mesenchymal stem cells (AD-MSCs) the promoter regions of the distal-less homeobox 5 (*DLX5*), *RUNX2*, bone gamma-carboxyglutamate protein (*BGLAP*) and *Osterix* genes were hypermethylated, leading to the suppression of these genes and the interruption of osteogenic differentiation (565). In a similar model, *Daniunaite et al.* (2015) found that during the osteogenic differentiation of AD-MSCs, the loss of 5mC marks was accompanied by a concomitant increase in TET1 expression (723) suggesting a pro-osteogenic role for TET1 in AD-MSCs. This finding has been mirrored in a study by *Yang et al.* (2018) where the knockout of TET1 and TET2 in BM-MSCs isolated from mice lead to a decrease in their differentiation capacity (724). Furthermore, a study by *Sepulveda et al.* (2020) demonstrated that a knockout of either TET1 or TET2 in uncommitted mesenchymal multipotent C3H cells resulted in a significant inhibition *Osx* gene transcription (725). However, other BM-MSC models of osteogenic differentiation have also shown TET1 to be repressed during osteogenesis and adipogenesis, whereas TET2 has promoted both processes (726) suggesting the need for validation when considering the role of TETs in umbilical cord mesenchymal stromal cells (UC-MSCs). Within the context of this study we saw enrichment of the top 100 dmCpGs associated with BMD at four years of age with the TET1 motif. Despite the enriched dmCpGs not being explicitly osteogenic, a study by *Sepulveda et al.* (2017) showed TET1 and TET2 can act as scaffolding proteins leading to the recruitment of epigenetic modulators to promoter sites (725) that may not be inherently pro-osteogenic. Conversely, the dmCpGs enriched for TET1 may be anti-osteogenic and their expression being actively suppressed. Although this is not likely as the top 100 dmCpGs associated with this analysis also showed an under-representation of H3K27me3 mark, the removal of which is typically associated with upregulated genes in MSCs post-commitment and not acquired on downregulated genes (727).

The transcription factor binding site motif enriched within the dmCpGs associated with BMD at six years of age was POU Class 5 Homeobox 1 (*POU5F1*) (NES=4.23). *POU5F1* encodes the octamer-binding transcription factor 4 (OCT4) protein. OCT4 has been shown to be essential for the self-renewal and maintenance of pluripotency for embryonic stem cells. OCT4 generates three main isoforms (OCT4A, OCT4B and OCT4B1). OCT4A has been seen to be expressed and support pluripotency in both umbilical cord blood MSCs and haematopoietic stem cells (728–731). Curiously, the dmCpGs associated with BMD at six years of age enriched for *POU5F1* (appendix

9.4.2.3.2) indicate no apparent functional role in the maintenance of multipotency or pluripotency. However, the B Cell Linker (*BLNK*) gene is critical in B cell development and the knockdown of Potassium Channel Tetramerization Domain Containing 9 (KCTD9) can disrupt NK cell development, raising the question as to whether OCT4 may be acting in a repressive manner to prevent activation of genes critical for development (732,733). *Liang et al.* (2008) have shown both NANOG and OCT4 associate with transcriptional repression complexes in embryonic stem cells (734). However further work by *Hammachi et al.* (2012) proposed transcriptional activation by OCT4 is sufficient for the maintenance and induction of pluripotency (735). Therefore to determine what effect OCT4 enrichment may be having for these dmCpGs associated with BMD at 6 years of age will take further investigation.

3.3.4 Histone mark and chromatin state analysis

Post-translational histone modifications influence gene expression through dynamic regulation of the chromatin structure and also play a role in DNA replication and repair, chromatin compaction and cell-cycle control. However, histone marks often do not act in isolation and can influence the activity of those around them. The chromHMM tool developed by the ENCODE project uses combinations of histone modifications in regions of DNA to annotate the genome into a number of predicted regulatory regions including promoters, enhancers, active poised or repressed chromatin and a number of other chromatin states. In the context of this study, the enrichment of these dmCpGs for these annotations could therefore provide some information about the functional state of the chromatin landscape surrounding the dmCpGs. Whilst dmCpGs associated with BMD at eight years of age did not show significant transcription factor motif or pathway enrichment, there was significant enrichment of the top 100 dmCpGs associated with the analysis overlapping the H3K4me1, H3K4me3, H3K27ac histone marks (Figure 26a). These dmCpGs were also found to be enriched in regions of the chromatin predicted to be an active TSS and under-represented for regions of quiescent or low activity (Figure 26b). Collectively this suggests a significant increase in transcriptional activity associated with the differential methylation of the top 100 dmCpGs associated with BMD at eight years of age.

3.3.5 Overlap between analyses

The overlap between differential methylation analyses within the same age group in this study was assessed to ascertain whether there may be differential methylation changes at common CpG sites associated with postnatal measures of both BMC and BMD. As the only BMC related differential methylation analysis to produce a methylation signal, BMC at four years of age was compared with BMD at four years of age demonstrating an overlap of 18% or 36 dmCpGs when comparing the top 100 dmCpGs associated with each analysis (Figure 27). These overlapping dmCpGs are not explicitly osteogenic so we may be observing methylation changes in genes functionally unrelated to osteogenic processes associated with BMC and BMD. However, whilst

potentially functionally unrelated, if reproduced and validated in other cohorts these markers could potentially act as biomarkers of either BMC or BMD at four years of age. Additionally, whilst the similarity in differential methylation changes is of interest, the lack of overlap between the methylation changes associated with the two analyses is also worthy of attention. If the non-overlapping dmCpGs associated with each postnatal bone measure are distinct and reproducible then their utility as a therapeutic or a prognostic marker will differ. BMD is and continues to be the primary criteria used for the diagnosis of osteoporosis and predicted risk of fracture, therefore the significant differential methylation changes associated with BMD may be very useful prognostic markers. Conversely, differential methylation changes associated with BMC may be a more effective targets for therapeutics as a low BMD for a given skeletal size will be due to low BMC.

Additionally, we aimed to ascertain whether maternal vitamin D status in late-stage pregnancy may influence the methylation signatures associated with bone outcomes through the adjustment of our differential methylation analysis regression models for MLSP D status. Comparison of each model to its respective MLSP D status adjusted model found an average overlap of 33.8% between the top 100 dmCpGs associated with each analysis. This suggests a common contingent of dmCpGs in umbilical cord blood associated with postnatal measures of BMC and BMD which are occurring independent of MLSP D status. These markers therefore could be taken forward for further analysis to identify what distinguishes them from the others in the analysis.

3.3.6 Future work

Within this study we identified significant differential methylation changes in umbilical cord blood DNA from the SWS study associated with both BMD at birth and eight years of age. Whilst the dmCpGs associated with BMD at eight years of age may make for useful prognostic biomarkers, this would require reproduction umbilical cord blood DNA from additional, larger cohorts. However, there also remains the potential for these genes to have a yet undiscovered functional relationship with skeletal development. The potential for this relationship could initially be addressed by the selective knockdown of these genes *in vitro*, within BM-MSCs undergoing osteogenic differentiation. The potential effect of selective knockdown or inhibition on the osteogenic differentiation of these cells could then be assessed by histochemical methods such as alizarin red, ALP or Von Kossa staining, the immunohistochemical or transcriptomic assessment of markers of differentiation such as RUNX2, collagen type I, osterix and osteocalcin.

Additionally, we saw no associations with BMC at birth, six or eight years of age and only weak associations with BMC at four years of age, BMD at four years of age and six years of age suggesting a lack of power in the study and the need for greater numbers. In addition, the lack of power in this study may be exacerbated by the number intergenic dmCpGs within enhancer regions on the EPIC array (677). Enhancer regions can often be large distances from the promoter

region with which they interact, meaning dmCpGs within enhancer regions are often not yet associated with a gene, making it difficult to determine the functional implications of their differential methylation. However, chromatin capture technologies can show the looping of chromatin that occurs to bring an enhancer region and the promoter of a target gene into close proximity. This technology could therefore help us to interpret the meaning of these intergenic methylation changes (736–738).

The differential methylation changes associated with BMD at eight years of age may potentially find utility as a prognostic biomarker for BMD at eight years of age, however the clinical utility of a biomarker for BMD at birth may be limited. This suggests investigation of the biological implications of these dmCpGs associated with BMD at birth may be more suitable. Although whilst umbilical cord blood DNA has been judged as a good surrogate for tissue derived from the mesoderm, umbilical cord blood is a heterogeneous mix of cell types. Therefore, we would need to conduct further functional studies *in vitro* utilising either isolated, purified UC-MSCs or primary BM-MSCs to attempt to ascertain the functional implications of these differential methylation changes as these are ultimately formed into osteoblasts *in vivo*.

Within this study we have identified significant enrichment of the top 100 dmCpGs associated with BMD at birth and four years of age for the TET1 and EZH2 transcription factor motifs. It has been suggested that demethylation and in particular TET1 plays a role in the differentiation of MSCs, however this has been seen to vary between studies (724–726). Therefore, to confirm the potential activity of TET1 in the UC-MSC cell type we would need to use an *in vitro* osteogenic differentiation model of UC-MSCs, utilising ChIP-seq to determine the binding of EZH2 and TET1 along with real time polymerase chain reaction (RT-PCR) to determine levels of transcript for each factor at given time points during differentiation. Additionally, the histone mark and chromatin state analysis performed within this study is done so with reference to the consolidated epigenome of human umbilical vein endothelial cells (HUVEC) cells. Whilst this is as similar an analogue to the umbilical cord blood epigenome as possible with the current data available, an *in vitro* model would also allow for the direct study of histone marks pertinent to the differentiation of UC-MSCs, such as those found by Wu *et al.* (2017) when studying chromatin dynamics in regard to the osteogenic differentiation of BM-MSCs (727). Also, to further understand the functional significance of transcription factor enrichment of the dmCpGs associated with each analysis, we would need to further analyse the function and potential associations between the dmCpGs enriched using a network or pathway analysis approach.

3.3.7 Limitations

This study has found a number of differential methylation changes in umbilical cord blood DNA associated with BMD at eight years of age that might have the potential, if replicated in other cohort to act as prospective biomarkers from birth. However, the potential biological implications

of for differential methylation changes such as those seen associated with BMD at birth may be difficult to determine. The EPIC array interrogated umbilical cord blood DNA which is a heterogenous mixture of cell types, therefore without further functional experimentation in other isolated, enriched UC-MSCs or BM-MSCs, it would be difficult to attribute these methylation changes to any one cell type. Furthermore, whilst it's generally understood how processes such as promoter methylation will affect transcriptional activity, it's difficult to infer how intragenic methylation will affect expression without further functional experimentation.

Additionally we saw no differential methylation changes associated with BMC at birth and only dmCpGs weakly associated with BMC at four years of age and BMD at four and six years of age. This may be an indication the study is underpowered and requires greater numbers, or alternatively the associations may not be there.

3.3.8 Conclusions

Work within this study has previously shown associations between perinatal methylation of the *RXRα* promoter at birth and postnatal BMC at four years of age. Whilst it wasn't possible to validate those CpGs on this EPIC array, we may have identified further significant differential methylation changes in umbilical cord blood DNA associated with both BMD at birth and eight years of age. A prognostic biomarker which could identify a reduced bone growth trajectory at eight years of age before puberty would be of substantial use in determining whether a therapeutic intervention, such as nutritional supplementation may be of benefit in childhood. As biomarkers these dmCpGs would require validation in umbilical cord blood DNA further cohorts, but this is becoming more viable with the advent of 450k CpG and 850k CpG methylation arrays.

4 Chapter 4 – Is DNA methylation at birth altered by maternal vitamin D supplementation or associated with maternal vitamin D status?

4.1 Introduction

An adequate maternal vitamin D status is critical during pregnancy for optimal maternal and foetal outcomes (739). Multiple observational studies around the world have shown an association between maternal vitamin D insufficiency during pregnancy and adverse neonatal outcomes (740–744). But the potential benefits of vitamin D supplementation during pregnancy in relation to postnatal offspring bone growth remains contentious. However, previous work within the Maternal Vitamin D Osteoporosis Study (MAVIDOS) randomised controlled trial (RCT) has shown that offspring born in the winter months had significantly greater bone measures at birth when born to mothers supplemented with vitamin D in comparison to a placebo (625). Furthermore, vitamin D supplementation reduced the perinatal methylation of cytosine-phosphate-guanine (CpG) sites within the promoter region of the retinoid X receptor- α (*RXR α*) gene, a factor required for the nuclear activity of vitamin D (745). In the Southampton Women's Survey (SWS) cohort perinatal differential methylation of a CpG site in this region was also associated with offspring bone mass at four years of age and maternal vitamin D status (3). This later work suggested a possible link between maternal serum 25-hydroxyvitamin D₃ (25(OH)D) concentration, epigenetic control of *RXR α* expression and offspring bone development. Therefore, we aimed to further expand on this work using umbilical cord tissue from the MAVIDOS RCT to conduct a genome wide scan for further potential associations between umbilical cord tissue DNA methylation and the supplementation of mothers with 1000 IU/day cholecalciferol (supplemental vitamin D₃) during pregnancy. Furthermore, we aimed to assess only those insufficient for vitamin D in the early stages of pregnancy to who vitamin D supplementation may confer the greatest increase in serum 25(OH)D over the course of pregnancy and so potentially demonstrate a more marked epigenetic signature.

DNA methylation is also largely cell-type and tissue specific (746,747). In contrast to the MAVIDOS RCT where umbilical cord tissue was collected, the SWS study collected umbilical cord blood instead of tissue, although it did also collect measures of maternal vitamin D status in early and late-stage pregnancy. Whilst mothers were not supplemented with vitamin D, this second dataset provides us with the opportunity to further explore whether maternal vitamin D status in late-stage pregnancy is associated with perinatal methylation changes in birth tissues.

4.1.1 Aims

The aims of this chapter are to investigate:

- i) Whether the supplementation of mothers with a 1000 IU/day placebo or vitamin D intervention during pregnancy altered umbilical cord tissue DNA methylation and in the MAVIDOS RCT.
- ii) Whether there is an association between cord tissue DNA methylation and mothers insufficient for vitamin D in the early stages of pregnancy receiving a 1000 IU/day placebo or vitamin D supplement intervention in the MAVIDOS RCT.
- iii) Whether associations exist between umbilical cord blood DNA methylation and maternal late-stage pregnancy vitamin D status in the SWS cohort similar to those induced by maternal vitamin D supplementation in the MAVIDOS RCT.

4.2 Results

To further identify potential differential methylation changes induced by maternal vitamin D supplementation and whether these differentially methylated cytosine-phosphate-guanine dinucleotides (dmCpGs) may be linked to offspring bone mineral content (BMC) or bone mineral density (BMD) in postnatal development would be of significant interest to support the hypothesis of this study. Therefore, DNA from 386 umbilical cord tissue samples from the MAVIDOS RCT were interrogated using the Illumina Infinium MethylationEPIC BeadChip (EPIC) array. Differential methylation analyses and comparisons made between analyses are detailed in (Figure 28).

4.2.1 Cohort characteristics

DNA was extracted from umbilical cord tissue (n=386) and methylation status interrogated (described in sections 2.3 and 2.4). Array data was available for 383 samples post-quality control (QC) and 371 samples after exclusion of premature infants (<37 weeks).

There were no significant differences between placebo and vitamin D intervention groups in measures of body mass index (BMI), the number of smokers or the proportion of those with a Caucasian ethnicity. Vitamin D status showed no significant difference (p -value=0.269) in early pregnancy between intervention groups; However, a significant difference was found between the placebo and vitamin D intervention groups with respect to late pregnancy vitamin D status (p -value= 1.78×10^{-60}). When stratified into vitamin D sufficient, insufficient and deficient groups a significant association (p -value= 9.63×10^{-21}) was found between the intervention groups, where the proportion of those insufficient or deficient for vitamin D decreased but the number of those sufficient for vitamin D increased (Table 15).

There were no significant differences in the offspring in both the placebo and vitamin D intervention groups showed for gestational age, age at dual energy X-ray absorptiometry (DXA), measures of BMC and BMD at birth and at four years of age, nor in season of birth. The vitamin D supplemented intervention group contained a greater number of males (p -value=0.03) than the placebo group (Table 16).

4.2.2 Pre-processing EPIC array samples

DNA was previously extracted from the umbilical cord tissue of 142 participants of the MAVIDOS RCT and underwent EPIC array methylation analysis. To increase the power of the analysis, DNA samples from a further 244 umbilical cord samples were also extracted as described in section 2.3.2. Quality and quantity were determined as in section 2.3.4. Tabulated quantitative data from extracted DNA (appendix 9.5.1) and gel electrophoresis (Figure 29) demonstrated the quality of the samples was such that 1.5µg of sample was sufficient for EPIC array analysis. Samples were dispatched for array analysis as described in section 2.3.2.2.

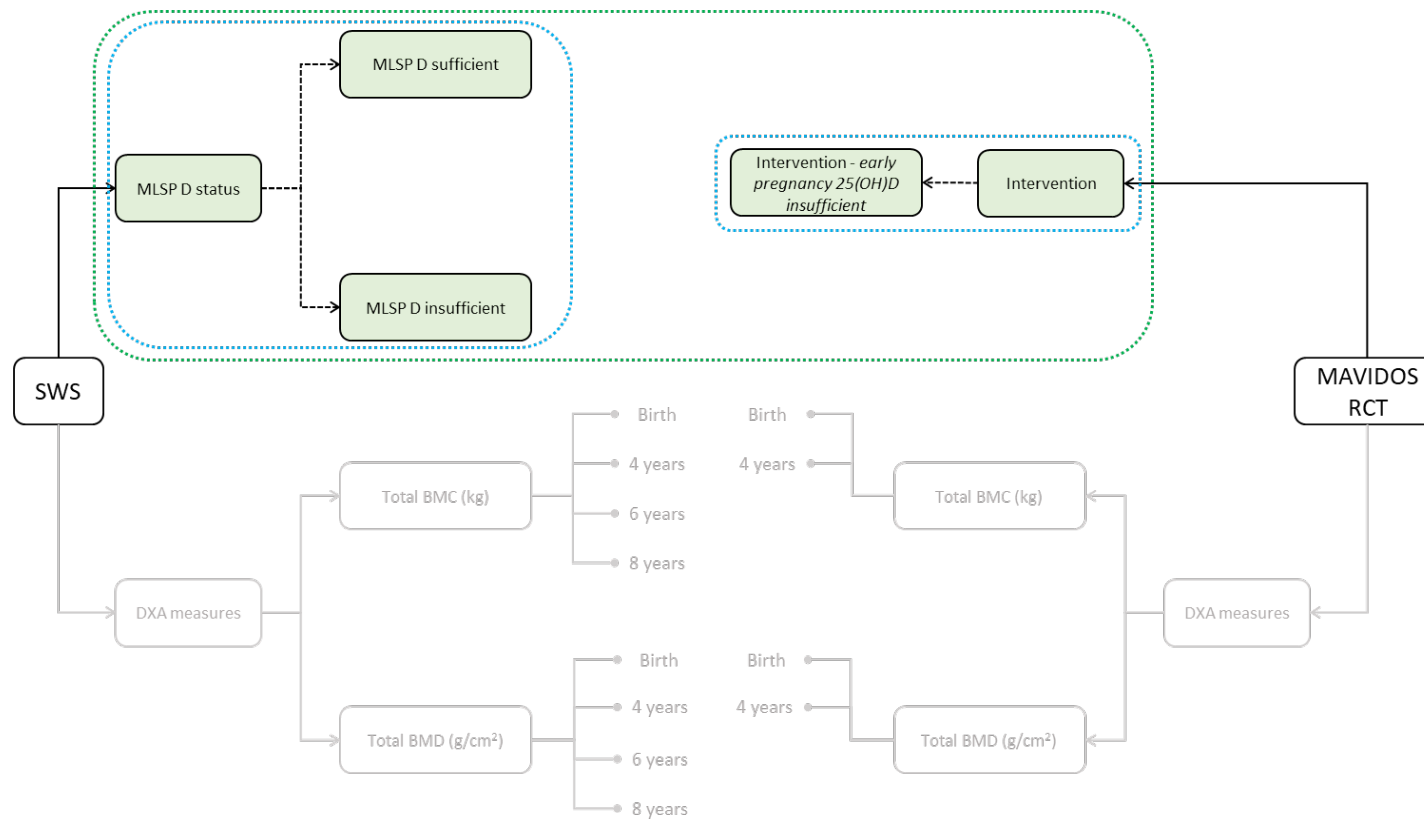


Figure 28. Differential methylation analyses and comparisons made between analyses with respect to maternal cholecalciferol supplementation during pregnancy in the MAVIDOS RCT and maternal 25(OH)D status in late pregnancy in SWS cohort

Within the MAVIDOS RCT, differential methylation analyses were conducted with respect to umbilical cord tissue DNA methylation and maternal cholecalciferol supplementation during pregnancy in both the whole study group (intervention) and the mothers insufficient for 25(OH)D in early pregnancy (intervention - early pregnancy 25(OH)D insufficient) (n=224). Furthermore, within the SWS study, differential methylation analyses were conducted with respect to umbilical cord blood DNA methylation and maternal late-stage pregnancy 25(OH)D (MLSP D) status, MLSP D status for those 25(OH)D insufficient in late pregnancy (<50nmol/L) (MLSP D insufficient), and those sufficient in late pregnancy (MLSP D sufficient) (≥50 nmol/L). A comparison of the top 100 dmCpGs when ranked by FDR was made between intervention-related analyses within the MAVIDOS RCT and between all MLSP D status related analyses within the SWS cohort, indicated in the figure by light blue boxes. In addition, comparisons were made between all MAVIDOS RCT intervention-related analyses and all SWS cohort MLSP D status-related analyses (green boxes).

4.2.3 MAVIDOS RCT QC

4.2.3.1 Beta values QC

Prior to differential methylation analysis, beta values generated for each sample were normalised as in section 2.4.1 and a series of QC steps undertaken (Figure 13). A scatter plot of the first two principal components (PCs) generated from the beta values indicated three samples with a sex classification contradicting phenotypic data (Figure 30a), indicating a sex mismatch and so were removed. A PC plot of beadchip showed evidence of a batch effect (Figure 30b), which was corroborated by a singular value decomposition (SVD) plot (Figure 31) suggesting the need for batch correction. The calculation of the median absolute deviation score (MADscore) found sixteen samples were outliers (MADscore < -5) and excluded from further analysis, leaving 367 samples in the dataset. Beta values were adjusted for batch using the ComBat function of the surrogate variable analysis (SVA) package (v3.20.0) to obtain batch-corrected beta values that could be used in further investigation.

4.2.3.2 Probes QC

Probes were excluded from further analysis based on detection value and bead count criteria as described in section 3.2.2.2. Further probes were removed based on their single-nucleotide polymorphism (SNP) content, ability to cross-hybridise, non-CpG relatedness or location on sex chromosomes as identified in *Zhou W et al.* (2017), *Pidsley et al.* (2016) and *Nordlund et al.* (2013) (645–647) (

4.2.3.3 Establishing model covariates

Differential methylation analysis required covariates to be established for each regression model. Covariates were selected initially based on biological relevance to the outcome variable, methylation. Smoking during pregnancy, sex and gestational age were all selected for their capacity to influence differential methylation changes at a number of CpG sites across the genome (680–684). Furthermore cellular composition was adjusted for using *Lin et al.* (2018) to ensure any difference in methylation between samples was not driven by cellular composition (649). Finally, to identify any other phenotypic variables potentially correlated with the outcome variable the 100,000 CpGs from the EPIC array with the greatest variance were used to generate a correlation matrix with the phenotypic data from this study. Sentries position was indicated a significant correlation (p -value=0.003) with the CpGs demonstrating the greatest variance and so was included a covariate in the differential methylation analysis regression models (Table 18).

4.2.3.4 Model QC

The quality of each differential methylation analysis model was assessed by the inflation factor (λ), a quantile-quantile plot (qqplot) and a p -value histogram. Those models considered inflated ($\lambda > 1.2$) were adjusted for inflation and bias using the bacon package (v.1.12.0) and newly generated p -values were adjusted for multiple testing (650). Models with a qqplot exhibiting a largely normal distribution and a p -value histogram with a sloping curve were taken forward for further analysis.

Table 15. Maternal cohort characteristics for the MAVIDOS RCT split by intervention group

Values are n (%), or median (5th, 95th percentile). P difference 25(OH)D at 34 weeks, cholecalciferol supplemented versus placebo group, $p < 0.001$. A sufficient early or late pregnancy serum 25(OH)D equates to a blood serum 25(OH)D concentration of ≥ 50 nmol/l, insufficient ≥ 25 nmol/l and < 50 nmol/l and deficient < 25 nmol/l. Group/number refers to the subgroup of a phenotypic characteristic group, or the number within the phenotypic characteristic group if no subgroups exist.

Phenotypic characteristic	Group/number	N (%) or median (5th, 95th percentile)		Test <i>p</i> -value
		Placebo n = 183	1000 IU/day 25(OH)D n = 188	
<i>Ethnicity</i>	White	172 (99.4)	172 (97.2)	0.105
	Other	1 (0.6)	5 (2.8)	
<i>Maternal age at birth of offspring</i>	363	31.06 (22.34, 39.38)	31.36 (21.77, 39.58)	0.947
<i>Smoking during pregnancy</i>	No	164 (90.1)	175 (94.1)	0.157
	Yes	18 (9.9)	11 (5.9)	
<i>Maternal BMI (kg/m²)</i>	349	25.74 (20.28, 35.43)	24.71 (19.94, 36.66)	0.096
<i>Early pregnancy: Serum 25(OH)D at 14 weeks (nmol/l)</i>	365	45.1 (20.15, 72.73)	43.8 (18.66, 66.7)	0.269
<i>Early pregnancy: Serum 25(OH)D at 14 weeks categories (nmol/l)</i>	Sufficient	66 (36.7)	64 (34.6)	0.872
	Insufficient	96 (53.3)	100 (54.1)	
	Deficient	18 (10)	21 (11.4)	
<i>Late pregnancy: Serum 25(OH)D at 34 weeks (nmol/l)</i>	360	42.42 (13.55, 79.64)	68.3 (31.22, 96.79)	1.78E-60
<i>Late pregnancy: Serum 25(OH)D at 34 weeks categories (nmol/l)</i>	Sufficient	58 (33)	152 (82.6)	9.63E-21
	Insufficient	75 (42.6)	24 (13)	
	Deficient	43 (24.4)	8 (4.3)	

Table 16. Offspring cohort characteristics for the MAVIDOS RCT split by intervention group

Group/number refers to the subgroup of a phenotypic characteristic group, or the number within the phenotypic characteristic group if no subgroups exist.

Phenotypic characteristic	Group/number	N (%) or median (5th, 95th percentile)		Test <i>p</i> -value
		Placebo n = 183	1000 IU/day 25(OH)D n = 188	
<i>Gestational age (weeks)</i>	368	40.43 (38.44, 41.86)	40.29 (38.71, 41.86)	0.80
<i>Sex</i>	Female	92 (50.5)	74 (39.6)	0.03
	Male	90 (49.5)	113 (60.4)	
<i>Season of birth</i>	Spring	46 (25.3)	41 (21.9)	0.78
	Summer	49 (26.9)	57 (30.5)	
	Autumn	54 (29.7)	52 (27.8)	
	Winter	33 (18.1)	37 (19.8)	
<i>Baby DXA: Age (days)</i>	341	4.5 (1, 14.65)	5 (1, 13.4)	0.64
<i>Baby DXA: Total BMC (g)</i>	326	61.5 (45.7, 79.61)	62.15 (44.92, 80.25)	0.83
<i>Baby DXA: Total BMD (g/cm²)</i>	326	0.21 (0.18, 0.23)	0.2 (0.18, 0.23)	0.65
<i>4-year DXA: Age (years)</i>	271	4.07 (3.99, 4.38)	4.07 (3.99, 4.34)	0.21
<i>4-year DXA: Subtotal BMC (g)</i>	245	349.34 (296.11, 425.67)	358.45 (291.53, 437.1)	0.24
<i>4-year DXA: Subtotal BMD (g/cm²)</i>	245	0.47 (0.42, 0.52)	0.47 (0.42, 0.54)	0.11

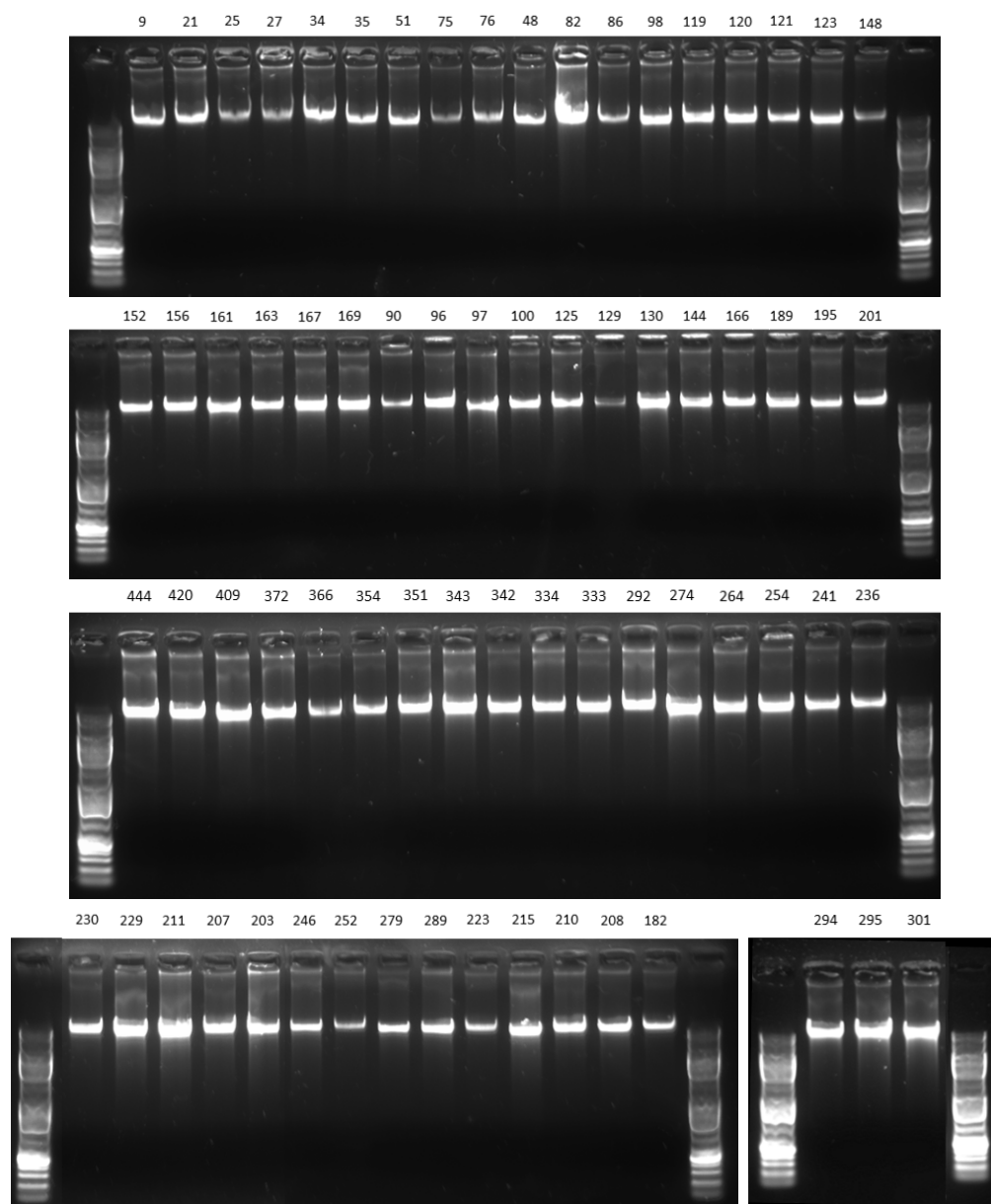


Figure 29. Agarose electrophoresis gels showing an example (n=70) of DNA quality post-phenol/chloroform extraction from crushed umbilical cord tissue taken from births within the MAVIDOS RCT for the purpose of EPIC array interrogation

Above, 70 of a total of 386 DNA samples which were all extracted from crushed umbilical cord tissue taken from births within the MAVIDOS RCT where mothers were supplemented with either cholecalciferol or a placebo during pregnancy. DNA extractions were performed using the phenol-chloroform technique as described in section 2.3.2.1. Once extracted, DNA quantity and quality was assessed using nanodrop spectrophotometer and 500ng assessed using agarose gel electrophoresis as described in section 2.3.4.

Chapter 4

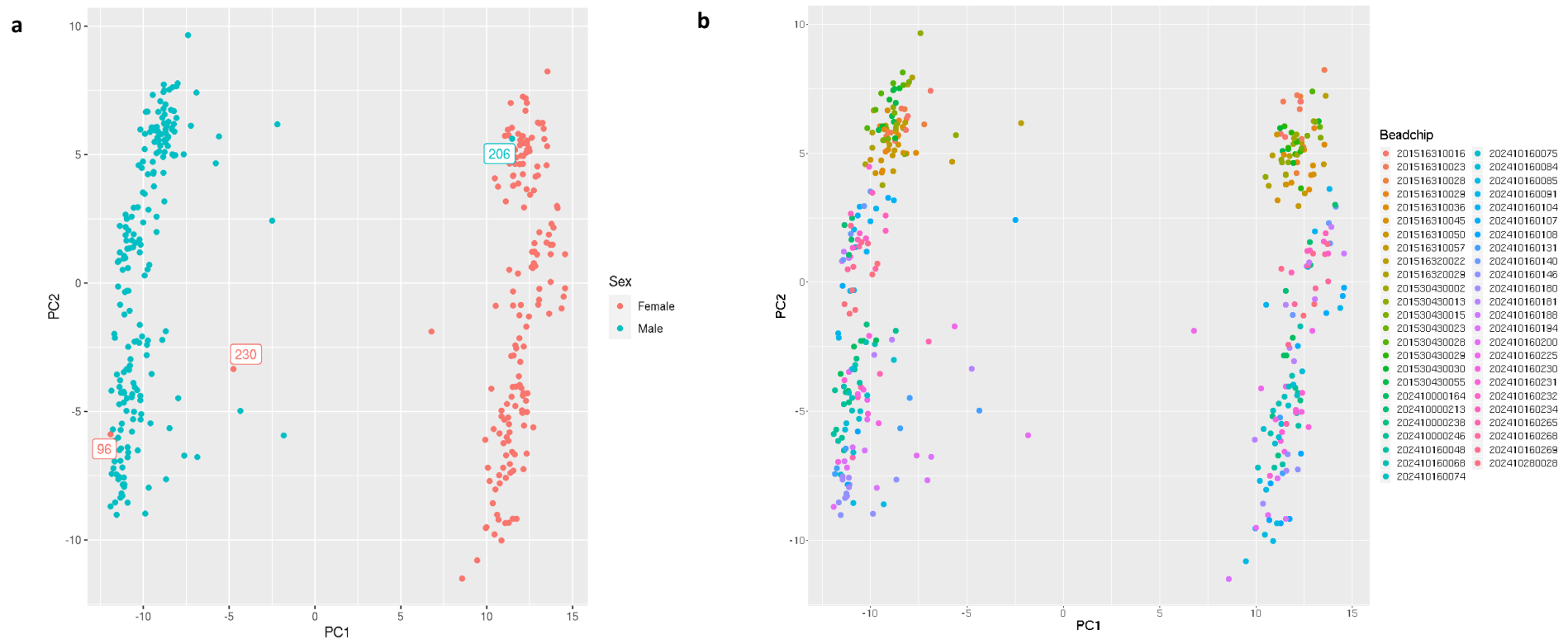


Figure 30. Scatter plots of the first two principal components generated from the principal component analysis of MAVIDOS RCT UCT DNA methylation data coloured by a) offspring sex and b) beadchip

Scatter plots of the first two principal component values generated for each sample by the principal component analysis of methylation data acquired from the EPIC array interrogation of MAVIDOS RCT umbilical cord tissue DNA, coloured by a) offspring sex and b) EPIC array beadchip. Plots of the first two principal components showed a) strong clustering based on sex indicating a strong influence of sex on methylation within the dataset and three samples, 206, 230 and 96 of which sex been misclassified. Similarly, b) clustering was seen with respect to beadchip, suggesting a need for the batch correction of the methylation data.

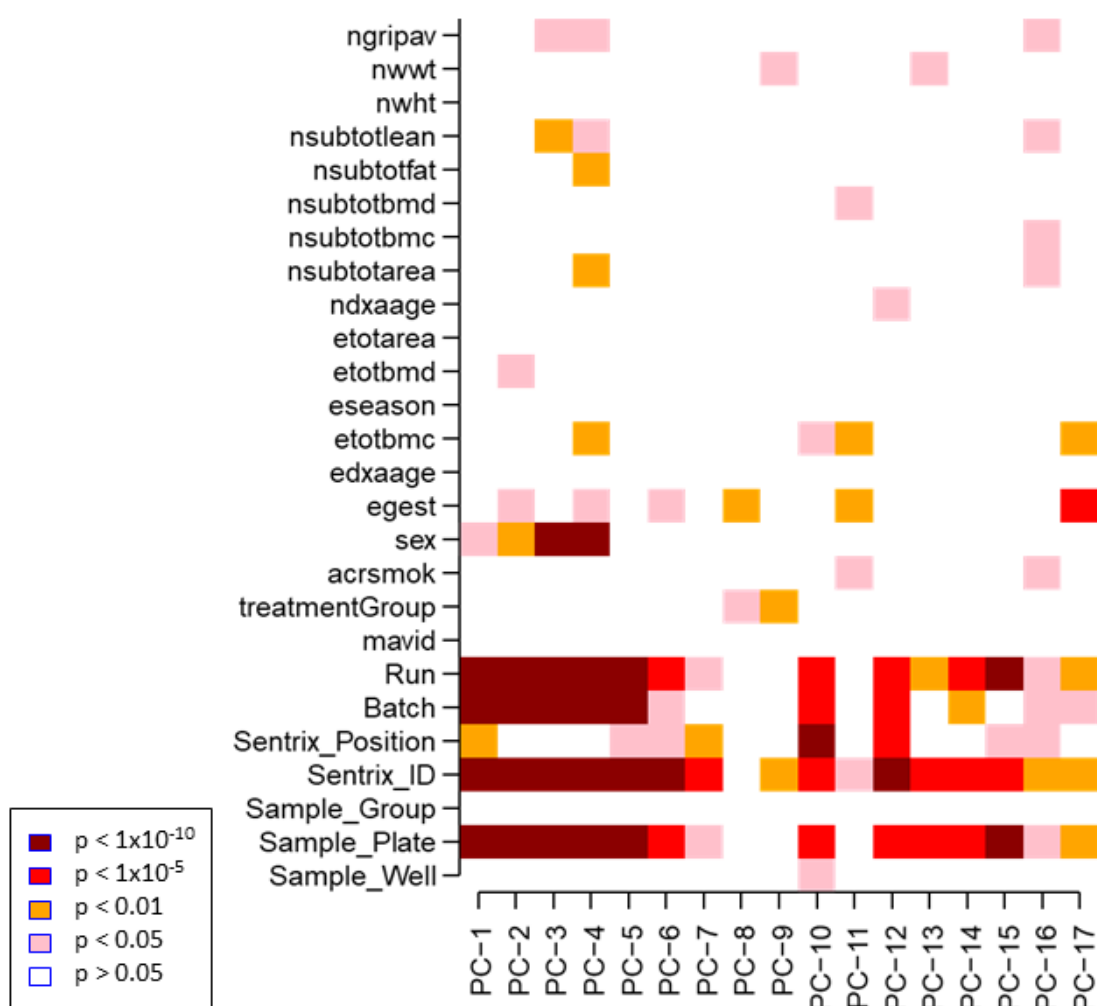


Figure 31. Singular value decomposition plot of post-normalisation of methylation data generated from the EPIC array interrogation of UCT DNA from the MAVIDOS RCT

The EPIC array interrogation of MAVIDOS RCT umbilical cord tissue DNA generated methylation data for each sample which post-QC was subjected to a singular value decomposition (SVD) analysis. SVD analysis allowed for the assessment of each phenotypic characteristic as a potentially significant source of variance in the methylation dataset. A post-normalisation plot MAVIDOS RCT methylation data indicated Sample plate, beadchip (indicated by Sentrix_ID), position on beadchip (indicated by Sentrix_position), batch, run, sex, gestational age (egest), BMD at birth (etotbmd) as significant (p -value <0.05) sources of variance in the methylation dataset when accounting for the first two principal components.

Table 17. The number of probes excluded from further analysis during probe QC of the MAVIDOS RCT dataset based on exclusion criteria described in section 3.2.2.2

Exclusion criteria	Number of probes excluded
Detection value >0.01	22839
Bead count <3 in 5% of samples	1316
SNP probes	93501
Cross-hybridising probes	3067
Non-CpG probes	2698
Sex chromosome	15978
Probes remaining post-QC	726468

Table 18. The covariates adjusted for in each regression model supplied to limma for differential methylation analysis in the MAVIDOS RCT

MAVIDOS RCT term	Definition
Sentrix_position	Position on chip
acrsmok	Smoking during pregnancy
sex	Sex
egest	Gestational age
Bcell	Estimated B cell composition
CD4T	Estimated CD4T cell composition
CD8T	Estimated CD8T cell composition
CD14	Estimated CD14 cell composition
Gran	Estimated granulocyte cell composition
NK	Estimated natural killer cell composition
Stromal	Estimated stromal cell composition
Endothelial	Estimated endothelial cell composition
Epithelial	Estimated epithelial cell composition
Mono	Estimated monocyte cell composition

4.2.4 Differential methylation analysis of supplementation with either a placebo or vitamin D intervention during pregnancy in the MAVIDOS RCT

4.2.4.1 Differentially methylated CpGs

To ascertain whether there were differential methylation changes induced by maternal vitamin D supplementation in MAVIDOS RCT umbilical cord tissue DNA, we conducted a differential methylation analyses with respect to intervention group and also an analysis of intervention group for mothers who were insufficient (<50 nmol/l) for vitamin D in the early stages of pregnancy. A differential methylation analyses interrogating the relationship between MAVIDOS RCT umbilical cord tissue DNA methylation and intervention group found 3 dmCpGs with a false discovery rate (FDR)<0.05. The most significant dmCpG associated with intervention group was an intergenic CpG, cg21023307 (FDR=0.007), the methylation of which was lower in the vitamin D supplemented intervention group (Figure 32).

Similarly, differential methylation analysis assessing the association between MAVIDOS RCT cord tissue DNA methylation and intervention group for mothers early-stage pregnancy vitamin D (MESP D) insufficient identified 7 dmCpGs with an FDR<0.05. Again, the most significant dmCpG was the intergenic CpG cg21023307 (FDR= 0.002). The methylation of cg21023307 was also lower in the vitamin D supplemented group (Figure 33).

The top dmCpGs (FDR <0.25) for both intervention and intervention MESP D insufficient analyses are listed in Table 19, with the top 100 dmCpGs for both analyses in appendix 9.4.3. A comparison of the top 100 dmCpGs from both analyses indicated an overlap of 14 dmCpGs (Figure 34), including dmCpG sites within the Solute Carrier Family 38 Member 10 (*SLC38A10*) gene, the Protein Tyrosine Phosphatase Receptor Type N2 (*PTPRN2*) gene and T-SNARE Domain Containing 1 (*TSNARE1*) gene.

4.2.4.2 Genomic locations of dmCpGs

The locations of the top 100 dmCpGs associated the intervention group and intervention MESP D insufficient group analyses were determined relative to CpG islands and genomic structure. Together, the top 100 dmCpGs associated with both analyses were most frequently located within an open sea region, less frequently in CpG islands in northern shores or shelves and least frequently in south shelves or shores. In terms of genomic structure, the largest proportion of the dmCpGs were located in the gene body, followed by the five prime untranslated region (5'UTR), first exon, 1500 bases upstream of the transcription start site (TSS) and finally the smallest proportion within the three prime untranslated region (3'UTR) (Figure 35).

Table 19. Results of differential methylation analysis investigating whether maternal vitamin D supplementation induces methylation changes in umbilical cord tissue DNA based on either intervention group, or intervention group only for mothers insufficient for vitamin D in the early stages of pregnancy in the MAVIDOS RCT

The differential methylation analysis investigating the induction of methylation changes in umbilical cord tissue (UCT) DNA based on intervention group found three significant (FDR<0.05) dmCpGs (n=339). The differential methylation analysis investigating the induction of methylation changes in UCT DNA based on intervention group MESP D insufficient found seven significant dmCpGs (n=213). DmCpGs associated with a known gene are represented by their respective associated gene symbol.

Model	Sample number	Lambda	FDR					Top 10 dmCpGs (FDR <0.25)
			<0.25	<0.20	<0.15	<0.10	<0.05	
Intervention	339	1.2	56	34	25	7	3	cg21023307, SLC38A10, TNC, ABCA7, KLK15, cg06629252, AMN1, ARL4C, NR2C1, cg00554871
Intervention MESP D insufficient	213	1.12	161	43	28	12	7	cg21023307, POLRMT, LOC102723376, TSNARE1, C14orf102, DCC, POLL, SUPT5H, cg20990091, APH1B

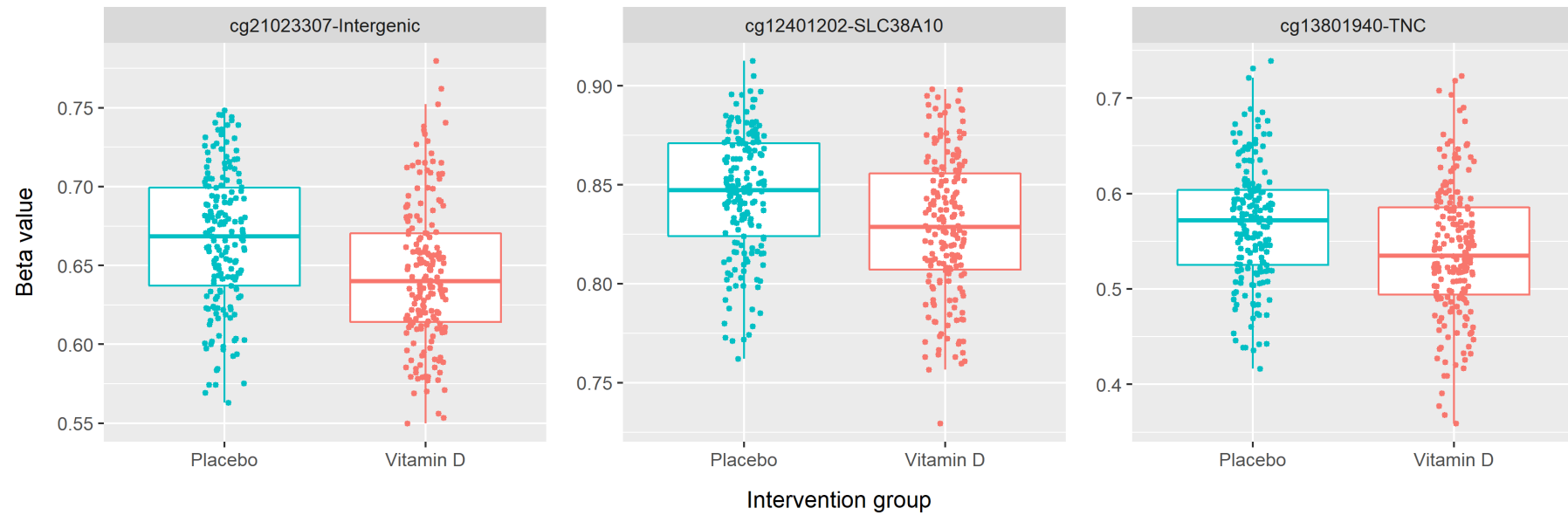


Figure 32. The association between the perinatal methylation of umbilical cord tissue at birth and maternal intervention group for the three dmCpGs significantly (FDR <0.05) differentially methylated within the intervention group differential methylation analysis in the MAVIDOS RCT

The differential methylation analysis investigating whether maternal vitamin D supplementation induced perinatal methylation changes present at birth within umbilical cord tissue DNA extracted from the MAVIDOS RCT based on intervention group (n=339) found methylation changes at cg21023307, cg12401202 and cg13801940, CpGs mapped to an intergenic region, the SLC38A10 gene and TNC gene respectively. A decrease in methylation was indicated for all significant dmCpGs within the vitamin supplemented group of mothers when compared to mothers supplemented with a placebo.

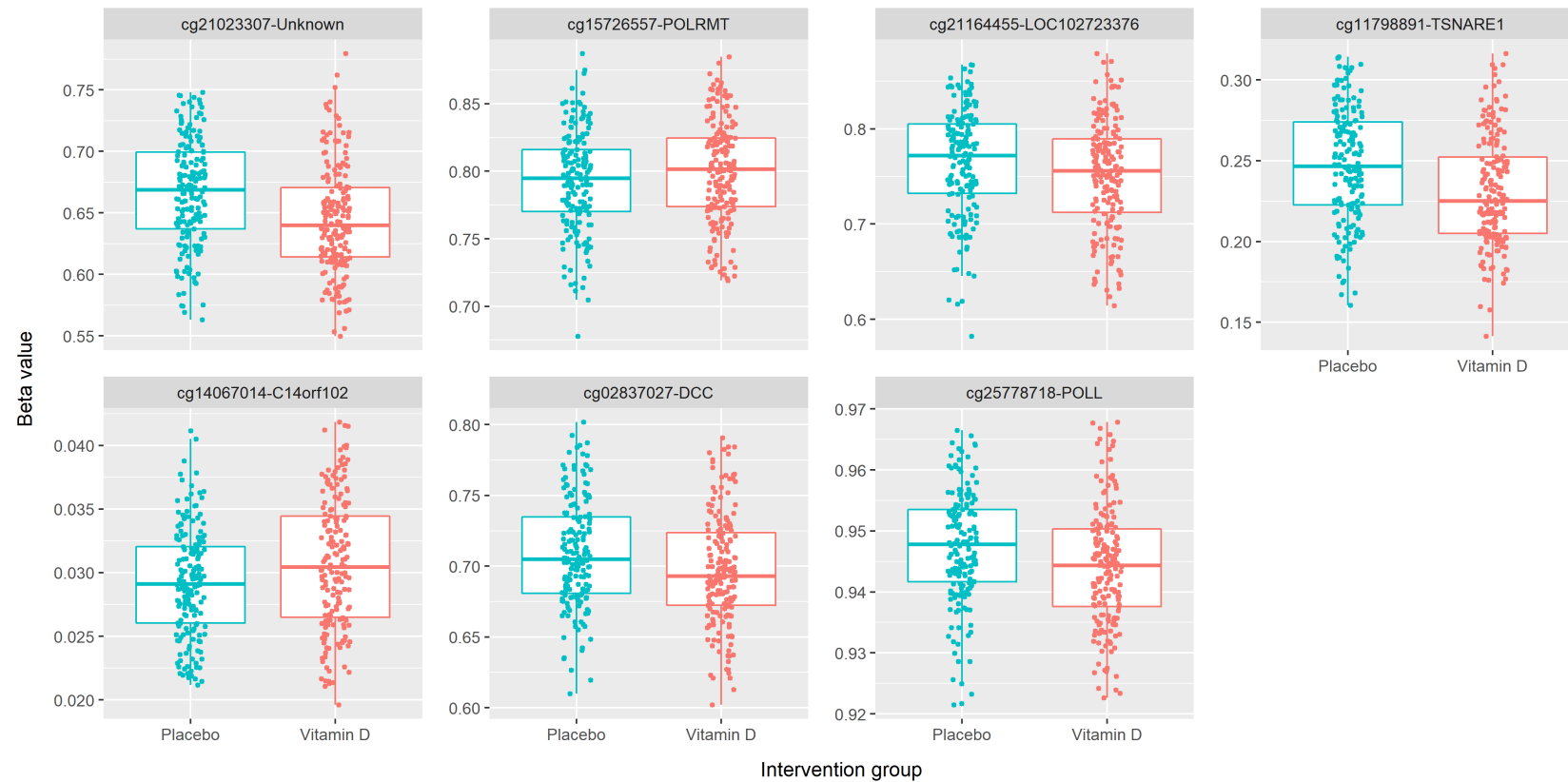


Figure 33. The association between methylation and intervention group for the seven dmCpGs significantly (FDR <0.05) differentially methylated in the intervention group MESP D insufficient differential methylation analysis within the MAVIDOS RCT

The differential methylation analysis investigating whether maternal vitamin D supplementation induced methylation changes within umbilical cord tissue DNA extracted from the MAVIDOS RCT based on intervention group MESP D insufficient (n=213) found methylation changes seven CpG sites. The most significant CpG site was mapped to an intergenic region, whereas the rest of the CpGs were mapped to a known gene. Five CpGs showed a decreased methylation in the vitamin D supplemented group of mothers, whereas two showed an increase in methylation.

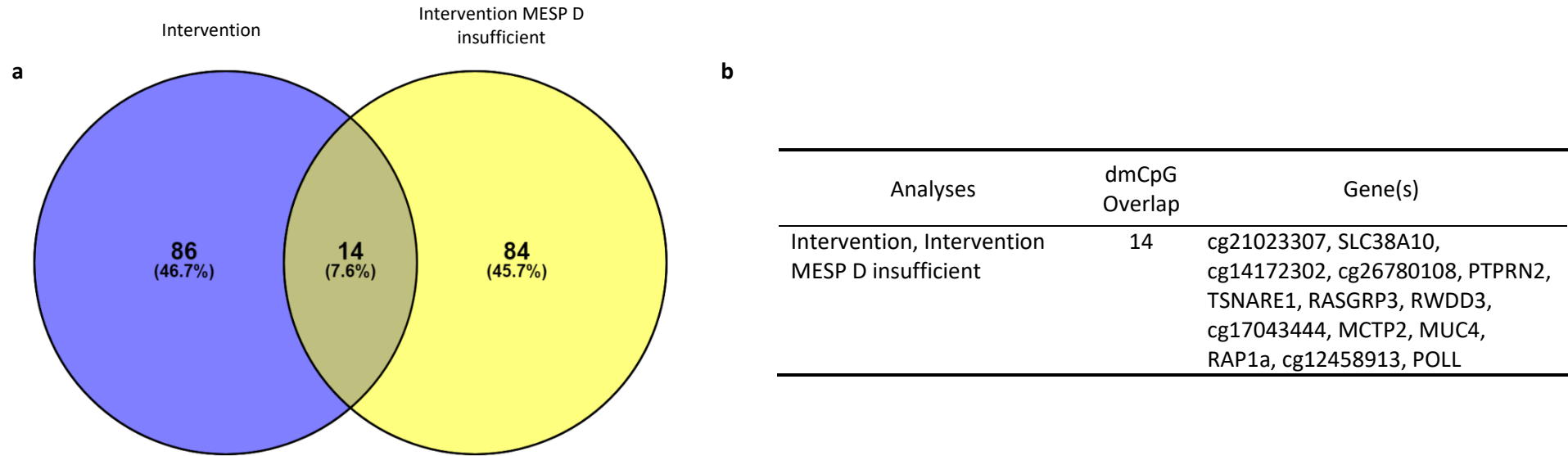


Figure 34. The a) venn diagram overlap between the top 100 dmCpGs associated with the intervention group and intervention group MESP D insufficient analyses and the b) associated identity of each overlapping dmCpG in the MAVIDOS RCT

A comparison of the top 100 dmCpGs when ranked by FDR was compared between differential methylation analyses investigating the relationship between umbilical cord tissue DNA methylation and receiving a cholecalciferol or placebo supplement intervention over the course of pregnancy and receiving a cholecalciferol or placebo supplement intervention over the course of pregnancy when initially insufficient for 25(OH)D in early pregnancy in the MAVIDOS RCT. A a) venn diagram depicts the overlap between dmCpGs taken from each analysis, and b) the overlapping dmCpG probe name, or the gene name if located within a known gene. Intervention, receiving a cholecalciferol or placebo supplement intervention over the course of pregnancy; Intervention MESP D insufficient, receiving a cholecalciferol or placebo supplement intervention over the course of pregnancy when initially insufficient for 25(OH)D in early pregnancy.

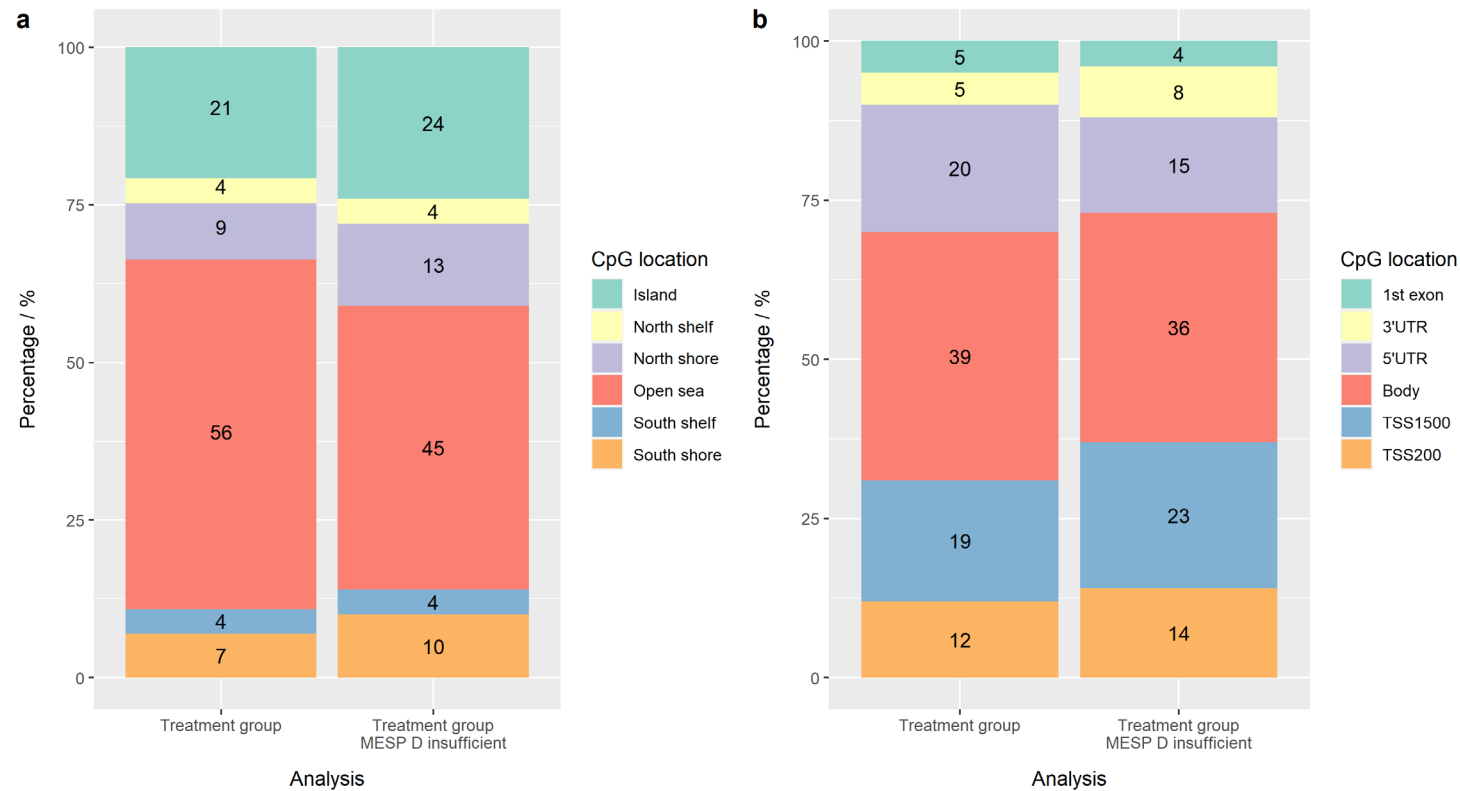


Figure 35. The location of the top 100 dmCpGs associated with intervention and intervention MESP D insufficient analyses relative to a) CpG islands and b) genomic structure

The top 100 dmCpGs associated with the differential methylation analysis investigating whether maternal vitamin D supplementation induces methylation changes in UCT DNA based on intervention group were largely in an open sea region in the body of a gene. In contrast, the top 100 dmCpGs associated with the intervention group MESP D insufficient analysis were largely in an open sea region, but up to 1500 bases upstream of a TSS. CpG shores, shelves and open sea regions are defined in appendix 9.3.

4.2.4.3 *Transcription factor enrichment analysis*

Transcription factor enrichment analysis of the top 100 dmCpGs associated with both intervention group and intervention group MESP D insufficient analyses found enrichment for 0 and 9 transcription factor binding motifs respectively. The three most significantly enriched transcription factor motifs (normalised enrichment score (NES)>4) for the dmCpGs associated with the intervention group MESP D insufficient analysis were the Retinoblastoma-Binding Protein 5 (RBBP5) motif (NES=5.01) in 16 genes, Transcription Factor 12 (TCF12) motifs (NES=4.84-4.38) in 16 genes and Myogenic Factor 6 (MYF6) motif (NES=4.43) in 16 genes (full results in Table 20). Full list of motif references, sequence logo graphs and enriched genes in appendix 9.4.3.2.2.

4.2.4.4 *Differentially methylated regions*

Analysis of umbilical cord blood DNA methylation with respect to both intervention group and intervention group for MESP D insufficient mothers indicated no significant regions of differential methylation.

4.2.4.5 *Pathway enrichment analysis*

4.2.4.5.1 *Cytoscape pathway analysis*

The Cytoscape clustering analysis of the top 100 dmCpGs associated with both intervention group and intervention group MESP D insufficient produced 2 and 1 clusters respectively (Table 21). Subsequent gene ontology (GO) term enrichment analysis showed the dmCpGs associated with intervention group were enriched for pathways such as vitamin binding (GO:19842, FDR=3.09e⁻²), epithelium development (GO:60429, FDR=3.08e⁻²) and muscle system processes (GO:3012, FDR=3.95e⁻²). Whereas GO term enrichment analysis of the dmCpGs associated with intervention group MESP D insufficient found enrichment for processes related to activity at the kinetochore (GO:776, FDR=1.93e⁻³) chromosome, centromeric region (GO:775, FDR=4.67e⁻³) and establishment or maintenance of cell polarity (Figure 36). Full ontology results are available in appendix 9.4.3.

4.2.4.5.2 *Ingenuity pathway analysis*

To elucidate further information about the functional significance of the methylation changes in umbilical cord tissue associated with vitamin D supplementation during pregnancy, a pathway and network analysis was also conducted using Ingenuity Pathway Analysis (IPA). Ingenuity Pathway Analysis identifies significant networks, upstream regulators, canonical pathways, networks, the diseases and functions associated with the differentially methylated CpGs based on the Ingenuity knowledge database. The Ingenuity knowledge database contains manually reviewed experimentally-derived data from scientific literature and third-party databases such as National Centre for Biotechnology Information, GO and Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotations (full list in appendix 9.7). The 'core analysis' function within IPA was used to

analyse the top 100 dmCpGs associated with the intervention group analysis alone due to licensing restrictions. Full canonical pathway analysis results in appendix 9.4.3.1.3.

The top 100 dmCpGs associated with intervention group were most significantly enriched for pathways with functions related to Epithelial Adherens Junction Signalling ($p=4.07e^{-4}$) in 5 genes, Rac signalling ($p=1.55e^{-3}$) in 4 genes and macrophage-stimulating protein recepteur d'origine nantais (MSP-RON) signalling in cancer cells pathway ($p=2.24e^{-3}$) in 4 genes (Figure 37). The most significant upstream regulators for the dmCpGs were miR-149-5p ($p=1.00e^{-4}$) in 2 genes, an mRNA suggested to regulate the switch between the adipogenic and osteogenic differentiation of BM-MSCs, tretinoin/all-trans-retinoic acid (ATRA) ($p=1.12e^{-4}$) in 18 genes, an agonist of RXR α and Nicastrin (NCSTN) ($p=4.38e^{-4}$), a subunit of the gamma secretase complex and central to the Notch signalling pathway (Table 22).

4.2.4.6 Histone mark and chromatin state analysis

The differential methylation analyses for intervention group and intervention group MESP D insufficient showed no enrichment of the top 100 dmCpGs for either analysis overlapping histone marks or a particular chromatin state in the genome.

Table 20. The transcription factor binding motifs enriched within the top 100 dmCpGs associated with intervention group MESP D insufficient mothers' analysis in the MAVIDOS RCT cohort

Transcription factor enrichment analysis of the top 100 dmCpGs associated with intervention group analysis and intervention group MESP D insufficient mothers analysis found enrichment for 0 and 9 transcription factor motifs respectively with a normalised enrichment score (NES) >4.

Analysis	Enriched TFs	Rank	TF	Normalised enrichment score	Motifs	Annotation	Multiple motifs enrichment range		
							Maximum NES	Minimum NES	Genes enriched
<i>Intervention group – Mothers insufficient for vitamin D in early pregnancy (<50nmol/l)</i>									
	9	1	RBBP5	5.01	1	Direct	NA	NA	16
		2	TCF12	NA	3	Direct	4.84	4.38	16
		3	MYF6	4.43	1	Direct	NA	NA	16
		4	NHLH1	NA	2	Direct	4.32	4.18	18
		5	HES5	4.27	1	Direct	NA	NA	9
		6	PTF1A	4.24	1	Inferred	NA	NA	21
		7	BHLHA15	4.11	1	Direct	NA	NA	12
		8	ASCL2	NA	3	Mixed	4.06	4.01	24
		9	HSF1	4.02	1	Direct	NA	NA	9

Table 21. Gene clusters produced during the pathway analysis of the top 100 dmCpGs associated with the intervention group and intervention group MESP D insufficient analyses

Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with the intervention group and intervention group analyses produced 2, and 1 distinct clusters of dmCpGs respectively. However only one cluster associated with the intervention group analysis and the cluster associated with the intervention group MESP D insufficient analysis saw significant enrichment of pathways with the involvement of three or more dmCpGs

Model	Cluster	Genes	Nodes	Edges	Score	Pathways FDR <0.05	Gene count ≥3
Intervention	1/2	TLN1, TEX14, GALNT18, VAV2, LMCD1, MAPK6, STAM2, FAM65A, EGFR, PCBP1, PLEKHA7, DOCK8, ARL4C, ADA, GTDC1, ZADH2, INPP5A, TNC, IL33, PAWR, MTR, GOT1, PC, E2F8, TPM4, TCF4, PPP2R5C, CYFIP1, CCL11, MOCS1, CPE, TLN2, MLLT4, RAP1GAP2	34	129	6.971	172	63
	2/2	MYBPC1, FGFR2, HOXC10, KCNQ3, PDE4D, EMX2, KCNQ5, PTPRN2	8	16	3.111	10	0
Intervention MESP vitamin D insufficient	1/1	CENPT, PHF3, RWDD3, PTPRN2, TSHZ3, MGRN1, IRS1, NDE1, PDS5B, SUPT5H, CELSR2, CENPA, LTBP1, ANK1, RASGRP3, GAK, CLIP1, PACS2, SCR1, TGFB1, RGL1, RALGDS	22	49	4.000	52	18



Figure 36. GO terms significantly enriched (adjusted p -value <0.05) for the top 100 dmCpGs associated with the intervention group and intervention group MESP D insufficient analyses in the MAVIDOS RCT
Gene ontology pathways seen to be significantly enriched in the dmCpG clusters produced from the clustering of the top 100 dmCpGs associated with the analyses for intervention group and intervention group MESP D insufficient. A larger point size and $-\log_{10}$ adjusted p -value indicates a greater significance and number of dmCpGs enriched for the pathway.



Figure 37. IPA pathway analysis indicting canonical pathways significantly enriched for the top 100 dmCpGs associated with differential methylation analysis for intervention group in the MAVIDOS RCT

The ‘core analysis’ function was used to determine the enrichment of canonical pathways in the the top 100 dmCpGs associated with the intervention group analysis in the MAVIDOS RCT. Pathways are derived from the Ingenuity Pathway Knowledge Base. A larger point size and $-\log p\text{-value}$ indicates a greater significance and number of dmCpGs enriched for the pathway.

Table 22. The top upstream regulators associated with IPA pathway analysis of the top 100 dmCpGs associated with the intervention group differential methylation analysis in the MAVIDOS RCT

Rank	Upstream Regulator	Molecule Type	<i>p</i> -value of overlap	Target Molecules in Dataset
1	miR-149-5p	Mature microRNA	1.00E-04	RAP1A, VAV2
2	tretinoin	Chemical - endogenous mammalian	1.12E-04	AFDN, ANXA6, CEBPZ, CHRM2, CPE, CSF1R, DLL4, EGFR, KCNQ5, MAPK6, MUC20, MUC4, NCOA3, NFATC1, NR2C1, TNC, TPM4, VAV2
3	NCSTN	Peptidase	4.38E-04	CSF1R, EGFR, GRN
4	Lh	Complex	6.06E-04	ARL4C, EGFR, MAPK6, PAWR, PDE4D, PPP2R5C
5	tacrolimus	Chemical drug	6.06E-04	CCR3, IL33, NFATC1, SLC17A6, TIAM1, TNC
6	RIPK1	Kinase	9.04E-04	EGFR, GRN, IL33
7	ETV6	Transcription regulator	1.08E-03	CSF1R, DLL4
8	TGFB1	Growth factor	1.44E-03	CCR3, CSF1R, EGFR, FGFR2, IL33, MAPK6, MUC4, NCOA3, NFATC1, PCBP1, PDE4D, PLAGL1, RAP1A, RASGRP3, TCF4, TNC, ZMIZ1
9	EDN1	Cytokine	1.47E-03	ANXA6, EGFR, GRN, TNC, TPM4
10	tazemetostat	Chemical drug	1.52E-03	E2F8, EGFR, EMX2, NFATC1, TIAM1, TNC
11	L-685,458	Chemical - protease inhibitor	1.70E-03	DLL4, EGFR
12	EIF4E	Translation regulator	1.74E-03	CSF1R, EGFR, HOXC10, IL33, TNC
13	tributyltin	Chemical reagent	1.84E-03	DOCK8, IL33, TNC, WNK4
14	QKI	Other	1.86E-03	CSF1R, EGFR, TNC
15	TCF3-PBX1	Fusion gene/product	2.19E-03	ARL4C, EXTL3
16	CCND1	Transcription regulator	2.64E-03	E2F8, EGFR, GPRIN2, NCOA3, TCF4, TIAM1
17	NUMB	Other	2.74E-03	DLL4, EGFR
18	EPHA2	Kinase	2.74E-03	EGFR, GRN
19	SPDEF	Transcription regulator	2.92E-03	CYFIP1, EGFR, TNC
20	IL3	Cytokine	2.97E-03	CCR3, CEBPZ, CSF1R, IL33, NFATC1, VAV2
21	HDAC7	Transcription regulator	3.35E-03	DLL4, NFATC1
22	SOX2	Transcription regulator	3.58E-03	C1QL1, CSF1R, EGFR, EMX2, RAP1A, SOX21, ZNF488
23	PARPBP	Other	3.68E-03	TIAM1,TNC
24	RBPJ	Transcription regulator	3.72E-03	DLL4, MAPK6, NFATC1, TNC
25	UCHL1	Peptidase	3.98E-03	ANXA6, MAPK6, RWDD3

4.2.5 Differential methylation analysis in relation to maternal late-stage pregnancy vitamin D status within the SWS cohort

4.2.5.1 Differentially methylated CpGs

Here, we aimed to ascertain whether associations exist between umbilical cord blood DNA methylation and maternal late-stage pregnancy vitamin D status in the SWS cohort that are similar to those induced by maternal vitamin D supplementation in the MAVIDOS RCT. Therefore, using umbilical cord blood from the SWS study conducted a genome wide scan to establish what associations exist between maternal late-stage pregnancy (MLSP) D status, MLSP D sufficiency ($25(\text{OH})\text{D} \geq 50 \text{ nmol/l}$) and MLSP D insufficiency ($25(\text{OH})\text{D} \geq 25 \text{ nmol/l}$ and $< 50 \text{ nmol/l}$) in this late stage of pregnancy and the methylation of umbilical cord blood DNA in the SWS cohort.

Differential methylation analysis of umbilical cord blood DNA methylation found 9 dmCpGs weakly associated ($\text{FDR} < 0.25$) maternal late-stage pregnancy vitamin D status. The most strongly associated dmCpG was cg26974062, a CpG located in the body of the Thioredoxin-interacting protein (*TXNIP*) gene ($\text{FDR} = 0.1821$).

In contrast, differential methylation analysis to assess the association between umbilical cord blood DNA methylation and MLSP D insufficiency found 12 dmCpGs with an $\text{FDR} < 0.05$. The most significant dmCpG associated with MLSP D insufficiency was cg19744478, located in the body of the Hexokinase1 (*HK1*) gene ($\text{FDR} = 0.005895$), the methylation of which showed a negative association with maternal vitamin D status (Figure 38). The *HK1* gene assists in the phosphorylation of glucose to produce glucose-6-phosphate, the first step in the majority of glucose metabolism pathways.

Finally, the assessment of any differential methylation changes in umbilical cord blood DNA found 26 dmCpGs weakly associated ($\text{FDR} < 0.25$) with MLSP D sufficiency. The dmCpG with the strongest association was cg01290714, a CpG found in the 3'UTR of the Arrestin Domain Containing 2 (*ARRDC2*) gene ($\text{FDR} = 0.1318$). The top ten dmCpGs for all three analyses ($\text{FDR} < 0.25$) are listed in Table 23, with the top 100 dmCpGs in appendix 9.4.4.

A comparison of the top 100 dmCpGs associated with MLSP D status, MLSP D insufficiency and MLSP D sufficiency was made to determine any common methylation changes occurring between the analyses. The comparison between MLSP D status and MLSP D sufficiency found an overlap of 12 dmCpGs, between MLSP D sufficiency and MLSP D insufficiency 1 dmCpG, whereas comparing the overlap between all analyses found 1 common dmCpG (Figure 39).

4.2.5.2 Genomic locations of dmCpGs

The dmCpGs associated with MLSP D status, MLSP D insufficiency and MLSP D sufficiency were each assessed for their location relative to both CpG islands and genomic structure. Collectively dmCpGs were located primarily within an open sea region, less so in a north shelf or shore or CpG

island and least frequently in a south shelf or shore. Relative to genomic structure, the largest proportion of the dmCpGs were located in the gene body, a lesser proportion in the 1500 bases upstream of the TSS, followed by the 5'UTR, 1st exon or 1st exon boundary. The dmCpGs were least frequently located in a 3'UTR (Figure 40).

Table 23. Results of differential methylation analysis investigating the association between UCB DNA methylation and maternal late-stage pregnancy vitamin D status (MLSP D), mothers insufficient (MLSP D insufficient) and sufficient (MLSP D sufficient) for vitamin D in late-stage pregnancy in the SWS cohort

The differential methylation analysis investigating the association between UCB DNA methylation with MLSP D status found one weakly associated dmCpG (FDR <0.20) (n=422). In contrast 12 dmCpGs were found to be significantly (FDR<0.05) associated with those MLSP D insufficient (25(OH)D $\geq$ 25 nmol/L & <50 nmol/L) (n=140) and 8 weakly associated with those MLSP D sufficient (25(OH)D $\geq$ 50 nmol/L) (n=282). Cgs associated with a known gene are represented by their associated gene symbol.

	Model	Sample number	Lambda	FDR					Top 10 dmCpGs (FDR <0.25)
				<0.25	<0.20	<0.15	<0.10	<0.05	
SWS	MLSP D	422	1.09	8	1	0	0	0	TXNIP, HMOX2, LRRC34, TXNIP, HMCN2, SLC6A18, cg15959921, cg04525002, CCDC49, PRSS21
SWS	MLSP D insufficient	140	1.12	240	106	38	18	12	HK1, cg08431985, cg18940084, SPECC1, SLC22A3, cg02379973, HCCA2, PDIK1L, NMU, GALNT13
SWS	MLSP D sufficient	282	1.02	10	8	8	0	0	ARRDC2, HCG27, DSEL, NEBL, cg11182160, cg12740011, cg13708613, BPI, LIG2, cg19090034

Chapter 4

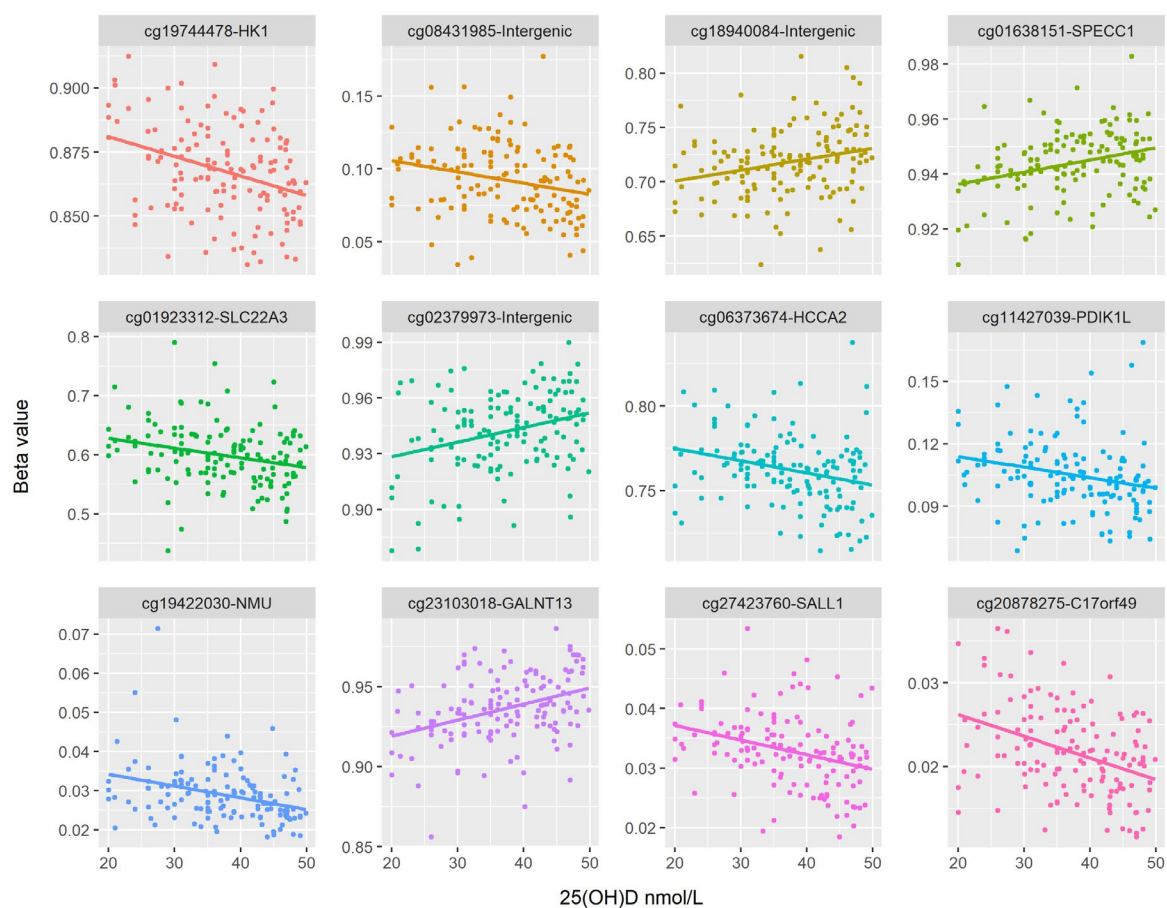


Figure 38. The association between methylation and maternal late-stage pregnancy serum 25(OH)D concentration for the 12 dmCpGs most significantly (FDR <0.05) associated with MLSP D insufficiency in the SWS cohort

The differential methylation analysis assessing the association between the methylation of 140 samples of UCB DNA and MLSP D insufficiency in the SWS cohort found significant (FDR <0.05) differential methylation of 12 CpGs, three of which were mapped to an intergenic region and 9 of which were mapped to known genes. The methylation of eight CpG sites was inversely associated with maternal serum 25(OH)D concentration and the methylation of four sites positively associated.

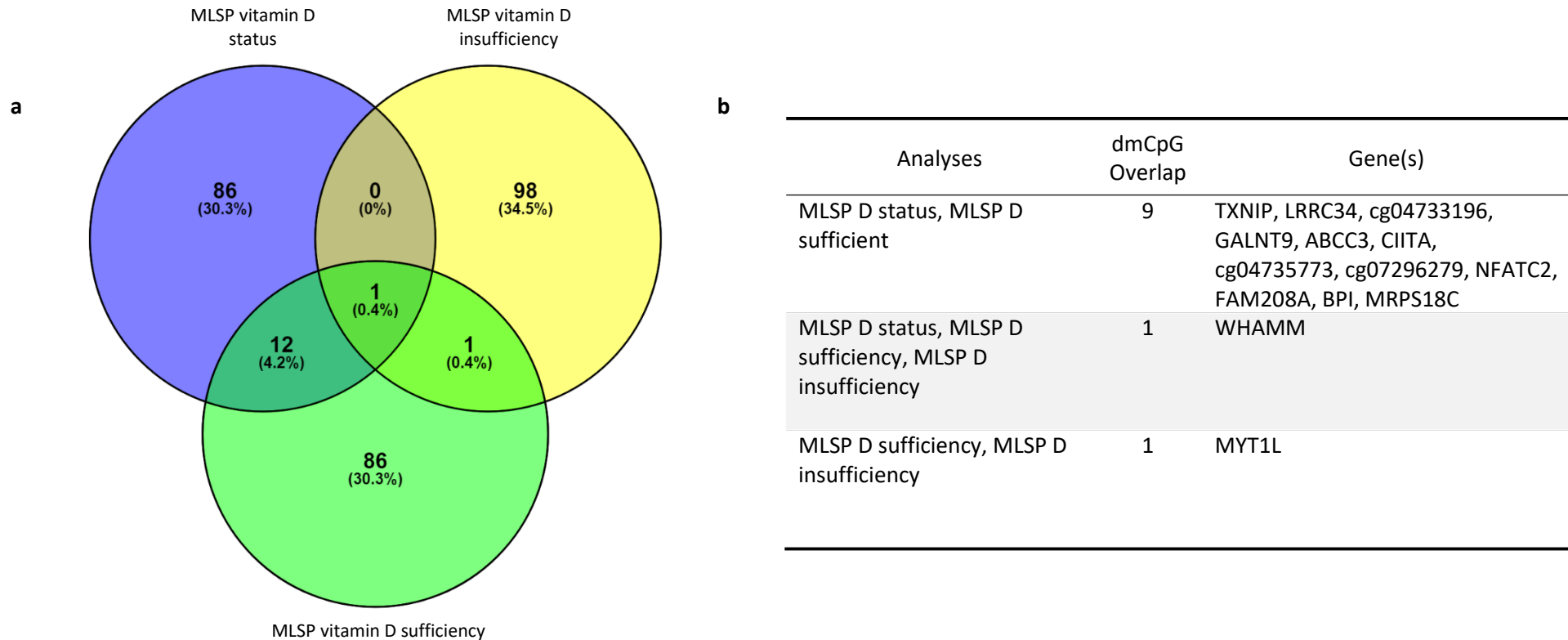


Figure 39. The a) venn diagram overlap between the top 100 dmCpGs MLSP D status, MLSP D sufficient and MLSP D insufficient analyses and the b) associated identity of each overlapping dmCpG
A comparison of the top 100 dmCpGs when ranked by FDR was compared between differential methylation analyses investigating the relationship between umbilical cord blood DNA methylation and measures of maternal late stage pregnancy (34 weeks) vitamin D status, maternal vitamin D status in those sufficient for vitamin D (≥ 50 nmol/L) in late pregnancy and maternal late stage pregnancy vitamin D status in those insufficient for vitamin D (< 50 nmol/L) in late stage pregnancy in the SWS cohort. A a) venn diagram depicts the overlap between dmCpGs taken from each analysis, and b) the overlapping dmCpG probe name, or the gene name if located within a known gene. MLSP D status, maternal late stage pregnancy (34 weeks) vitamin D status; MLSP vitamin D insufficiency, maternal vitamin D status in those sufficient for vitamin D (≥ 50 nmol/L) in late pregnancy; MLSP vitamin D sufficiency, maternal late stage pregnancy vitamin D status in those insufficient for vitamin D (< 50 nmol/L) in late stage pregnancy.

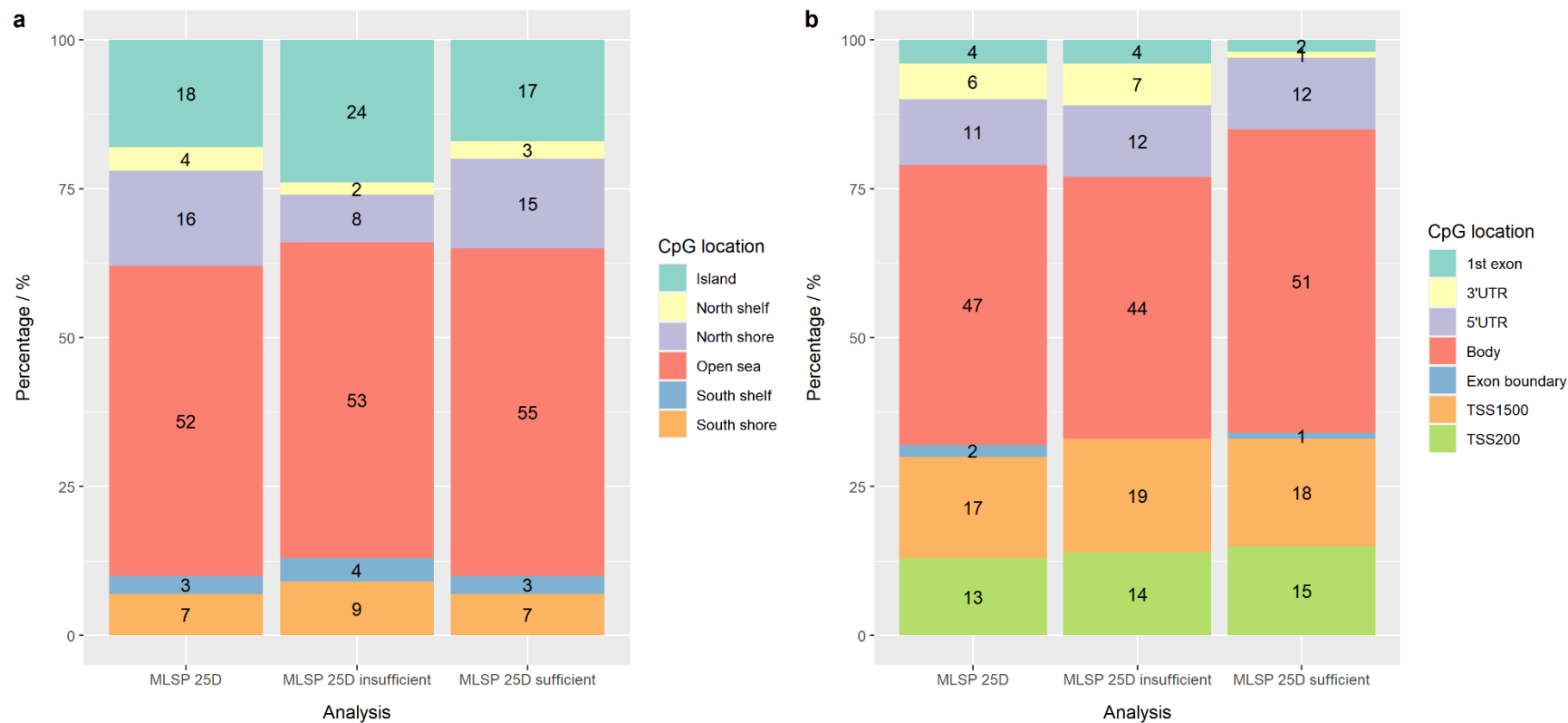


Figure 40. The location of the top 100 dmCpGs associated with MLSP D status, MLSP D insufficiency and MLSP D sufficiency relative to a) CpG islands and b) genomic structure
The top 100 dmCpGs associated with the differential methylation analysis investigating the association between UCB DNA methylation and MLSP D status, MLSP D insufficiency and MLSP D sufficiency in the SWS cohort were shown to be largely in an open sea region and predominantly within the body of a gene. CpG shores, shelves and open sea regions are defined in appendix 9.3.

4.2.5.3 *Transcription factor enrichment analysis*

Transcription factor enrichment analysis of the top 100 dmCpGs associated with MLSP D status, MLSP D insufficiency and MLSP D sufficiency showed enrichment for 4, 1 and 12 transcription factor binding motifs respectively. The three motifs showing the greatest enrichment for the dmCpGs associated with MLSP D status were Ovo Like Transcriptional Repressor 1 (OVOL1) motifs (NES=5.02-4.2) in 17 genes, the T-Box Transcription Factor 18 (TBX18) motif (NES=4.3) in 10 genes and T-Box Transcription Factor 20 (TBX20) motifs (NES=4.29-4.08) in 10 genes. With respect to MLSP D insufficiency, dmCpGs were shown to be enriched for the NK2 Homeobox 5 (NKX2-5) motif (NES = 4.26) in 18 genes. Transcription factor enrichment analysis for MLSP D sufficiency showed enrichment for Zinc Finger And SCAN Domain Containing 23 (ZSCAN23) motifs (NES=6.85-5.78) in 16 genes, Forkhead Box M1 (FOXM1) motifs (NES=4.69-4.25) in 21 genes and the Nuclear Factor I C (NFIC) motif (NES=4.65) in 10 genes (Table 24). Full list of motif references, sequence logo graphs and enriched genes in appendix 9.4.4.

4.2.5.4 *Differentially methylated regions*

DMR analysis of the dmCpGs produced from the differential methylation analysis of MLSP vitamin D status, MLSP vitamin D insufficiency and MLSP vitamin D sufficiency indicated no significant regions of differential methylation.

4.2.5.5 *Pathway / network analysis*

4.2.5.5.1 *Gene-associated dmCpG associated pathways*

Clustering of the top 100 dmCpGs associated with MLSP D status, MLSP D insufficiency and MLSP D sufficiency analyses produced 1, 2 and 0 clusters respectively (Table 25). Post-clustering GO term enrichment analysis showed the dmCpGs associated with MLSP D status were enriched for pathways involved in activity within the nuclear envelope (GO:5635, FDR = $4.54e^{-2}$) and neuron projection (GO:43005, FDR = $4.54e^{-2}$). The dmCpGs associated with MLSP D insufficiency pathway were enriched for ribonucleotide binding (GO:32553, FDR = $4.92e^{-2}$), purine ribonucleotide binding (GO:32555, FDR = $4.92e^{-2}$) and adenosine triphosphate (ATP) binding (GO:5524, FDR = $4.92e^{-2}$) (Figure 41). Full ontology results for each model in appendix 9.4.4.

4.2.5.6 *Histone mark and chromatin state analysis*

Histone mark and chromatin state enrichment analysis indicated a significant under-representation of the top 100 dmCpGs associated with MLSP D sufficiency overlapping the H3K27ac (FDR=0.0004), H3K4me3 (FDR=0.0034), H3K9ac (FDR=0.005) and H3K4me1 (FDR=0.0371) histone marks (Figure 42). The dmCpGs associated with MLSP D status and MLSP D insufficiency indicated no significant enrichment for either histone marks or chromatin state.

4.2.6 Overlap between the MAVIDOS RCT and SWS cohort study analyses

The MAVIDOS RCT demonstrated maternal supplementation with vitamin D was beneficial for a winter birth in relation to offspring postnatal measures of BMC, BMD or bone area. One factor that may have contributed to this finding is a lack of sunlight in the winter months, leading to an increase in maternal vitamin D insufficiency in the placebo group during the late 2nd to 3rd trimesters of foetal development when the mineralisation of the foetal skeleton occurs (690,691). Therefore, an aim of this study was to assess whether associations exist between umbilical cord blood DNA methylation and maternal late-stage pregnancy vitamin D status in the SWS cohort similar to those induced by maternal vitamin D supplementation in the MAVIDOS RCT. An assessment of the top 100 dmCpGs from each analysis found no overlap.

Table 24. The transcription factor binding motifs enriched within the top 100 dmCpGs associated with MLSP D status, MLSP D insufficient and MLSP D sufficient differential methylation analyses in the SWS cohort

Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at birth, four, six and eight years of age found the enrichment of 4, 1 and 12 transcription factor motifs respectively with a normalised enrichment score (NES) >4.

Analysis	Enriched TFs	Rank	TF	Normalised enrichment score	Motifs	Annotation	Multiple motifs enrichment		
							range		Genes enriched
							Maximum NES	Minimum NES	
Maternal late-stage pregnancy vitamin D									
	4	1	OVOL1	NA	2	Mixed	5.02	4.2	17
		2	TBX18	4.3	1	Direct	NA	NA	10
		3	TBX20	NA	2	Direct	4.29	4.08	10
		4	ZNF8	4.01	1	Inferred	NA	NA	11
Maternal late-stage pregnancy vitamin D insufficient (≥25 nmol/l and <50nmol/l)									
	1	1	NKX2-5	4.26	1	Inferred	NA	NA	18
Maternal late-stage pregnancy vitamin D sufficient (≥50nmol/l)									
	12	1	ZSCAN23	NA	2	Direct	6.85	5.78	16
		2	FOXM1	NA	2	Direct	4.69	4.25	21
		3	NFIC	4.65	1	Direct	NA	NA	10
		4	IRF8	NA	2	Direct	4.43	4.29	20
		5	ZNF816	4.27	1	Direct	NA	NA	21
		6	NKX3-2	4.21	1	Direct	NA	NA	13
		7	STAT5A	4.16	1	Direct	NA	NA	11
		8	ZNF85	4.13	1	Direct	NA	NA	10
		9	FOXG1	4.09	1	Direct	NA	NA	10
		10	NFATC1	4.09	1	Direct	NA	NA	16
		11	POU6F2	4.07	1	Direct	NA	NA	12
		12	RUNX1	4.07	1	Direct	NA	NA	9

Table 25. Gene clusters produced during the pathway analysis of the top 100 dmCpGs associated with MLSP D status and MLSP D insufficiency differential methylation analyses in the SWS cohort
 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with MLSP D status, MLSP D insufficiency and MLSP D sufficiency produced 1, 2 and 0 distinct clusters of dmCpGs respectively. The cluster associated with MLSP D status and only one cluster associated with MLSP D insufficiency saw significant enrichment of pathways with the involvement of three or more dmCpGs

Model	Cluster	Genes	Nodes	Edges	Score	Pathways FDR <0.05	Gene count ≥3
MLSP D status	1/1	RANBP2, ZCCHC7, ANXA3, TNKS, TMEM176B, FAM208A, CAPN15, MYLK, ROBO1, NKAIN2, MAPK10, EP400, RPRD1B, NOS1	14	28	3.753	44	2
MLSP D insufficient	1/2	SPAG9, DAZAP2, NFIX, ATP5A1, HNRNPU, UNC5C, TACR1, C8A, DERA, PNRC2, LETM1, NIN, DDAH1, NMU, KIF13A, PIR, ULK2, HOXB1, CDK13, ROS1, GLUL, CBLN2	22	50	4.174	38	12
	2/2	RASA1, DCTN4, TMEM267, L3MBTL1, CUX2, RHOJ, GFPT1, DAPK1, EIF4E3, HAS2, NRG1, UBE2L6, SPECC1, PTK2B, NMUR2	15	34	4.125	0	0

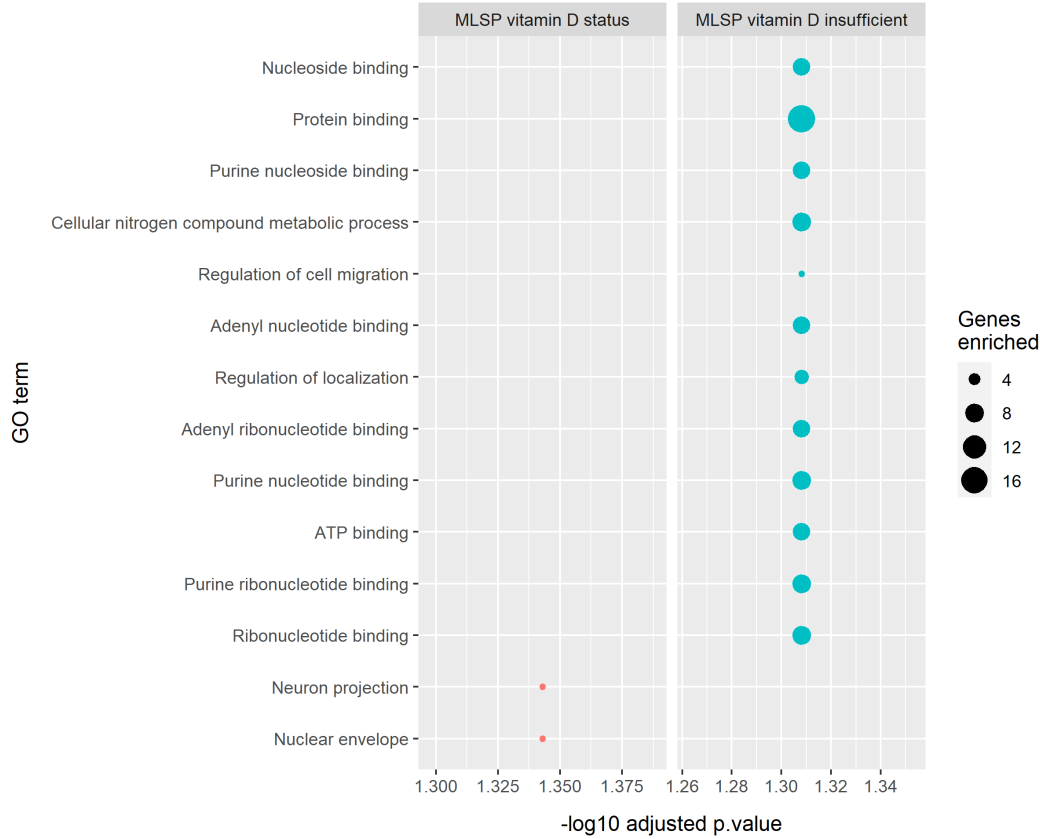


Figure 41. GO terms significantly enriched (adjusted p-value <0.05) for the top 100 dmCpGs associated with MLSP D status and MLSP D insufficiency in the SWS cohort

Gene ontology pathways shown to be significantly enriched in the dmCpG clusters produced from the clustering of the top 100 dmCpGs associated with MLSP D status and MLSP D insufficiency. The dmCpGs associated with MLSP D sufficiency produced no clusters and so no significant gene ontology pathway enrichment was identified. A larger point size and $-\log_{10}$ adjusted p -value indicates a greater significance and number of dmCpGs enriched for the pathway.

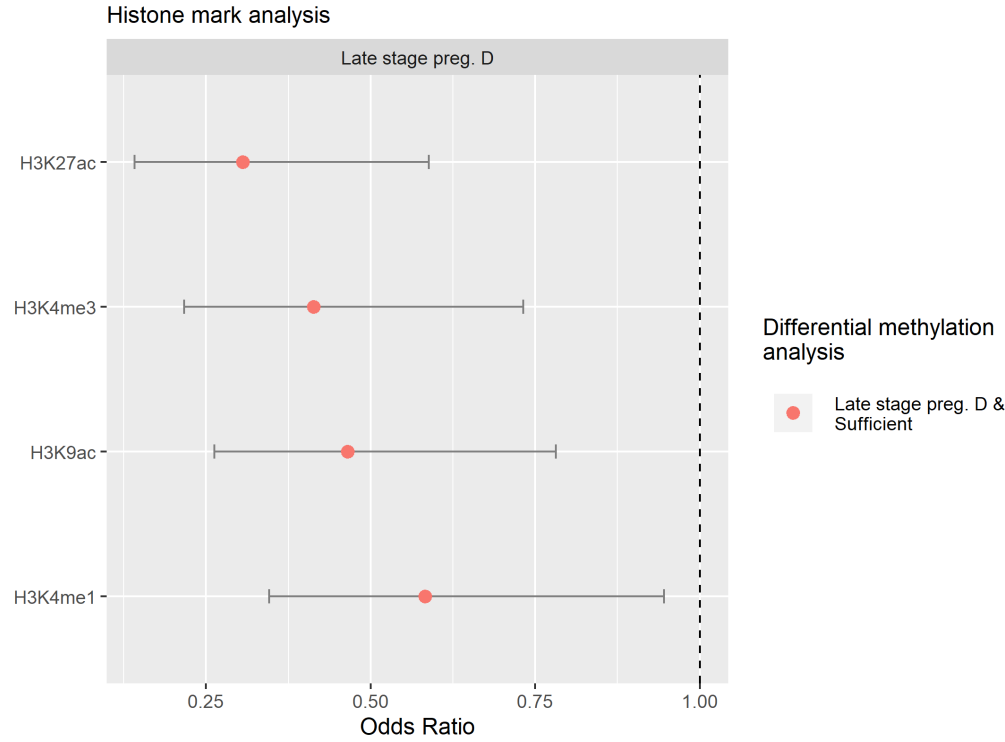


Figure 42. Odds ratio graph produced from the histone mark analysis of the top 100 dmCpGs associated with MLSP D sufficient analysis in the SWS cohort overlapping histone marks in the genome
The histone mark enrichment analysis of the top 100 dmCpGs associated with the differential methylation analyses of MLSP D sufficiency showed significant (FDR <0.05) under-representation (odds ratio <1) of H3K27ac, H3K4me3, H3K9ac and H3K4me1 histone marks associated with MLSP D sufficiency.

4.3 Discussion

4.3.1 Maternal vitamin D supplementation during pregnancy in the MAVIDOS RCT

4.3.1.1 Summary

Work previously conducted in the MAVIDOS RCT demonstrated that maternal vitamin D supplementation during pregnancy influences offspring whole-body BMC, bone area and BMD in a season of birth dependant manner. Furthermore, maternal vitamin D supplementation was shown to lead to perinatal differential methylation changes in the promoter region of *RXRα* gene in umbilical cord tissue DNA, a region in which differential methylation was also found to be predictive of postnatal measures of BMC in the SWS cohort study (3,745). Therefore, we aimed to further expand on this work by interrogating umbilical cord tissue DNA with the EPIC array. Indeed, within the intervention group analysis we found significant maternal vitamin D supplementation-induced differential methylation changes in umbilical cord tissue DNA which may potentially be linked to skeletal development, one of which, *SLC38A10*, overlapped with the analysis assessing intervention group for mothers insufficient for vitamin D in early pregnancy. However, the analysis for intervention group MESP D insufficient mothers also indicated a greater number of significant dmCpGs as a result of vitamin D supplementation and overall, not a large amount of overlap with intervention group analysis was seen. This difference in the strength of the epigenetic signal due to supplementation may be due to the nature of baseline vitamin D concentration being one of the key factors in mediating an individuals response to supplementation (748–752). The hepatic hydroxylation of vitamin D has been suggested to be a process which can become saturated and so limit any potential rise in serum 25(OH)D as a result of supplementation (632). Therefore, if in the vitamin D supplemented group, those in the intervention analysis with a greater serum 25(OH)D concentration in early pregnancy may have seen a i) limited increase in 25(OH)D over the course of pregnancy, or ‘benefit’ of supplementation or ii) activity in vitamin D-related pathways possibly yet to be activated by vitamin D supplementation in those insufficient in early pregnancy. Alternatively, this may be an indication of a lack of power in the study reflected more acutely in the intervention group and a need for a larger study. Nonetheless, these analyses provide us with dmCpGs we can take forward for further analysis to assess the effects of differential methylation on the transcriptional regulation of gene expression. However, due to the heterogenous mix of cell types in the umbilical cord tissue, if we wish to look at biological relevance of these methylation changes in relation to skeletal development, we will need to investigate in either isolated and enriched UC-MSCs or BM-MSCs, the precursor cell to osteoblasts *in vivo*.

Cytoscape pathway enrichment analysis of the top dmCpGs associated with the intervention group analysis found enrichment for a broad range of processes including vitamin binding, muscle system processes and immunity-related pathways. Whereas IPA analysis found enrichment for

pathways integral to skeletal and general development such as Wnt/ β -catenin, Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signalling and lipid metabolism, all of which have the potential be influenced by vitamin D (753–755). In addition, IPA analysis identified vitamin A as a potential upstream regulator of genes seen to have differentially methylated CpGs, however this would take extensive validation to confirm. The enriched pathways associated with intervention group MESP D insufficient were largely cell-cycle related, potentially reflective of the anti-proliferative effects of vitamin D (756). No histone mark enrichment or regions of differential methylation were identified for either analysis but the dmCpGs associated with the intervention MESP D insufficient analysis showed significant transcription factor enrichment.

4.3.1.2 Top hits

Interrogation of the MAVIDOS RCT found significant differential methylation changes in umbilical cord tissue DNA associated with both intervention group and intervention group MESP D insufficient analyses. The most significant dmCpG induced by supplementation within the intervention group analysis, cg21023307 was intergenic and therefore would require further investigation to reveal its potential functional significance. The other significant dmCpGs associated with this analysis were cg12401202, located in the body of Solute Carrier Family 38 Member 10 (*SLC38A10*, FDR=0.02741) gene which showed a decreased methylation in the vitamin D supplemented group and cg13801940, located in the 5'UTR of the Tenascin C (*TNC*, FDR=0.02741) gene which also showed a decrease in methylation in the vitamin D supplemented group.

The solute carrier (SLC) superfamily is the largest group of transporters in the human genome. The SLC superfamily are membrane-bound and typically function as ATP-independent transporters of a wide range of potential substrates. Whilst *SLC38A10* is typically associated with the transport of neurotransmitters (757), as a conditional knockout of *SLC38A10* in mice conducted by the International Knockout Mouse Consortium program found significant metabolic and skeletal abnormalities in a homozygous mouse model. Knockout of *SLC38A10* led to issues such as increased oxygen consumption and energy expenditure, decreased albumin and amylase but also a decreased body weight, BMC and body length with a weak, brittle bone matrix phenotype characteristic of osteogenesis imperfecta (758,759). *Bassett et al.* (2012) proposed the transporter may serve a crucial role in linear bone growth during development by regulating cell volume expansion during hypertrophic differentiation of chondrocytes, citing mutations in similar transporters such as *SLC25D1* and *SLC26A3* which have been seen to cause skeletal and chondrodysplasias respectively (27,759–761). This points to a potential role for *SLC38A10* in the early stages of bone development. However, within the context of this study the methylation of the CpG in the *SLC38A10* gene body decreased in the vitamin D supplemented group. A reduction of methylation within a gene body or intragenic region can be considered as a transcriptionally

repressive (762–766), suggesting vitamin D supplementation may reduce the transcriptional activity of this gene. But in contrast to our understanding of the effect of promoter methylation on gene transcription, the role of intragenic methylation is not yet entirely clear. It has been suggested intragenic methylation may allow for a cell-context specific regulation of alternative promoters in the gene body, prevent spurious intragenic transcription in highly expressed genes and subsequent production of potentially harmful, aberrant proteins, or facilitate alternative splicing. Therefore interpretation of methylation changes in the gene body would have to be interpreted with caution and as with other proposed changes in transcriptional activity, would need to be validated experimentally (767–771).

TNC is an extracellular matrix glycoprotein expressed abundantly during embryogenesis, peaking shortly after birth and decreasing into adulthood. TNC is primarily expressed in tendon tissue but during development has been found to be an early marker of tendon, ligament and bone formation and suggested to have a role in the regulation of osteoblast or osteoclast function (772–774). Transforming growth factor β 1 (TGF β 1) is a critical regulator of bone homeostasis and disruption of TGF β 1 signalling has been shown to have significant consequences on bone mass and quality (775). *In vitro* TGF β 1 has been seen to enhance TNC expression in rat osteoblast cells, whereas *Chen et al.* (2016) showed knockdown of TNC in mice suppresses osteoblast differentiation and promotes an osteoporotic phenotype through inhibition of the Wnt signalling pathway (276,776,777). Within the context of this study a dmCpG site within the 5'UTR of the *TNC* gene also saw an overall lower methylation in the vitamin D supplemented group when compared to placebo. As a part of the potential promoter region, this methylation change would generally correspond with an increase in transcriptional activity. Studies have shown interactions between vitamin D and TNC activity. Both *González-Sancho et al.* (1998) and *Alvarez-Dolado et al.* (1999) have shown supplementation of cells with 1 α ,25-dihydroxyvitamin D3 (1,25(OH) $_2$ D) *in vitro* can have a repressive effect on the transcription of TNC, however these experiments were conducted on unrelated mammary epithelial cells and rat glioma cells respectively so should be treated with caution when comparing to the birth tissues evaluated in this study (778,779).

The most significant dmCpGs associated with those intervention group MESP D insufficient were RNA Polymerase Mitochondrial (*POLRMT*) gene, responsible for mitochondrial gene expression, the T-SNARE Domain Containing 1 (*TSNARE1*) involved vesicle targeting and function and Chromosome 14 open reading frame 102 (*C14orf102*) thought to be potentially involved in the nuclear export and surveillance of mRNA. To date these genes have not been reported to have a link to bone development, however vitamin D has classically been seen to be anti-proliferative, halting the cell cycle at the G0/G1 phase and shown to influence both transcription and translation of mRNA (756). Mitochondrial gene expression is essential to proliferation, and the nuclear export of mRNA necessary for transcription and translation, therefore depending on the influence differential methylation has on the transcriptional activity of these genes this may be an

indication of a vitamin D-induced shift towards greater differentiation and less proliferation within these samples.

4.3.1.3 Pathway enrichment analysis

Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with intervention found significant enrichment for pathways regulating cellular signalling processes, tissue morphogenesis and cellular proliferation. Additionally, we also found enrichment for specific process such as vitamin binding, muscle system processes, muscle contraction and epithelial development (Figure 36). Enrichment of these specific pathways highlights the broad number of extra-skeletal potential physiological roles for vitamin D in the body, where for example vitamin D deficiency has been seen to both impede skeletal muscle development and reduce muscle contraction strength (780). Also, treatment of primary cultures of human bronchial epithelial cells with vitamin D has been seen to alter airway epithelial cell morphology and stimulate epithelial cell proliferation *in vitro* (781,782). Vitamin D interactions are multiple and complex, therefore the strongest effect of supplementation may not always be on the skeleton. In contrast, pathway enrichment analysis of the intervention MESP vitamin D insufficient analysis largely showed enrichment for cell-cycle related processes. This may reflect the potential for vitamin D to halt progression of the cell cycle and potentially enhance differentiation. Additionally, this difference in enriched pathways may re-iterate the potential for supplementation to activate different pathways for those who were initially insufficient, vs. those who were initially potentially sufficient.

In addition to Cytoscape analysis, dmCpGs associated with intervention were assessed for pathway enrichment using IPA which utilises a comprehensive database to indicate instances of gene activity in pathways which have been documented in scientific literature. Interrogation of the IPA database found maternal vitamin D supplementation during pregnancy lead to differential methylation changes in genes enriched for a number of different pathways, potentially highlighting the pleiotropic role of vitamin D as a factor in a range of biological processes. However, these results did highlight predominant themes within the results, including enrichment for a number of signalling pathways including the Wnt/ β -catenin, NF- κ B and phosphatase and tensin homolog (PTEN) pathways, each of which play a fundamental role in bone formation. IPA pathway analysis also identified enrichment for epithelial adherens junction signalling and a number of other immune and cancer related pathways (Figure 37). Whilst on first inspection this range of pathways may seem spurious, the functional range of the pathways enriched in this IPA analysis may be reflective of the broad functional effects of vitamin D in human physiology. Vitamin D has the capacity to interact with or modulate the activity of the all three potentially bone-related pathways (783–785), has a well-established role in immune regulation (786) and cancer (787) and as mentioned previously, can play a role in altering epithelial morphology.

In addition, there is an established relationship between both bone biology and the immune system where factors secreted by bone cells may influence the immune system and vice versa, as seen with the bone remodelling process (section 1.3.2.1.3.4). Here, IPA pathway analysis of the top 100 dmCpGs associated with intervention also showed enrichment for a range of immune-related pathways, including nuclear factor kappa B (NF- κ B) signalling. The NF- κ B family of transcription factors are master regulators of both adaptive and innate immune responses and inflammatory processes in response to both injury and infection (788). In the context of bone, NF- κ B has been seen to have roles in skeletal development, endochondral ossification, osteoblast and osteoclast biology and common bone diseases (789). It has previously been identified that time and stage-specific inhibition of NF- κ B signalling in differentiated osteoblasts can enhance bone matrix formation and bone mineral density in postnatal growth, whereas the inverse effect is seen with stimulation of the pathway. Subsequently, as may be expected Chang *et al.* (2013) found inhibition of NF- κ B signalling significantly enhanced MSC-mediated bone formation, suggested to be due to the prevention of NF- κ B mediated β -catenin ubiquitination and degradation through induction of Smurf1 and Smurf2 (790). Significantly 1,25(OH) $_2$ D has also been identified to suppress NF- κ B activity *in vitro* in several human cell types including macrophages, fibroblasts, keratinocytes, pancreatic islet cells, and adipocytes (791–795). However a recent human vitamin D supplementation RCT by Mousa *et al.* (2017) showed no effect on the expression of NF- κ B in response to 25(OH)D supplementation, although expression was assessed in PBMCs (796). Nonetheless, this suggests the need initially for further investigation into the impact of 1,25(OH) $_2$ D on the expression of NF- κ B in MSCs and the impact that may have on osteogenic differentiation of UC-MSCs.

Conversely however, we did also see the more specific enrichment of the PPAR α /RXR α activation pathway. Whilst peroxisome proliferator-activated receptor gamma (PPAR γ)/RXR α activity will enhance the differentiation of adipocytes often at the expense of osteoblast growth, the PPAR α /RXR α heterodimer is primarily involved in the regulation of lipid metabolism and homeostasis (797–799). There have been suggestions vitamin D insufficiency may be causally associated with adiposity in both adults and children. Previous work in the SWS cohort has also shown an insufficient maternal late-stage pregnancy vitamin D status was associated with a greater postnatal fat mass at four and six years of age and perinatal methylation changes have been seen in the RXR α gene associated with adiposity at nine years of age in a separate cohort (583,800). Supplementation with a vitamin D may therefore potentially enhance lipid metabolism and homeostasis via PPAR α /RXR α activation pathway, however this would need to be further experimentally validated. Furthermore, osteoblasts oxidise fatty acids as an energy source during normal bone acquisition and conditions characterised by lipid dysregulation such as atherosclerosis are often seen with disorders of bone metabolism such as osteoporosis are

frequently seen together (801–804). Therefore if vitamin D supplementation does indeed enhance lipid metabolism, this may indirectly favour bone development.

4.3.1.3.1 *Top upstream regulators*

The top dmCpGs associated with intervention were further analysed using IPA to predict significant upstream regulators (Table 22). This provides the opportunity to identify factors which may potentially act synergistically alongside vitamin D or interact in some manner to influence biological activity. However, as with our most significant dmCpGs, we would need to either validate these findings in enriched UC-MSC cells or ultimately BM-MSCs to make inferences about the possible biological implications of the enrichment of these upstream regulators. IPA was performed on the intervention analysis alone due to licencing restrictions.

The most significant upstream regulator enriched for the dmCpGs associated with intervention was microRNA 149-5p (miR-149-5p) (p value=1.00e⁻⁰⁴). The human miR-149 hairpin gives rise to a “guide strand” miR-149-5p and the sister “passenger” strand miR-149-3p, each with a distinct sequence and differing biological function (805). Whilst miR-149-3p has been implicated in regulating the switch between adipogenic and osteogenic differentiation of BM-MSCs, the role of miR-149-5p is not yet clear (806).

Another significant upstream regulator, Tretinoin, also known as all-*trans*-retinoic acid (807) is the major biologically active metabolite of vitamin A and also a significant upstream regulator (p -value=1.12e⁻⁰⁴). Vitamin A is a critical micronutrient for pregnancy essential for maternal and foetal ocular health, development of the foetal skeleton, maintenance of the foetal immune system and other organs (808–811). Both vitamin D and vitamin A employ the same heterodimerisation partner, RXR, for binding to the response elements of target genes (812,813). This is of significant functional interest as it has been suggested there is an inter-relationship between vitamin A and vitamin D in the regulation bone formation. This was supported by a study by Joo *et al.* (2015) that found in a cross-sectional study of 6481 subjects that among men with a serum 25(OH)D of <50 nmol/L (vitamin D insufficient), both the top and bottom tertiles of vitamin A dietary intake had a lower BMD than the middle group. When >50 nmol/L (vitamin D sufficient) there were no differences in BMD by vitamin A intake (814). This finding has been reproduced in 75,747 postmenopausal women 50–79 y of age participating in the Women’s Health Initiative Observational Study in the United States, where a high vitamin A status and low vitamin D status has been associated with a low BMD and increased risk for fracture (815). It has been hypothesised that each factor may act as a competitive inhibitor to the others activity (816). Within the context of this study all-*trans*-retinoic acid was a significant upstream regulator of eighteen dmCpGs associated with receiving a vitamin D intervention during pregnancy (Table 22). These dmCpGs may therefore provide a potential starting point for investigation for points of interaction between both vitamins A and D, and the potential biological repercussions of any

interaction. Within this study, the dmCpGs regulated by ATRA included nuclear factor of activated T cells 1 (NFATC1), a the master transcription factor of osteoclastogenesis which has been suggested to be antagonised both by retinoic acid and 1,25(OH)₂D (817,818).

In addition, both the SRY-Box Transcription Factor 2 (SOX2) (p -value=3.58e⁻⁰³) and TGFβ1 (p -value=1.44e⁻⁰³) were shown to be significant upstream regulators of the top 100 dmCpGs associated with the intervention analysis. SOX2 is a major factor required to maintain MSCs in a proliferative and pluripotent state (819). There is currently no known association between SOX2 and vitamin D supplementation, although it could be hypothesised vitamin D is most likely to suppress SOX2 activity as a factor which is ostensibly pro-osteogenic in human MSCs (368–370,372,374,820,821). TGFβ1 has been seen previously to interact with vitamin D both synergistically and antagonistically each other in regards to bone growth (822,823). The dmCpGs enriched for these upstream regulators may provide initial points of investigation to explore the potential, synergistic, or antagonistic interaction of the upstream regulator and vitamin D supplementation.

4.3.2 *Maternal late-stage vitamin D status in the SWS cohort*

4.3.2.1 *Summary*

The MAVIDOS RCT previously identified maternal vitamin D supplementation in the winter months was beneficial for postnatal measures of BMC, BMD and bone area in offspring when compared to a placebo. This may potentially be due to the increase in maternal vitamin D insufficiency in the placebo group during the late 2nd to 3rd trimesters of foetal development when the mineralisation of the foetal skeleton occurs (317,318). Therefore here, we aimed to use SWS cohort study methylation array data to assess whether similar differential methylation changes exist between intervention group in the MAVIDOS RCT and MLSP D status in the SWS cohort. Additionally, we aimed to assess whether MLSP D insufficiency or sufficiency would have a differential effect on the CpGs differentially methylated and the pathways enriched in terms of vitamin D-related function. However no overlap was found with the intervention group analyses conducted for the MAVIDOS RCT and MLSP D status analyses conducted using SWS array data. This may be likely due to the differences in tissue types in the analyses as methylation can be largely cell-type specific and it has been reported the methylation profiles of these tissues are largely distinct (458,693).

Furthermore, analysis of the SWS cohort study found weak associations (FDR <0.25) between umbilical cord blood DNA methylation and MLSP D status, significant associations (FDR <0.05) with MLSP D insufficiency, but only weak associations with MLSP D sufficiency. This may be an indication maternal serum vitamin D concentration in late pregnancy may have had a more profound effect with respect to methylation when the mother was insufficient. Pathway enrichment analysis for MLSP D status showed enrichment for neuron projection and nuclear

envelope pathways, whereas the MLSP D insufficient analysis showed enrichment for a number of transcription-related pathways and no enrichment was identified for those MLSP D sufficient. This apparent difference in enrichment for MLSP D status and MLSP D insufficient analyses may be due to the difference in the number of genes used in the pathway enrichment analysis, where 14 genes were used in the MLSP D status analysis and 22 used in the MLSP D insufficient analysis. However, this analysis result also suggests maternal vitamin D insufficiency may indeed lead to the differential methylation of a small functional network of genes with involvement in similar processes such as nucleoside, nucleotide and ribonucleotide binding in umbilical cord blood, whereas this biological change simply does not occur in umbilical cord blood DNA when mothers have a normal range of serum vitamin D in late pregnancy. No differentially methylated regions (DMRs) were identified for any analysis, histone mark enrichment was only indicated for MLSP D status however all three analyses showed significant transcription factor enrichment. Overlap between the analyses was minimal.

4.3.2.2 Top hits

The dmCpG most strongly associated with MLSP D status, MLSP D insufficient and MLSP D sufficient analyses were cg26974062, in the body of the *TXNIP* gene (FDR=0.1821), cg19744478 in the body of the *HK1* gene (FDR= 0.005895) and cg01290714 in the 3'UTR of the Arrestin Domain Containing 2 (*ARRDC2*) gene (FDR=0.1318) respectively.

4.3.2.2.1 *HK1* and *TXNIP*

The dmCpGs most strongly associated with MLSP D status (FDR <0.25) included two dmCpGs located in the body of the *TXNIP* gene, cg26974062 (TXNIP_1, FDR = 0.1821) and cg02988288 (TXNIP_2, FDR = 0.2218). Functionally, TXNIP acts on both a systemic and cellular level to regulate glucose production and metabolism, where TXNIP deficiency is associated with an increased cellular glucose uptake and metabolism (824,825). Subsequently, overexpression of TXNIP in animal models has been seen to induce apoptosis of pancreatic β -cells, reduce insulin sensitivity in certain tissues and decrease energy expenditure (826–829). In contrast, vitamin D has been suggested to improve glucose homeostasis and improve insulin sensitivity (830). Accordingly, vitamin D supplementation has indeed been seen to improve glucose tolerance and insulin sensitivity in the elderly, although the role of vitamin D supplementation in glucose homeostasis is contentious (831–836). However a recent meta-analysis of vitamin D supplementation RCTs in pregnant women showed a significant decrease in insulin resistance (837). Vitamin D deficiency has also been shown to have similar effects to TXNIP overexpression including an impaired pancreatic β -cell function, insulin resistance and a higher risk of diabetes (831,838). The mirrored roles of these factors with respect to glucose homeostasis and insulin sensitivity indicates a potential functional relationship between both TXNIP and vitamin D. Indeed, a study by *Abu el Maaty et al.* (2018) found long term 1,25(OH)₂D treatment induced TXNIP expression in HL-60

cells and increased TXNIP stability in a glucose dependant manner (839). Within our study, the TXNIP_1 dmCpG overlapped both the MLSP D status and MLSP sufficient analyses, in which the dmCpG showed an increase in methylation within the gene body. A methylation increase in the body of a gene has been associated with an increase in expression of the gene. Our observations, if validated, along with those of *Abu el Maaty et al.* (2018) suggests an antagonistic relationship between the factors, where a replete or vitamin D sufficient status may lead an increase in TXNIP expression to maintain glucose homeostasis. The methylation changes associated with TXNIP should be interpreted with caution however, as a weak association with MLSP D status (FDR <0.2) suggests this may not be strong association despite evidence of vitamin D-mediated TXNIP induction in the literature (840,841).

Interestingly, the most significant dmCpG associated with the MLSP D insufficient analysis was cg19744478 in the body of the *HK1* gene. HK1 is a ubiquitous hexokinase localised to the outer membrane of mitochondria which catalyses the first, rate limiting step in glucose metabolism (842). Methylation of the CpG was decreased in the body of the gene, indicating a potential reduction in transcriptional activity. This could indicate a lower vitamin D status is associated with a potential downregulation of glucose metabolism, an observation which follow with suggestions of vitamin D as a factor to improve glucose homeostasis (830) and the potential associations seen with TXNIP in both the MLSP D and MSLP D sufficient analyses. In addition, a recent study by *Seipelt et al.* (2020) showed the offspring of vitamin D deficient mice fed a high fat diet, disrupted glucose homeostasis and adiposity in male offspring (843). However methylation changes in the body of a gene must be treated with caution as mentioned previously.

Whilst the potential influence of vitamin D status on the metabolism of glucose is not necessarily within the scope of this study, the possible role of vitamin D status to maintain an optimum insulin sensitivity may be an interesting avenue of investigation, particularly in relation to bone formation. Diabetes, where systemic glucose metabolism is dysregulated can lead to increased bone frailty an osteoporotic fractures (844). Furthermore, insulin acts as an anabolic agent in bone, regulating both bone formation and resorption. Insulin receptors are expressed on both the osteoblastic and osteoclastic cell surfaces and are required for osteoblast proliferation, survival and differentiation (845,846). Therefore we may be observing methylation changes which are a result of vitamin D activity in one of many extra-skeletal roles to improve glucose homeostasis and maintain an appropriate insulin sensitivity, but indirectly conferring a benefit for bone maintenance and formation.

4.3.2.3 Transcription factor enrichment

Transcription factor enrichment of the top 100 dmCpGs associated with MLSP D status, MLSP D insufficient and MLSP D sufficient mothers found enrichment for 4, 1 and 12 transcription factor motifs respectively (Table 24).

The transcription factor motif most significantly enriched for the dmCpGs associated with MLSP D status was OVOL1 (NES=5.02-4.2). OVOL1 has been identified by a number of studies as integral to the development and differentiation of epithelial tissue including in hair follicles, kidney and germline epithelium. Although in addition, a study by *Min et al.* (2018) found not only that OVOL1 is expressed during osteoblastic differentiation but knockdown attenuated osteoblastic differentiation, overexpression enhanced the expression of osteogenic genes such as runt-related transcription factor 2 (*RUNX2*) and alkaline phosphatase (*ALP*) and enhanced matrix mineralisation (847). There is no currently directly understood interaction between vitamin D and OVOL1, but the targets enriched with the OVOL1 motif could be a starting point by which to elucidate whether a connection exists between the factors.

The transcription factor binding motif most significantly enriched for the dmCpGs associated with MLSP D insufficient mothers was NK2 homeobox 5 (*NKX2-5*) (NES=4.26). *NKX2-5* is required for development of both the heart and spleen (848). Vitamin D also has role in maintaining cardiac health and potential roles in development so there may be a potential relationship between the two factors, but this would be outside the remit of this study (849).

The dmCpGs associated with MLSP D sufficient mothers showed significant enrichment for the Zinc Finger and SCAN Domain Containing 23 (*ZSCAN23*) transcription factor motif (NES=6.85-5.78). *ZSCAN23* does not currently have any understood function but also enriched was the NFATC1 transcription factor (NES=4.09), the master regulator of osteoclast differentiation. Expression of NFATC1 can be at least in part regulated through cell to cell interaction, an example of which is presented during the bone remodelling process. During the bone remodelling process cell to cell interaction leads to bidirectional signalling between the ephB4 receptor, expressed on osteoblasts and the ephrin-B2 ligand, expressed on osteoclast precursors to allow for regulation of bone resorption and formation activities. Whilst forward signalling through ephB4 receptor allows for the stimulation of osteoblastic differentiation, reverse signalling through the ephrin-B2 ligand stimulates the expression of both NFATC1 and c-Fos, leading the inhibition of osteoclastic differentiation and so reducing bone-resorptive activity. Where vitamin D has been seen to promote the differentiation of bone-forming osteoblast progenitor cells, it may also be expected to suppress the differentiation of bone-resorptive osteoclast progenitor cells. However, a study by *Kogawa et al.* (2010) found that in the presence of physiological levels of 25(OH)D, the metabolism of 25(OH)D into 1,25(OH)D₃ by human osteoclast precursors resulted in the increased expression of a number of important osteoclast-associated genes, including NFATC1. In addition, 25(OH)D dose-dependently reduced the resorptive capacity peripheral blood mononuclear cell-derived osteoclasts without compromising cell viability, suggesting vitamin D may increase the differentiation capacity osteoclasts, but reduce their resorptive capacity. This was reinforced by the finding that osteoclasts formed from splenocytes of vitamin D receptor-null mice had a greater resorptive capacity when compared to wild-type cells (204).

The RUNX1 TF binding site was also a factor identified as significantly enriched. RUNX1, with the coordinated activity of RUNX2 or RUNX3 have also been identified, depending on the skeletal element as potentially crucial for earlier stages of chondrogenesis in endochondral ossification (69,70), but also in lineage specification. RUNX1 conditional knockout mice generated by Tang *et al.* (2021) exhibited decreased osteogenesis and increased adipogenesis and through further work determined RUNX1 may act to promote osteogenic commitment and inhibit adipogenesis through modulation of the bone morphogenic protein (BMP) and Wnt/ β -catenin signalling pathways (850). However, an association between vitamin D and RUNX1 activity has not yet been established and so would require further work to establish any relationship with vitamin D status.

4.3.3 Further work

4.3.3.1 MAVIDOS RCT

Within this study we have found vitamin D-induced differential methylation changes in MAVIDOS RCT umbilical cord tissue DNA. The next steps to further this work would be to ascertain the potential biological relevance of the most significant differential methylation changes in relation to skeletal development. These assays were conducted on umbilical cord tissue samples which contains a heterogenous mix of DNA from a number of cell-types, therefore we would aim to replicate this study, or further probe the individual differential methylation changes in isolated and enriched UC-MSCs as a surrogate of BM-MSCs, or ideally in primary BM-MSC samples. One of the primary concerns is to establish how these differential methylation changes affect the transcriptional activity of the genes with which they are associated. Within this study we have shown significant differential methylation of CpG sites in the body of the *SLC38A10* gene and the 5'UTR of the *TNC* gene in umbilical cord tissue to be linked to receiving a vitamin D supplement intervention during pregnancy. Whilst the effect of 5'UTR methylation in the *TNC* gene would be most commonly associated with an increase in transcriptional activity, the potential effect of differential methylation within the body of the *SLC38A10* gene is not clear. Techniques such as ribonucleic acid sequencing (RNA-seq) alongside a further EPIC array on the same, vitamin D-treated UC-MSCs or BM-MSCs would provide data for both the methylation state and transcriptional activity of these genes. Furthermore, techniques such as single-cell nucleosome, methylation and transcription sequencing (scNMT-seq) allows for chromatin accessibility, DNA methylation and transcriptome profiling to be evaluated on a single cell level in unison. This would not only provide a comprehensive analysis of transcriptional activity of these cells, but allow for discrimination between any underlying differences in cell-type (851).

The differential methylation analyses interrogating umbilical cord tissue DNA for potential associations with either intervention group or intervention group for those MESP D insufficient returned the same intergenic CpG as the most significantly differentially methylated in both analyses (Table 19). Whilst the EPIC array covers large number of CpGs, a number of these sites

are within intergenic enhancer regions (677). Enhancer regions by their nature are typically *cis* acting but have been seen to be located up to 1 Mb proximal or distal to the promoter of the regulated gene. This means CpGs within these regions are unlikely to be annotated with any associated gene within the array and so the effect of their differential methylation is difficult to determine. However, enhancers function by creating chromatin loops that bring the enhancer region and the promoter of the target gene into close proximity. This looping, or chromatin conformation can be studied by chromatin capture methods, which may therefore provide an avenue for further investigation for this CpG significant in both analyses in the future (736–738).

Cytoscape and IPA pathway enrichment analysis of differentially methylated CpGs produced from both the intervention and intervention MESP D analyses indicated the significant enrichment of a large number of pathways. These enriched pathways could provide for further avenues of investigation into the role of vitamin D supplementation during pregnancy, however before doing so it would be prudent to validate these results. To ensure the bioinformatic tools used to conduct pathway enrichment analyses were robust, validation of pathway analysis results could be achieved by assessing the same set of genes associated with differentially methylated CpGs produced from each differential methylation analysis with a further pathway enrichment tools such as g:Profiler or TopGO (852,853). Results from these tools could then be cross-referenced with the original results to ensure they are in concordance with one another.

4.3.3.2 SWS

Whilst the significant differential methylation changes in umbilical cord blood DNA were seen to be associated with MLSP D insufficient mothers, both the MLSP D status and MLSP D sufficient analyses showed only weak associations, indicating a potential lack of power in the dataset and a potential need for assessment in a larger cohort. Although similar to the MAVIDOS RCT, the EPIC array analysis of SWS samples was conducted on umbilical cord blood DNA which contained a heterogeneous mix of cell types. Therefore, to address the aims of this study and understand biological implications of these methylation changes we would need establish *in vitro* an isolated and enriched UC-MSC or BM-MSC model. This model could also be utilised to assess the potential metabolic effects of vitamin D supplementation. The dmCpG most strongly associated with MLSP D status and the most significant dmCpG associated with MLSP D insufficiency were genes associated with glucose homeostasis. To ascertain whether vitamin D potentially affects metabolic characteristics such as mitochondrial respiration, glycolysis and ATP production in osteoblastic precursor cells, we could stimulate either isolated enriched UC-MSCs or BM-MSCs with vitamin D and assess their metabolic activity with an Agilent Seahorse analyser. These cells could then be further differentiation into along an osteogenic differentiation pathway to assess the differentiation potential and phenotypic characteristic of these cells.

4.3.4 Limitations

4.3.4.1 MAVIDOS RCT EPIC array

The MAVIDOS RCT demonstrated the beneficial effects of vitamin D supplementation on postnatal BMC and BMD when births occur in the winter and spring months of the year. *Cooper et al.* (2016)

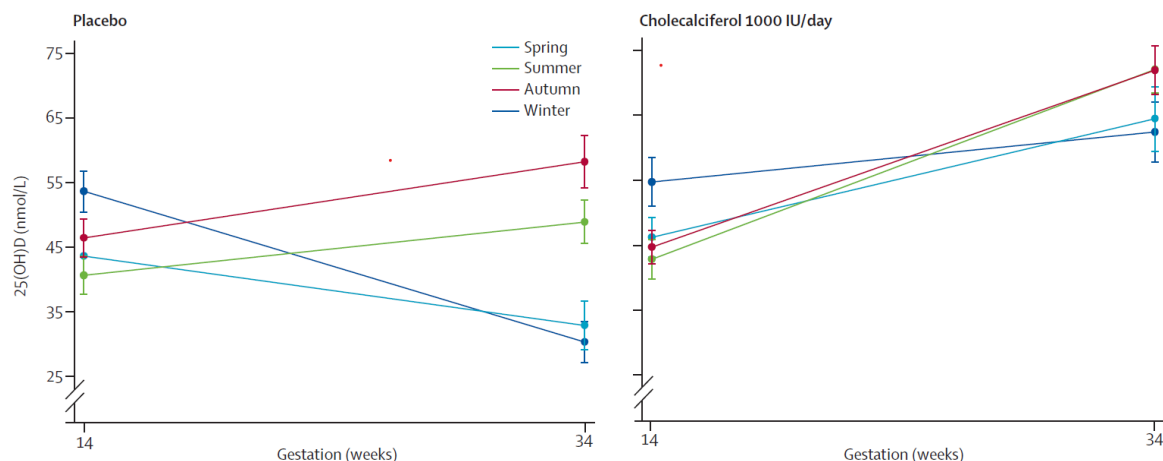


Figure 43. Maternal 25(OH)D status at baseline (14 weeks' gestation) and 34 weeks' gestation by intervention group and season of birth within the MAVIDOS RCT

These graphs taken from *Cooper et al.* (2016) showed that despite a lack of vitamin D supplementation, mothers within the placebo group with pregnancies ending in the Summer and Autumn months indicated a similar rise in serum 25(OH)D concentration when compared to those with births in the Winter and Spring months (625).

showed a demonstrable increase in maternal blood serum 25(OH)D concentration associated with vitamin D supplementation, however within the placebo group summer and autumn births also demonstrated an increase in maternal serum 25(OH)D concentration without the aid of a 1000 IU/day vitamin D supplement (625) (Figure 43), most likely as a result of increased sun exposure. This may have potentially diluted the strength of the epigenetic signal produced from the differential methylation analysis interrogating umbilical cord tissue DNA methylation with respect to intervention group. We cannot adjust the model for season as an interaction between supplement and season was seen to be what lead to greater measures of BMC, BMD and bone area postnatally. We could perform a differential methylation analysis with respect to intervention group in the winter months alone where the benefit to supplementation was seen, however this study was not sufficiently powered to do so, therefore ultimately, we need to repeat this analysis with greater number in the study. Umbilical cord tissue methylation has also been shown to exhibit a high inter-individual variability (458,649) which may dilute the signal further, reinforcing the need for greater numbers. A number of other factors can affect individual response to vitamin D supplementation, however again this study is not powered to account for all these variables.

4.3.4.2 SWS EPIC array

The potential comparison to be made between SWS umbilical cord blood DNA methylation and MAVIDOS RCT umbilical cord tissue DNA methylation is limited by tissue type. Methylation is often, but not always cell and tissue-type specific. In addition, both umbilical cord tissue and umbilical cord blood have been seen to have largely distinct methylomes which may be reflective of their respective mesenchymal and haematopoietic origins. This may therefore have limited the potential for the identification of common methylation changes (458,649).

4.3.5 Conclusions

Previous work within the MAVIDOS RCT has shown the maternal vitamin D supplementation-induced perinatal methylation changes within potentially bone-related biomarkers such as RXRa (745). Whilst RXRa is not present on the EPIC array, we have been able to identify further significant perinatal differential methylation changes within potentially pro-osteogenic genes in umbilical cord tissue DNA associated with receiving a 1000 IU/day vitamin D supplement intervention during pregnancy in the MAVIDOS RCT. Whilst these dmCpGs wouldn't be useful biomarkers, we can move forward to investigate the potential biological implications of these differential methylation changes with respect to change in transcriptional activity of the genes in isolated, enriched UC-MSCs or a BM-MSC *in vitro* model.

In addition, we've seen significant differential methylation changes in umbilical cord blood DNA associated with maternal vitamin D insufficiency in late-stage pregnancy within the SWS cohort. These analyses reflected the potential role of vitamin D in bolstering metabolic activities such as glucose homeostasis and should certainly be followed up to assess the broader effect this may have on bone development.

5 Chapter 5 – DNA methylation at birth is associated with BMC and BMD in infancy and childhood in the MAVIDOS RCT

5.1 Introduction

Whilst we have previously identified associations between umbilical cord blood DNA methylation and bone measures in the Southampton Women's Survey (SWS) cohort (section 3.2), the Maternal Vitamin D Osteoporosis Study (MAVIDOS) randomised controlled trial (RCT) provides another valuable dataset from which to further expand on this work and explore the associations between the methylation of birth tissues and postnatal measures of bone mineral content (BMC) or bone mineral density (BMD). Furthermore, it allows us to, within the same cohort, ascertain whether any commonality is seen between differential methylation changes induced by vitamin D supplementation and differential methylation changes associated with postnatal measures of BMC or BMD.

5.1.1 Aims

This chapter aims to further expand on the work within the SWS cohort (section 3.2) to identify DNA methylation signatures produced from the Illumina Infinium MethylationEPIC BeadChip (EPIC) array interrogation of MAVIDOS RCT umbilical cord tissue DNA associated with postnatal bone outcomes at the ages of birth and four years of age.

5.2 Results

To further investigate the relationship between birth tissue DNA methylation state and postnatal measures of BMC and BMD, methylation data acquired from the EPIC array interrogation of MAVIDOS RCT cord tissue was used to investigate associations between umbilical cord tissue DNA methylation and measures of BMC and BMD at birth and four years of age in the MAVIDOS RCT. Differential methylation analyses and comparisons made between analyses are detailed in Figure 44.

5.2.1 Cohort characteristics

DNA was extracted from cord tissue (n=386) and the methylation state of the DNA interrogated with the EPIC methylation array as described in section 2.3.2. Array data was available for 383 samples post-quality control (QC) and 371 samples post removal of premature infants (<37 weeks).

Mothers in the cohort were predominantly white (98.3%), 92.1% did not smoke during pregnancy and had a median age of 31.23 years at the birth of their offspring. Of the samples remaining for analysis post-QC 50.7% were from mother-child pairs previously randomised to the placebo intervention group. The median maternal serum 25-hydroxyvitamin D3 (25(OH)D) concentration

increased from 44.45 nmol/l in early pregnancy (14 weeks) to 55.85 nmol/l in late pregnancy (34 weeks). When stratified into sufficient, insufficient and deficient vitamin D status groups, the number of those sufficient increased from 35.6% in early pregnancy to 58.3% in late pregnancy, 53.7% were insufficient in early pregnancy and 27.5% insufficient in late pregnancy and the number of those deficient increased from 10.7% in early pregnancy to 14.2% in late pregnancy (Table 26).

The offspring had a median gestational age of 40.43 weeks and were 55% male. Within the cohort 28.7% of the offspring were born in the summer, 28.7% in the autumn, 23.6% in the spring and 19% in the winter. Table 27 provides further details of the median offspring age at dual energy X-ray absorptiometry (DXA) and accompanying median BMC and BMD measures at the ages of birth and four years of age.

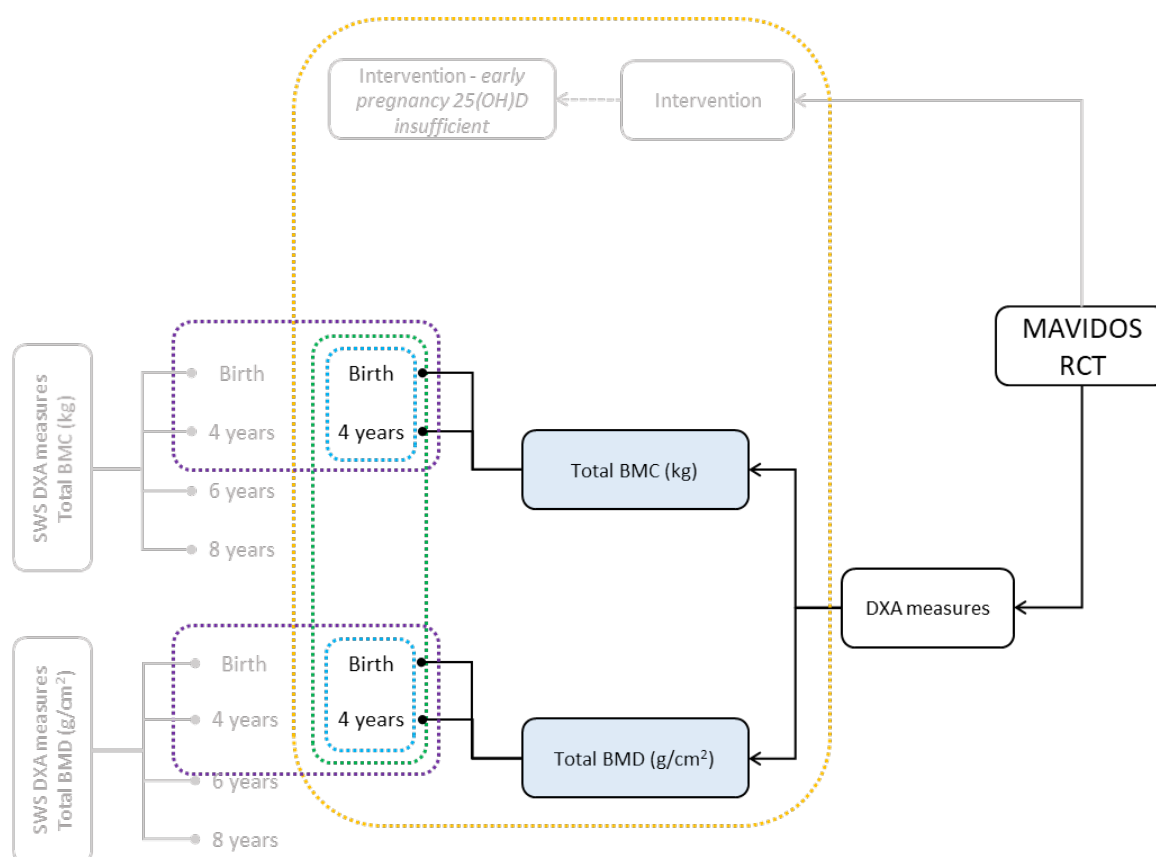


Figure 44. Differential methylation analyses and comparisons made between analyses with respect to DXA bone measures of both total body BMC and BMD at birth and four years of age in the MAVIDOS RCT

Within the MAVIDOS RCT, differential methylation analyses were conducted with respect to umbilical cord tissue DNA methylation and DXA measures of total BMC (kg), total BMD (g/cm²) at birth and four years of age. A comparison of the top 100 dmCpGs when ranked by false discovery rate (FDR) was made within BMC or BMD related analysis groups between birth and four years of age, indicated by the light blue boxes. Comparisons between BMC and BMD related analyses were made between measures taken at the same age, where dmCpGs were produced in both analyses with an FDR<0.25 (green box). Comparisons were also made between BMC and BMD analyses and maternal cholecalciferol supplementation during pregnancy related analyses (yellow box). Finally, in this chapter, comparisons were made between measures of BMC and BMD in both the MAVIDOS RCT and SWS cohort, where measures were taken at the same age and differentially methylated CpGs (dmCpGs) were produced in both analyses with an FDR<0.25 (purple boxes).

Table 26. Maternal cohort characteristics for the MAVIDOS RCT

Values are n (%), or median (5th, 95th percentile). A sufficient early or late pregnancy serum 25(OH)D equates to a blood serum 25(OH)D concentration of ≥ 50 nmol/l, insufficient ≥ 25 nmol/l and < 50 nmol/l and deficient < 25 nmol/l.

Phenotypic characteristic	Group/number	N (%) or median (5th, 95th percentile)
<i>Ethnicity</i>	White	344 (98.3)
	Other	6 (1.7)
<i>Maternal age at birth of offspring</i>	363	31.23 (21.8, 39.51)
<i>Smoking during pregnancy</i>	No	339 (92.1)
	Yes	29 (7.9)
<i>Maternal BMI (kg/m²)</i>	349	25.06 (20.06, 36.12)
<i>Intervention group</i>	Placebo	188 (50.7)
	1000 IU/day 25(OH)D	183 (49.3)
<i>Early pregnancy: Serum 25(OH)D at 14 weeks (nmol/l)</i>	365	44.45 (19.63, 71.2)
<i>Early pregnancy: Serum 25(OH)D at 14 weeks categories (nmol/l)</i>	Sufficient	130 (35.6)
	Insufficient	196 (53.7)
	Deficient	39 (10.7)
<i>Late pregnancy: Serum 25(OH)D at 34 weeks (nmol/l)</i>	360	55.85 (16.95, 92)
<i>Late pregnancy: Serum 25(OH)D at 34 weeks categories (nmol/l)</i>	Sufficient	210 (58.3)
	Insufficient	99 (27.5)
	Deficient	51 (14.2)

Table 27. Offspring cohort characteristics for the MAVIDOS RCT

Phenotypic characteristic	Group/number	N (%) or median (5th, 95th percentile)
<i>Gestational age (weeks)</i>	368	40.43 (38.57, 41.86)
<i>Sex</i>	Male	203 (55)
	Female	166 (45)
<i>Season of birth</i>	Spring	87 (23.6)
	Summer	106 (28.7)
	Autumn	106 (28.7)
	Winter	70 (19)
<i>Baby DXA: Age (days)</i>	341	5 (1, 14)
<i>Baby DXA: Total BMC (g)</i>	326	61.61 (45.5, 79.82)
<i>Baby DXA: Total BMD (g/cm²)</i>	326	0.2 (0.18, 0.23)
<i>4-year DXA: Age (years)</i>	271	4.07 (3.99, 4.36)
<i>4-year DXA: Total BMC (g)</i>	245	352.99 (291.56, 429.02)
<i>4-year DXA: Total BMD (g/cm²)</i>	245	0.47 (0.42, 0.53)

5.2.2 Differential methylation analysis in relation to BMC

5.2.2.1 Differentially methylated cytosine-phosphate-guanine dinucleotides (CpGs)

Analysis of the relationship between methylation in umbilical cord tissue DNA and BMC at birth found no differentially methylated cytosine-phosphate-guanine dinucleotides (dmCpGs) with a false discovery rate (FDR)<0.25. In contrast, six dmCpGs were found to be significantly associated with BMC at four years of age with an FDR<0.05 and a further 167 dmCpGs found to be more weakly associated with BMC at four years of age with an FDR<0.25. The top ten dmCpGs (FDR<0.25) associated with BMC at four years of age are listed in Table 28 and the top 100 dmCpGs in appendix 9.4.5.1.1.

The most significant dmCpG associated with BMC at four years of age was cg21542248, its methylation was positively associated with BMC and located in the body of the chromosome 14 open reading frame 23 (*C14orf23*) gene (FDR = 0.00136). *C14orf23* produces a long intergenic non-protein coding RNA, the function of which has not yet been identified. Of the remaining dmCpGs significantly associated with BMC at four years of age cg02405318, associated with Transmembrane and Coiled-Coil Domain Family 3 (*TMCC3*) gene and cg20098255, associated with POU Class 6 Homeobox 1 (*POU6F1*) showed a positive relationship between their methylation state and BMC at 4 years of age. However, the dmCpGs cg02973930 an intergenic CpG, cg24384472, associated with CUB and Sushi Multiple Domains 3 (*CSMD3*) gene and cg25723331, associated with the cluster of differentiation 82 (*CD82*) gene showed a negative relationship between methylation and BMC at four years of age (Figure 45).

Table 28. The results of differential methylation analysis interrogating the association between umbilical cord tissue (UCT) DNA methylation and BMC at four years of age in the MAVIDOS RCT

The differential methylation analysis investigating the association between UCT DNA methylation and BMC at four years of age found six significant dmCpGs (FDR<0.05) (n=147). No notable inflation was observed $\lambda=1.03$. DmCpGs associated with a known gene are represented by their respective associated gene symbol.

Model	Sample number	Lambda	FDR					Top 10 dmCpGs (FDR <0.25)
			<0.25	<0.20	<0.15	<0.10	<0.05	
4 years	147	1.03	173	76	68	11	6	C14orf23, cg02973930, CSMD3, TMCC3, POU6F1, CD82, FER1L5, DAB2IP, cg16702610, cg25106913

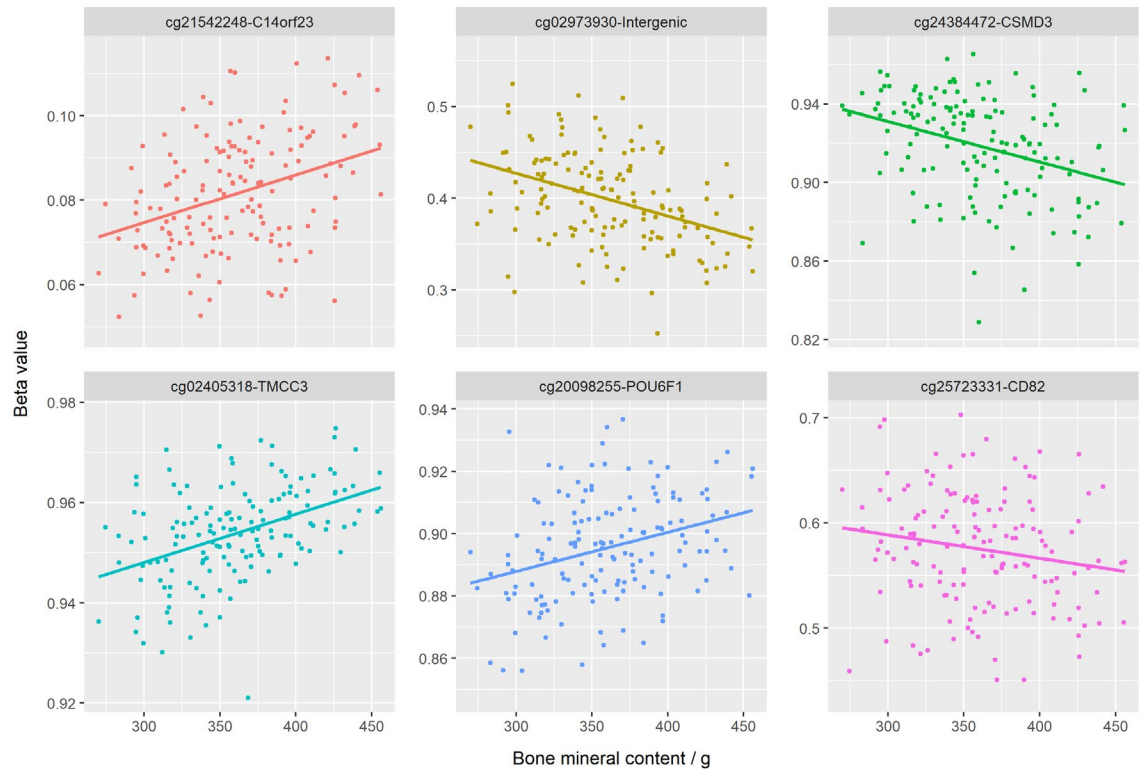


Figure 45. Scatter plots indicating the association between umbilical cord tissue DNA methylation and total body BMC at four years of age for the six dmCpGs found to be significantly ($FDR < 0.05$) associated with BMC at four years of age in the MAVIDOS RCT

Methylation data produced from the EPIC array interrogation of MAVIDOS RCT umbilical cord tissue (UCT) DNA was used to conduct a differential methylation analysis investigating the association between UCT DNA methylation and DXA measurements of total body BMC at four years of age for offspring within the MAVIDOS RCT. The differential methylation analysis assessing the association between UCT DNA ($n=147$) methylation and BMC at four years of age in the MAVIDOS RCT found significant ($FDR < 0.05$) differential methylation of six CpGs cg21542248, cg0297390, cg24384472, cg02405318, cg20098255 and cg25723331 which were mapped to the C14orf23 gene, an intergenic region and the CSMD3, TMCC3, POU6F1 and CD82 genes respectively. Methylation of the three CpG sites cg21542248, cg02405318 and cg20098255 were shown to increase with an increasing offspring BMC at four years of age, whereas the methylation of the CpG sites cg02973930, cg24384472 and cg25723331 were shown to decrease with a decreasing offspring BMC at four years of age.

5.2.2.2 *Genomic locations of dmCpGs associated with BMC at four years of age*

The locations of the top 100 dmCpGs associated with BMC at four years of age were determined relative to both CpG islands and genomic structure. The dmCpGs associated with BMC at four years of age were most commonly located within open sea regions (72% of associated dmCpGs), 12% were located within CpG islands and 16% in CpG shores or shelves. Relative to genomic structure 52% of the dmCpGs were located in the gene body, 27% in the five prime untranslated region (5'UTR) and the first exon or exon boundary, 19% were located up to 1500 bases upstream of the transcriptional start site (TSS) and 2% were located in a three prime untranslated region (3'UTR). The distribution of the top 100 dmCpGs in the genome relative to both CpG islands and genomic structure for the analysis of BMC at four years of age is shown in Figure 46.

5.2.2.3 *Transcription factor enrichment analysis of dmCpGs associated with BMC at four years of age*

Transcription factor enrichment analysis demonstrated the enrichment of 4 transcription factor motifs for the top 100 dmCpGs associated with BMC at four years of age. The dmCpGs associated with BMC at four years of age demonstrated enrichment for the FOS Like 1, activator protein-1 (AP-1) Transcription Factor Subunit (FOSL1) motif (normalised enrichment score (NES)=5.12) in 13 genes, TATA-Box Binding Protein (TBP) motifs (NES=4.38-4.16) in 16 genes, the Zinc Finger and BTB Domain Containing 20 (ZBTB20) motif (NES=4.19) in 11 genes and finally the POU Class 3 Homeobox 2 (POU3F2) motif (NES=4.03) in 13 genes (Table 29). Full list of motif references, sequence logo graphs and enriched genes in appendix 9.4.5.1.2.

5.2.2.4 *Differentially methylated regions associated with BMC at four years of age*

A regional analysis of the dataset showed no differentially methylated regions (DMRs) with significant differential methylation associated with BMC at four years of age with a Stouffer's value (SV)<0.05.

5.2.2.5 *Pathway enrichment analysis of dmCpGs associated with BMC at four years of age*

5.2.2.5.1 *Cytoscape pathway enrichment analysis*

Clustering analysis of the top 100 dmCpGs associated with BMC at four years of age produced four clusters (Table 30). Gene ontology (GO) term enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age showed enrichment for pathways involved in the transmission of nerve impulses (GO:19226, FDR = $2.17e^{-2}$), signal transduction (GO:7165, FDR = $2.17e^{-2}$) and signalling process (GO:23046, FDR = $2.20e^{-2}$) (Figure 47). Full ontology results for BMC at four years of age in appendix 9.4.5.1.3.

5.2.2.6 Enriched histone mark and chromatin states associated with BMC at four years of age

Histone mark and chromatin state analysis showed a significant under-representation of the top 100 dmCpGs associated with BMC at four years of age overlapping both the H3K4me3 (FDR=0.0008) and H3K9ac (FDR=0.0075) histone modifications (Figure 48).

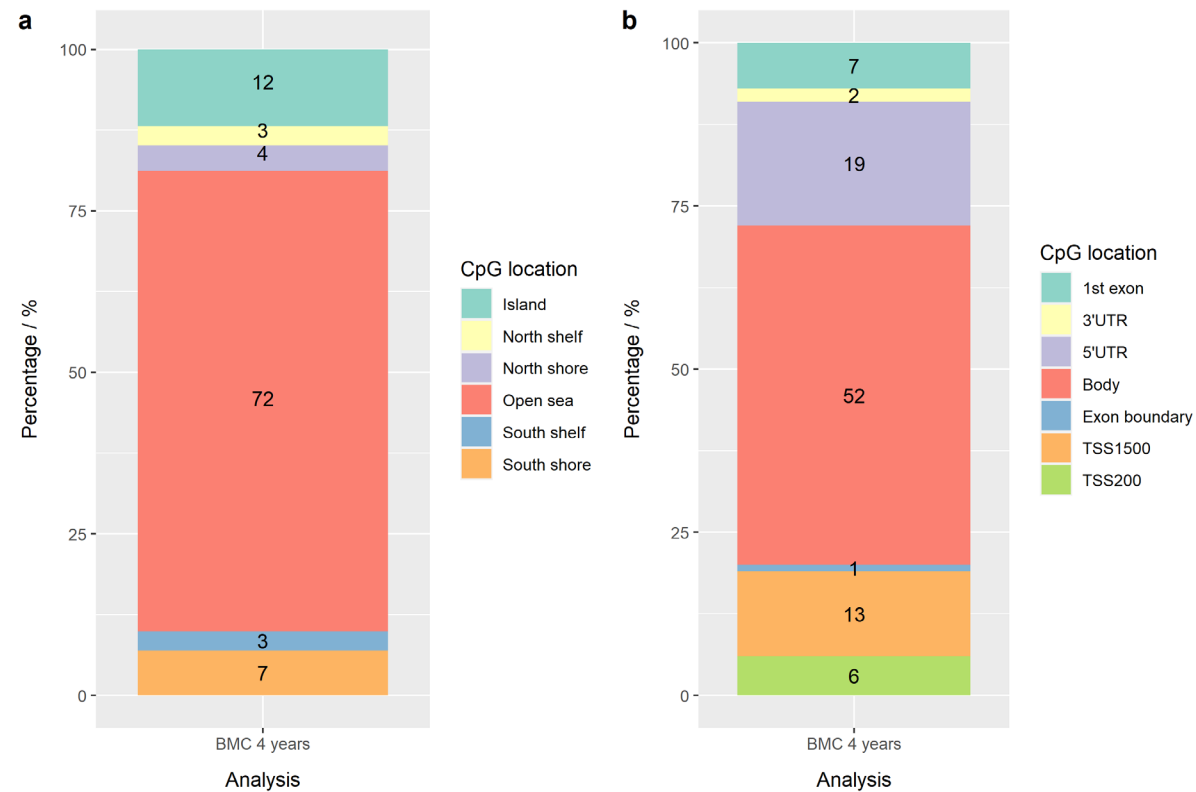


Figure 46. The location of the top 100 dmCpGs associated with BMC at four years of age relative to a) CpG islands and b) genomic structure

The top 100 dmCpGs associated with the differential methylation analysis investigating the association between UCT DNA methylation and BMC at four years of age in the MAVIDOS RCT were shown to be largely in an open sea region and predominantly within the body of a gene. CpG shores, shelves and open sea regions are defined in appendix 9.3.

Table 29. The transcription factor binding motifs enriched within the top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT
Transcription factor enrichment analysis of the top 100 dmCpG associated with BMC at four years of age in the MAVIDOS RCT found the enrichment of four transcription factor motifs with a normalised enrichment score (NES) >4.

Analysis	Enriched TFs	Rank	TF	Normalised enrichment score	Motifs	Annotation	Multiple motifs enrichment range		
							Maximum NES	Minimum NES	Genes enriched
<i>BMC at 4 years</i>									
	4	1	FOSL1	5.12	1	Direct	NA	NA	13
		2	TBP	NA	4	Inferred	4.38	4.16	16
		3	ZBTB20	4.19	1	Direct	NA	NA	11
		4	POU3F2	4.03	1	Direct	NA	NA	13

Table 30. Gene clusters produced during the pathway analysis of the top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT

Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT produced four distinct clusters of dmCpGs. However only clusters 1 and 2 saw significant enrichment of pathways with the involvement of three or more dmCpGs.

Model	Cluster	Genes	Nodes	Edges	Score	Pathways FDR <0.05	Gene count ≥3
BMC at 4 years	1/4	ATP8A2, DYSF, DAB2IP, GMDS, HTR1E, GABRB3, ARHGAP15, CNTNAP2	8	18	4.000	31	5
	2/4	MPP7, PLCB1, DLG2, FBXL7, SRGAP1, PARK2	6	15	3.714	40	3
	3/4	CSMD3, SAMD4A, LRFN2, DDHD2, RCAN1, FAM107B, STIM1, EYA2, MAFF, NAV2, WNT11, DNAJC13, USP12	13	27	3.571	0	NA
	4/4	PRDM2, PPP2R5C, PTPRM, ARHGAP22, CLASP1, TMEM117, CNTN4	7	15	3.250	0	NA

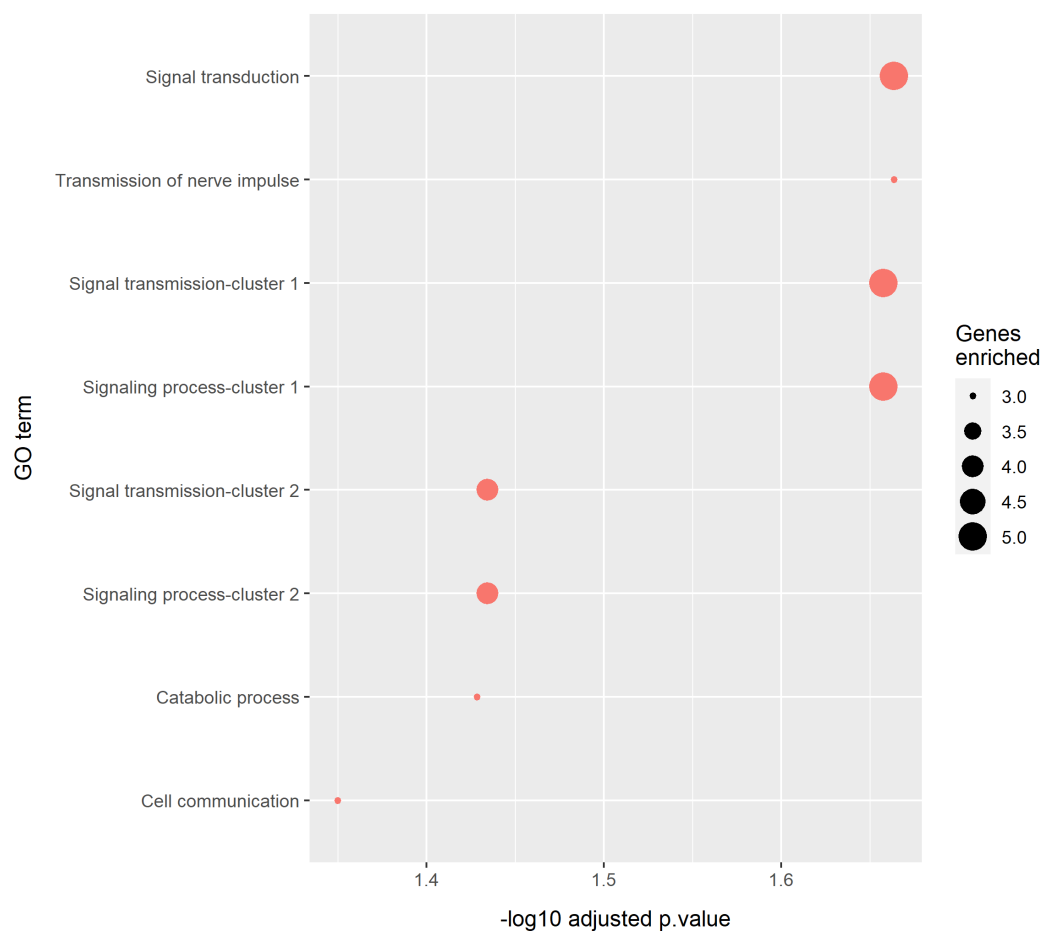


Figure 47. GO terms significantly enriched for the top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT
Gene ontology pathways shown to be significantly enriched in the dmCpG clusters produced from the clustering of the top 100 dmCpGs associated with BMC at four years of age. A larger point size and $-\log_{10}$ adjusted p -value indicates a greater significance and number of dmCpGs enriched for the pathway.

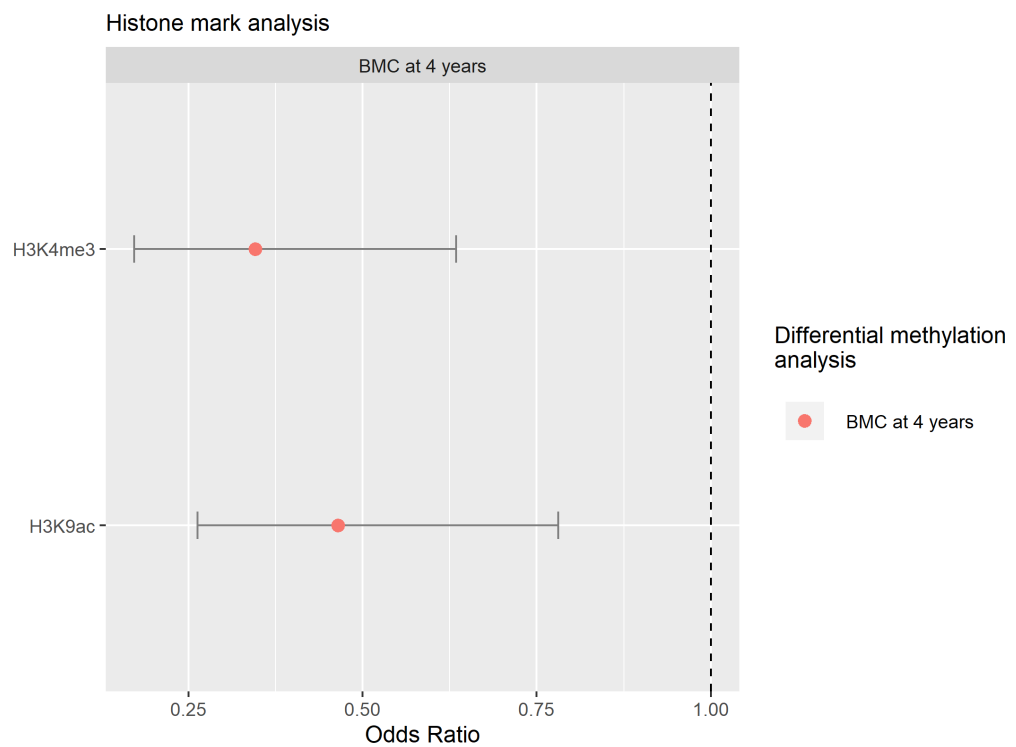


Figure 48. Odds ratio graph produced from histone mark analysis of the top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT overlapping histone marks in the genome

The histone mark enrichment analysis of the top 100 dmCpGs associated with the differential methylation analyses of BMC at four years of age in the MAVIDOS RCT showed significant (FDR < 0.05) under-representation (odds ratio < 1) of H3K4me3 and H3K9ac histone marks associated with BMC at four years of age.

5.2.3 Differential methylation analysis in relation to BMD

5.2.3.1 Differentially methylated CpGs

The relationship between umbilical cord tissue DNA methylation and BMD at the ages of birth and four years was also investigated. Analysis of the relationship between DNA methylation in umbilical cord tissue and BMD at birth and four years of age found 6 and 22 dmCpGs respectively with an $FDR < 0.05$. The dmCpG-associated genes associated with BMD at birth and four years of age are listed in Table 31 and the top 100 dmCpGs for each analysis in appendix 9.4.6.

The most significant dmCpGs associated with BMD at birth cg05138702 was located in the 5'UTR of the C-Terminal Binding Protein 2 (*CTBP2*) gene ($FDR = 0.007972$) and its methylation was negatively associated with BMD at birth (Figure 49). The *CTBP2* gene produces alternative transcripts, encoding two distinct proteins. One is a transcriptional repressor seen to act as a NADH-dependent repressor of the p300-runt-related transcription factor 2 (*RUNX2*) transcriptional complex which plays a role in bone formation, the other is a major component of synaptic ribbons. Similarly, all other significant dmCpGs ($FDR < 0.05$) showed a negative relationship between their methylation and BMD (Figure 49). The most significant dmCpG associated with BMD at four years of age was cg24384472 was located in the gene body of the CUB and Sushi Multiple Domains Protein 3 (*CSMD3*) gene ($FDR = 0.001049$) which plays a role in dendrite development. The methylation of the dmCpG cg24384472 was negatively associated with BMD at four years of age, similarly 16 of the remaining significant dmCpGs were negatively associated with BMD at four years of age, whereas 5 showed a positive relationship (Figure 50). In addition, a comparison of the top 100 dmCpGs associated with both BMD at birth and four years found an overlap of a single dmCpG-associated gene, *CSMD3* (Figure 51).

Table 31. Results of differential methylation analysis interrogating the association between UCT DNA methylation and BMD at birth and four years of age in the MAVIDOS RCT

The differential methylation analysis investigating the association between UCT DNA methylation with BMD at birth found six significant dmCpGs (FDR<0.05) (n=274). Furthermore, 22 dmCpGs were found to be significantly associated with BMD at four years of age (n=147). DmCpGs associated with a known gene are represented by their respective associated gene symbol.

Model	Sample number	Lambda	FDR					Top 10 dmCpGs (FDR <0.25)
			<0.25	<0.20	<0.15	<0.10	<0.05	
Birth	274	1.07	29	23	14	6	6	CTBP2, NEUROD4, cg22209601, TOP3A, VAV2, cg07524558, cg15020206, SLFN12, CST5, C15orf27
4 years	147	1.10	559	279	170	60	22	CSMD3, cg26257545, PTPRM, FERMT2, cg25193525, GABRB3, cg16702610, RAB37, TIGAR, HIVEP1

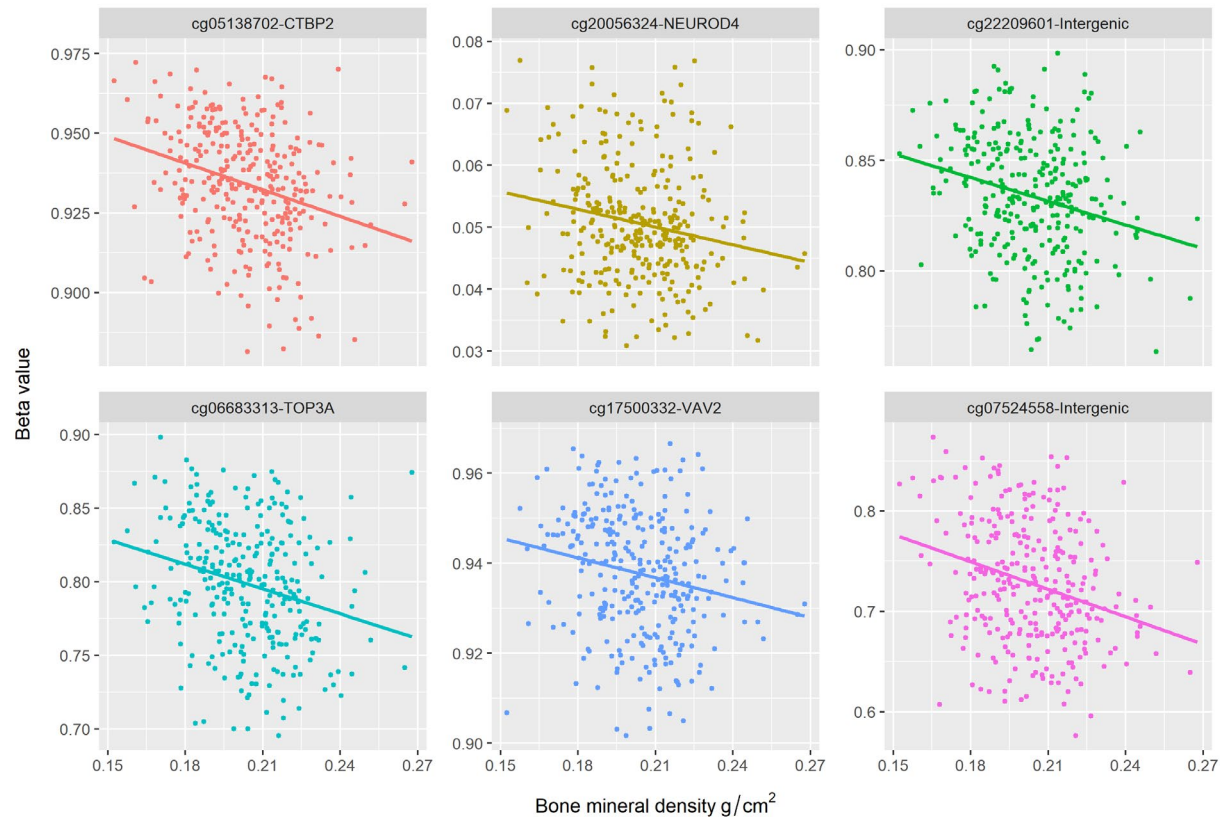


Figure 49. The association between the methylation and BMD for the six dmCpGs significantly (FDR<0.05) associated with BMD at birth in the MAVIDOS RCT

The differential methylation analysis assessing the association between the methylation of 274 samples of UCT DNA and BMD at birth in the MAVIDOS RCT found significant (FDR <0.05) differential methylation of CpGs cg05138702, cg20056324, cg22209601, cg06683313, cg17500332 and cg07524558 which were mapped to the CTBP2 gene, the NEUROD4 gene, an intergenic region, the TOP3A and VAV2 genes and finally an intergenic region respectively. All dmCpGs showed an inverse association between methylation and BMD at birth.

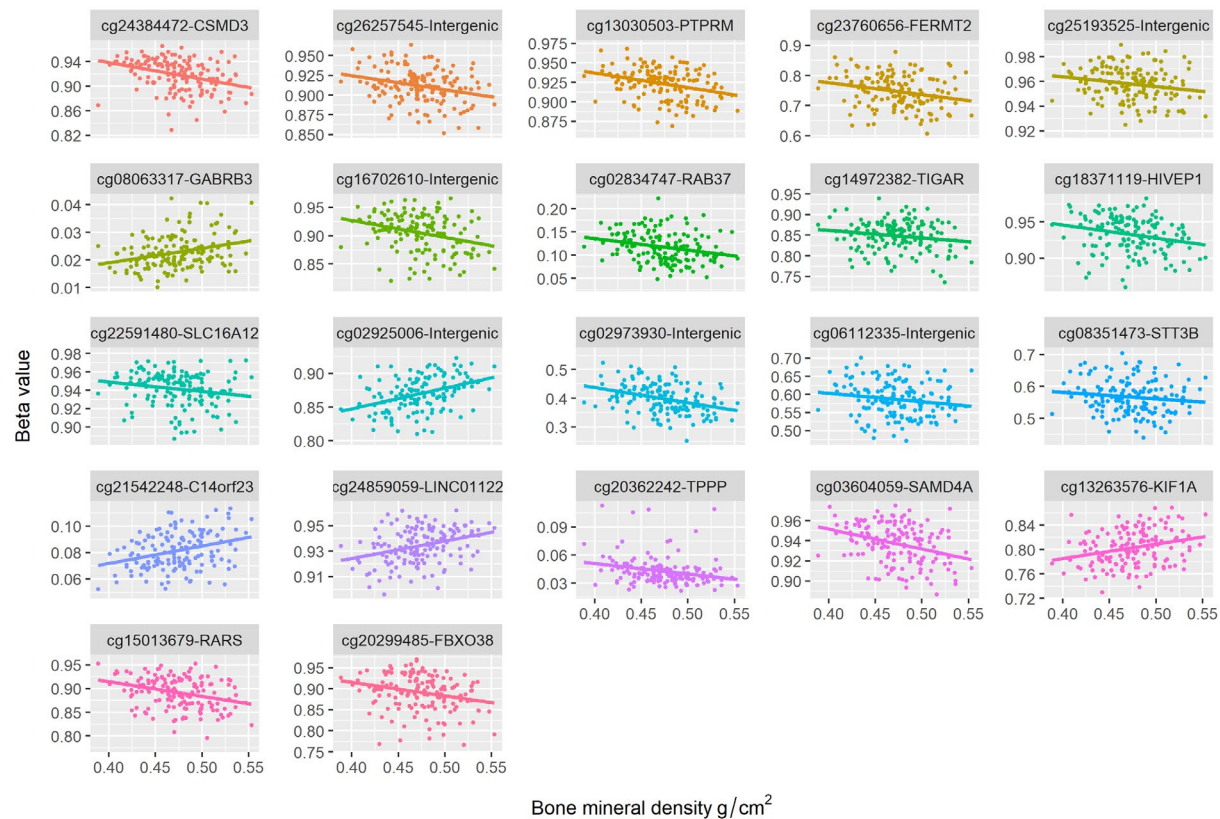


Figure 50. The association between methylation and BMD for the 22 dmCpGs significantly (FDR < 0.05) associated with BMD at four years of age in the MAVIDOS RCT

The differential methylation analysis assessing the association between the methylation of 147 samples of UCT DNA and BMD at four years of age in the MAVIDOS RCT found significant (FDR < 0.05) differential methylation of 22 CpGs, six of which were mapped to an intergenic region and 16 of which were mapped to known genes. The methylation of 17 CpG sites was inversely associated with BMD at four years of age and the methylation of five sites positively associated.

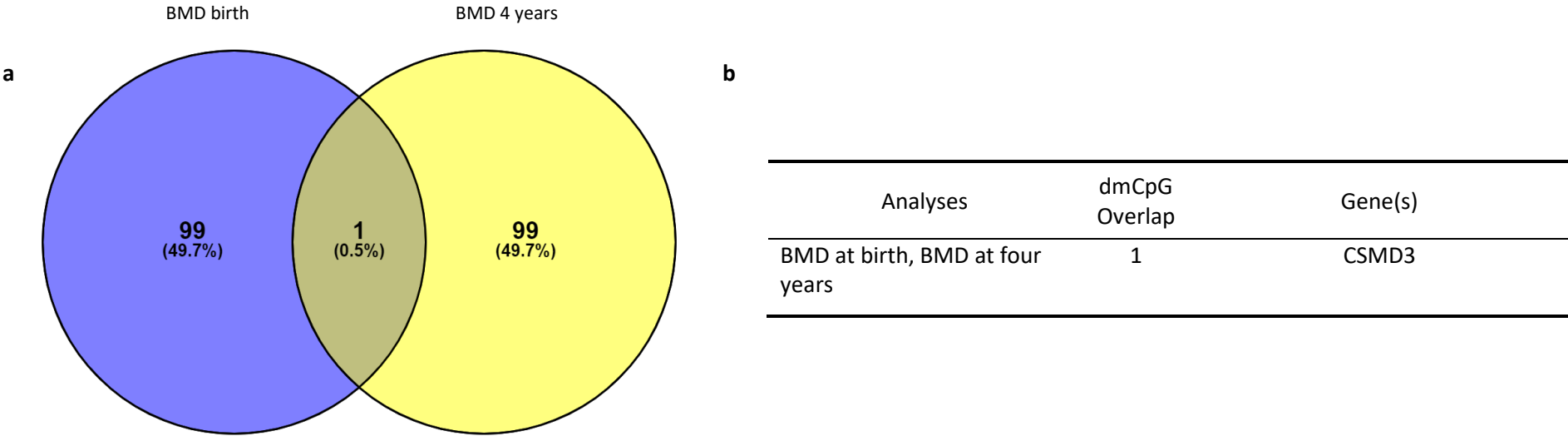


Figure 51. The a) venn diagram overlap between the top 100 dmCpGs associated with BMD at birth and BMD at four years of age in the MAVIDOS RCT and the b) associated identity of each overlapping dmCpG

A comparison of the top 100 dmCpGs when ranked by FDR was compared between differential methylation analyses investigating the relationship between umbilical cord tissue DNA methylation and measures of BMD at birth and four years of age in the MAVIDOS RCT. A a) venn diagram depicts the overlap between dmCpGs taken from each analysis, and b) the overlapping dmCpG probe name, or the gene name if located within a known gene.

5.2.3.2 Genomic locations of dmCpGs

The differential methylation analysis of BMD at birth and four years of age produced dmCpGs predominantly within an open sea region, less frequently in a shore or shelf surrounding a CpG island and least frequently in a CpG island. In regard to genomic structure, dmCpGs were most commonly positioned within the gene body, whereas fewer were found upstream of the TSS, in the first exon or exon boundary, non-coding UTR regions and least commonly within the 3'UTR. The distribution of the top 100 dmCpGs in the genome relative to both CpG islands and gene structure for analyses of BMD at birth and four years of age are shown in Figure 52.

5.2.3.3 Transcription factor enrichment analysis

Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at both birth and four years of age indicated enrichment for 3 and 2 transcription factor motifs respectively. The transcription factor motifs enriched for BMD at birth were the Sp1 transcription factor (SP1) motif (NES=4.42) in 10 genes, the Zinc Finger Protein 665 (ZNF665) motif (NES=4.37) in 10 genes and Zinc Finger Protein 568 (ZNF568) motif (NES=4.14) in 30 genes. Whereas in regard to BMD at four years of age, enrichment was shown for the SRY-Box Transcription Factor 30 (SOX30) motif (NES = 4.37) in 19 genes and (TEAD1) motifs (NES = 4.16-4.02) in 15 genes (Table 32). Full list of motif references, sequence logo graphs and enriched genes in appendix 9.4.6.

5.2.3.4 Differentially methylated regions

Assessment of the differential methylation analyses for DMRs found no regions (SV <0.05) of significant differential methylation associated with BMD at birth or four years of age.

5.2.3.5 Pathway enrichment analysis

5.2.3.5.1 Cytoscape pathway enrichment analysis

Clustering of the top 100 dmCpGs associated with BMD at birth and four years of age produced 1 and 4 clusters respectively (Table 33). GO term enrichment analysis demonstrated enrichment of the top 100 dmCpGs associated with BMD at four years of age for protein serine/threonine kinase activity (GO:4674, FDR = $5.56e^{-11}$), protein kinase activity (GO:4672, FDR = $3.42e^{-10}$) and protein amino acid phosphorylation (GO:6468, FDR = $7.35e^{-10}$) (Figure 53). Full ontology results for each analysis in appendix 9.4.6.

5.2.3.6 Histone mark and chromatin state analysis

Histone mark and chromatin state analysis for the top 100 dmCpGs associated with BMD at birth showed no significant enrichment. Conversely there was a significant under-representation of the top 100 dmCpGs associated with BMD at four years of age overlapping both H3K4me3 (FDR=0.0152) and the H3K9ac (FDR=0.0152) histone marks (Figure 54), however no chromatin state enrichment was identified.

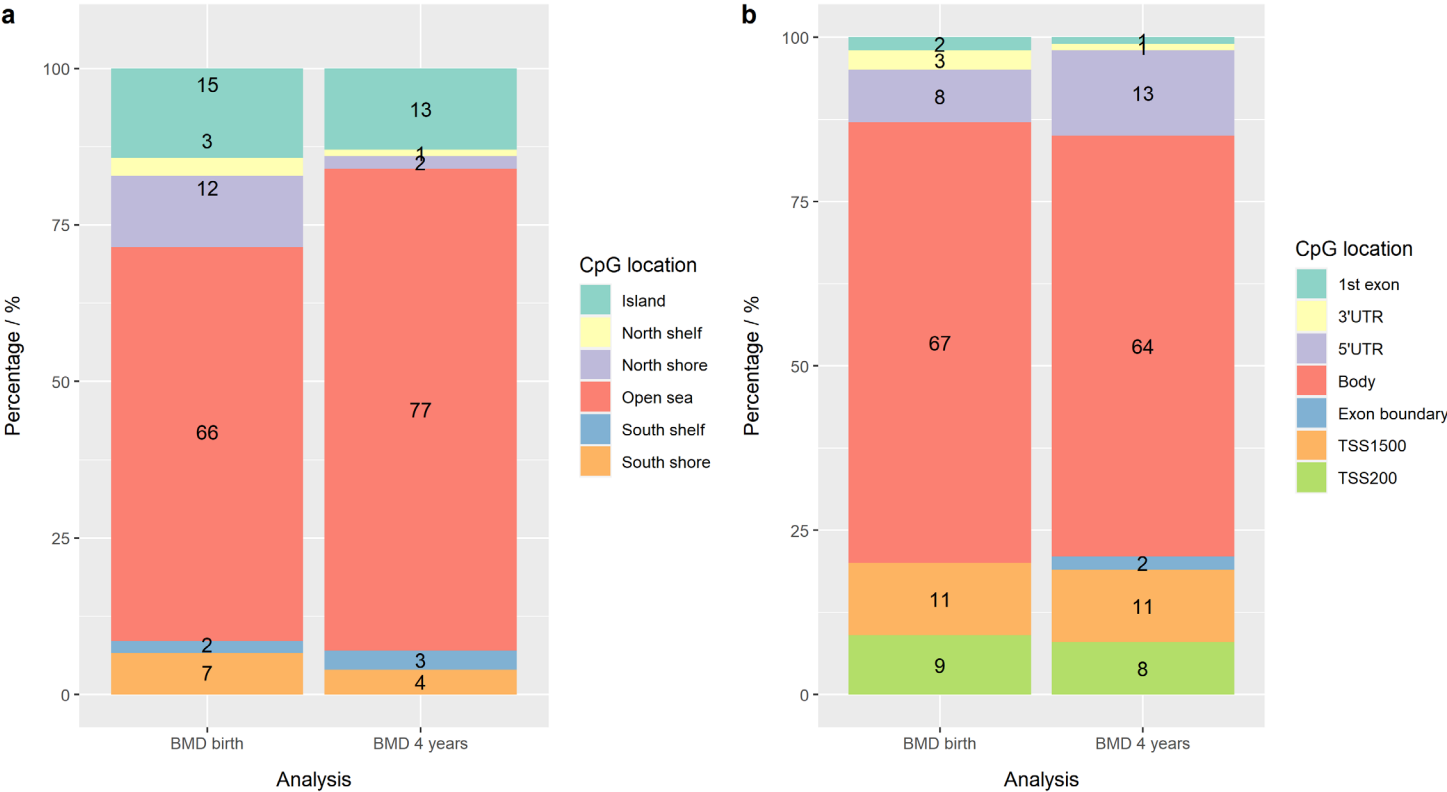


Figure 52. The location of the top 100 dmCpGs associated with BMD at birth and four years of age relative to a) CpG islands and b) genomic structure
The top 100 dmCpGs associated with the differential methylation analysis investigating the association between UCT DNA methylation and both BMD at birth and four years of age in the MAVIDOS RCT were shown to be largely in an open sea region and predominantly within the body of a gene. CpG shores, shelves and open sea regions are defined in appendix 9.3.

Table 32. The transcription factor binding motifs enriched within the top 100 dmCpGs associated with BMD at birth and four years of age for the MAVIDOS RCT

Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at birth and four years of age found the enrichment of 3 and 2 transcription factor motifs respectively with a normalised enrichment score (NES) >4.

Analysis	Enriched TFs	Rank	TF	Normalised enrichment score	Motifs	Annotation	Multiple motifs enrichment range		
							Minimum NES	Maximum NES	Genes enriched
<i>BMD at birth</i>									
		1	SP1	4.42	1	Direct	NA	NA	10
		2	ZNF665	4.37	1	Direct	NA	NA	10
		3	ZNF568	4.14	1	Direct	NA	NA	30
<i>BMD at 4 years of age</i>									
		1	SOX30	4.37	1	Inferred	NA	NA	19
		2	TEAD1	NA	2	Inferred	4.02	4.16	15

Table 33. Gene clusters produced during the pathway analysis of the top 100 dmCpGs associated with BMD at birth and four years of age in the MAVIDOS RCT

Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMD at birth and four years of age produced 1 and 4 distinct clusters of dmCpGs respectively. However no significant pathway enrichment was seen for the cluster produced from the top 100 dmCpGs associated with BMD at birth, but cluster 1 and 4 produced from the clustering of the top 100 dmCpGs associated with BMD at four years of age saw significant enrichment of pathways with the involvement of three or more dmCpGs.

Model	Cluster	Genes	Nodes	Edges	Score	Pathways FDR <0.05	Gene count ≥3
BMD at birth	1/1	IGF-1, KCNAB2, FIBCD1, PDIA5, HTRA1, AUTS2, NOSTRIN, MYO10, TMEM141, KCNB2, CTBP2	11	22	3.333	0	NA
BMD 4 years	1/4	IKBKB, MAP2K1, MAP2K2, CHUK, EIF2AK3, MAPK1, NLK, MAPK3	8	39	6.222	345	331
	2/4	TTC17, MCCC1, CYP19A1, HTR1E, CNTNAP4, KIT, GPHN, ERC1, APAF1, CREB5, ZDHHC18, FBXL7, FUT5, GABRB3	14	35	4.400	0	NA
	3/4	GLRB, COL5A1, MYCBP2, HIVEP1, DBT, TMCC3, PLA1, ACACA, C5orf24, FGGY, GMDS, CADM1, EPB41, GPR1, STT3B, HOOK3, DAB2IP, SAMD4A	18	36	3.684	0	NA
	4/4	CLIC5, PLA2G12A, PINX1, RSF1, MED12L, MKLN1, TERT, KCNMA1, TLL2, CSMD3, KIF1A, GPC6, CLASP1, FERMT2	14	23	3.067	59	10

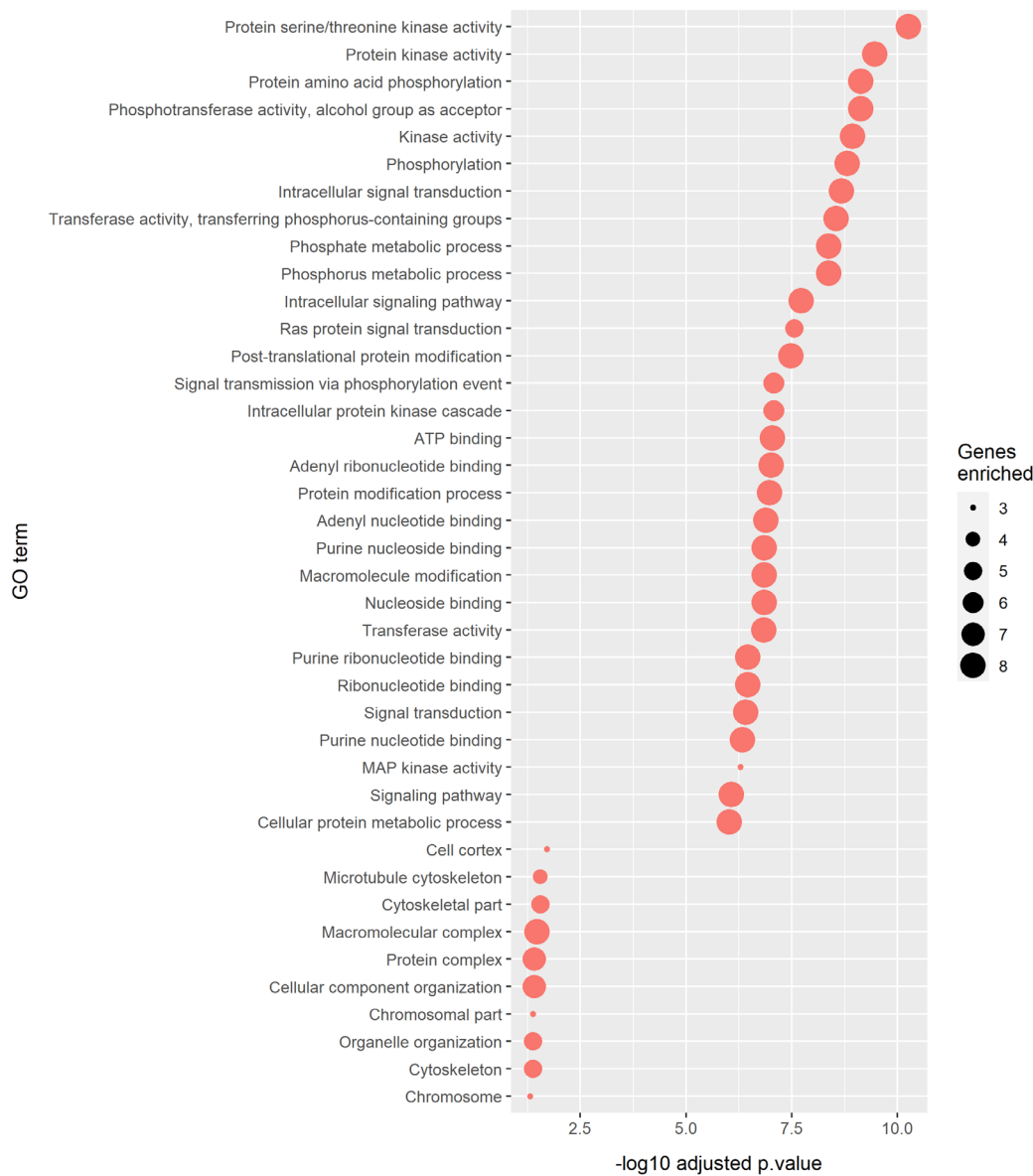


Figure 53. GO terms significantly enriched for the top 100 dmCpGs associated with BMD at four years of age in the MAVIDOS RCT

Gene ontology pathways shown to be significantly enriched in the dmCpG clusters produced from the clustering of the top 100 dmCpGs associated with BMD at four years of age. The dmCpGs associated with BMD at birth produced one cluster, but no significant gene ontology pathway enrichment was identified. A larger point size and $-\log_{10}$ adjusted p -value indicates a greater significance and number of dmCpGs enriched for the pathway.

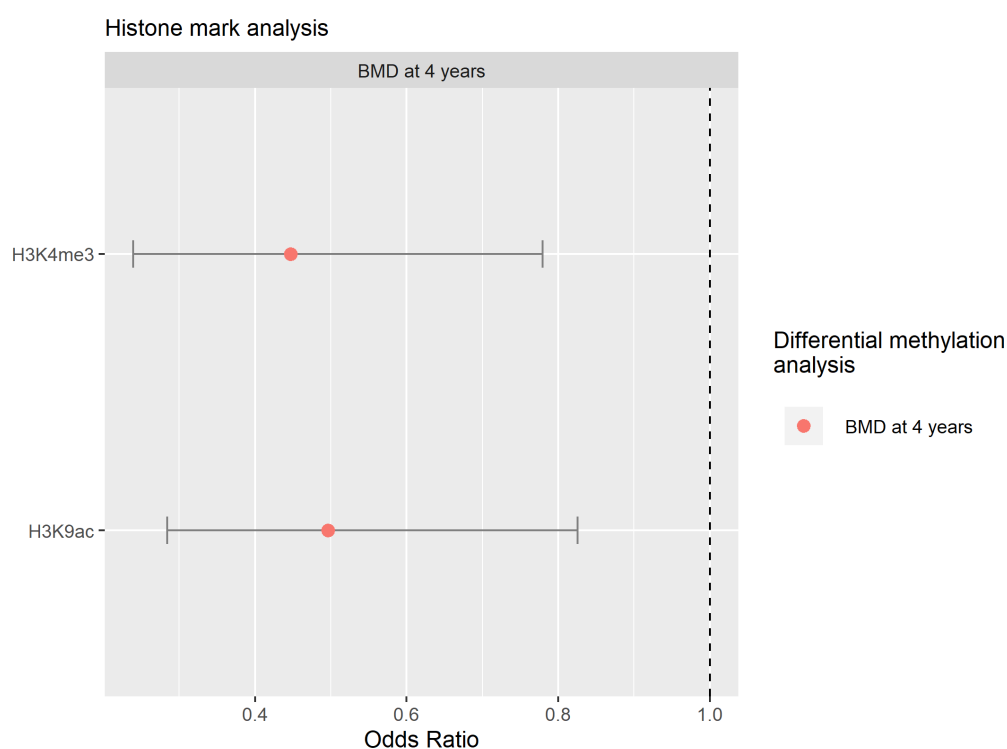


Figure 54. Odds ratio graph produced from histone mark analysis of the top 100 dmCpGs associated with BMD at four years of age in the MAVIDOS RCT overlapping histone marks in the genome

The histone mark enrichment analysis of the top 100 dmCpGs associated with the differential methylation analyses of BMD at four years of age in the MAVIDOS RCT showed significant (FDR <0.05) under-representation (odds ratio <1) of H3K4me3 and H3K9ac histone marks associated with BMD at four years of age. No significant under or over representation was found for BMD at birth.

5.2.4 *Overlap between dmCpGs associated with BMC and BMD in the MAVIDOS RCT*

5.2.4.1 *dmCpG overlap within age groups*

To determine whether there were common methylation changes associated with either BMC or BMD in the MAVIDOS RCT, the overlap between the top 100 dmCpGs associated with each differential methylation analyses of the same age group was determined. An assessment of the overlap between the top 100 dmCpGs associated with BMC at the age of four years and BMD at four years of age found an overlap of 32 dmCpGs (Figure 55).

5.2.5 *Does adjustment for vitamin D intervention potentially influence the methylation changes associated with offspring postnatal bone measures?*

A summary of the findings of the MAVIDOS RCT by Cooper *et al.* (2016) found no association between maternal vitamin D supplementation and whole-body BMC at four years of age when compared to placebo. However a significant interaction was found between treatment group and season, where neonates born in the winter had better bone measures of BMC, BMD and bone area when born to mothers were supplemented with vitamin D (625). Furthermore, a candidate gene approach found significant differential methylation in the promoter region of the retinoid X receptor- α (*RXR α*) gene associated with maternal vitamin D supplementation, the methylation of which has been seen to associate with BMC content in childhood within the SWS study and when agonism of RXR in mouse models has shown to lead to irregular development of the long bones (3,745,854). This suggests that maternal vitamin D supplementation within this study may potentially alter any associations between cord tissue DNA methylation and BMC at birth and four years of age, therefore we repeated each differential methylation analysis with correction for intervention as a covariate in the regression model (full results in Table 34). The adjustment of the models for intervention group allowed us to account for any potential association between maternal supplementation with 1000 IU 25(OH)D a day and prenatal bone growth.

Differential methylation analyses were repeated for BMC at four years of age and BMD at birth and four years of age with adjustment for maternal late-stage pregnancy vitamin D (MLSP D) status (full results in Table 34). A comparison between the top 100 dmCpGs associated with the differential methylation analyses of BMC at four years of age, with and without intervention group as a covariate found 60 of a combined total of 200 dmCpGs in common. A comparison of the top 100 dmCpGs associated with the differential methylation analyses of BMD at birth with and without intervention group as a covariate found 61 of 200 dmCpGs in common and similarly at four years of age the addition and removal intervention group as a covariate found 50 of 200 dmCpGs in common.

5.2.6 *Are there differential methylation changes shared between the differential methylation analyses of the MAVIDOS RCT and SWS cohorts in respect to postnatal bone measures?*

A comparison was made between the dmCpG-associated genes associated with the MAVIDOS RCT and SWS cohorts. The comparison effectively enlarges the pool of data at our disposal when investigating the relationship between DNA methylation of birth tissues and postnatal bone measures. We can also potentially identify dmCpGs either unique or common to both tissues where dmCpGs occurring in either one, or multiple tissues could potentially indicate methylation changes with a more pervasive or substantial relationship with measures of postnatal bone growth.

A comparison of differential methylation analyses of umbilical cord tissue DNA and umbilical cord blood DNA from the MAVIDOS RCT and SWS studies respectively found an overlap of one dmCpG with respect to BMC at four years of age, but no overlap in dmCpGs was found between the studies for either BMD at birth or four years of age. The dmCpG overlapping the analyses of BMC at four years of age was found within the body of the HECT C2 and WW Domain Containing E3 Ubiquitin Protein Ligase 1 (*HECW1*) gene, however both showed opposing changes in direction of methylation.

5.2.7 *Are there differential methylation changes shared between the differential methylation analyses of the MAVIDOS RCT with respect to both postnatal bone measures and vitamin D supplementation?*

One aim of this work was to ascertain whether we saw differential methylation changes induced by maternal vitamin D supplementation in genes which may also be potential biomarkers of postnatal bone growth as seen previously in the SWS cohort with respect to RXRa. A comparison of the top 100 dmCpGs associated with the differential methylation analyses of umbilical cord tissue DNA with respect to BMC at four years of age, BMD at birth, intervention group and intervention group for maternal early-stage pregnancy vitamin D (MESP D) insufficient mothers found a number of differential methylation changes within the body of the Protein Tyrosine Phosphatase Receptor Type N2 (*PTPRN2*) gene. Both the intervention group and intervention group MESP D insufficient analyses saw a decrease in the methylation of cg12144852 in the vitamin D supplemented group. However the analysis of BMC at four years of age showed a decrease in the methylation of cg21160944 and the analysis of BMD at birth showed a decrease in methylation of cg07624226 with increasing BMC and BMD respectively. Furthermore, an increase in the methylation of cg09086678 was seen in the vitamin D supplemented group associated with the MESP D insufficient analysis and a decrease in the methylation of cg22075154 associated with an increase BMD at four years within the body of the B-Raf Proto-Oncogene, Serine/Threonine Kinase (*BRAF*) gene (Figure 56).

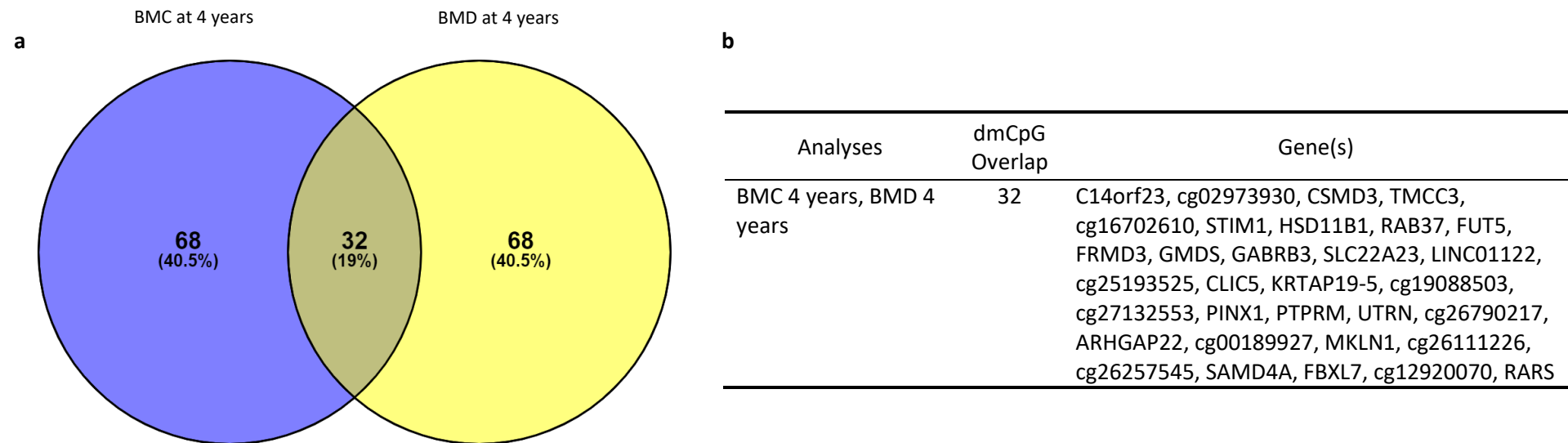
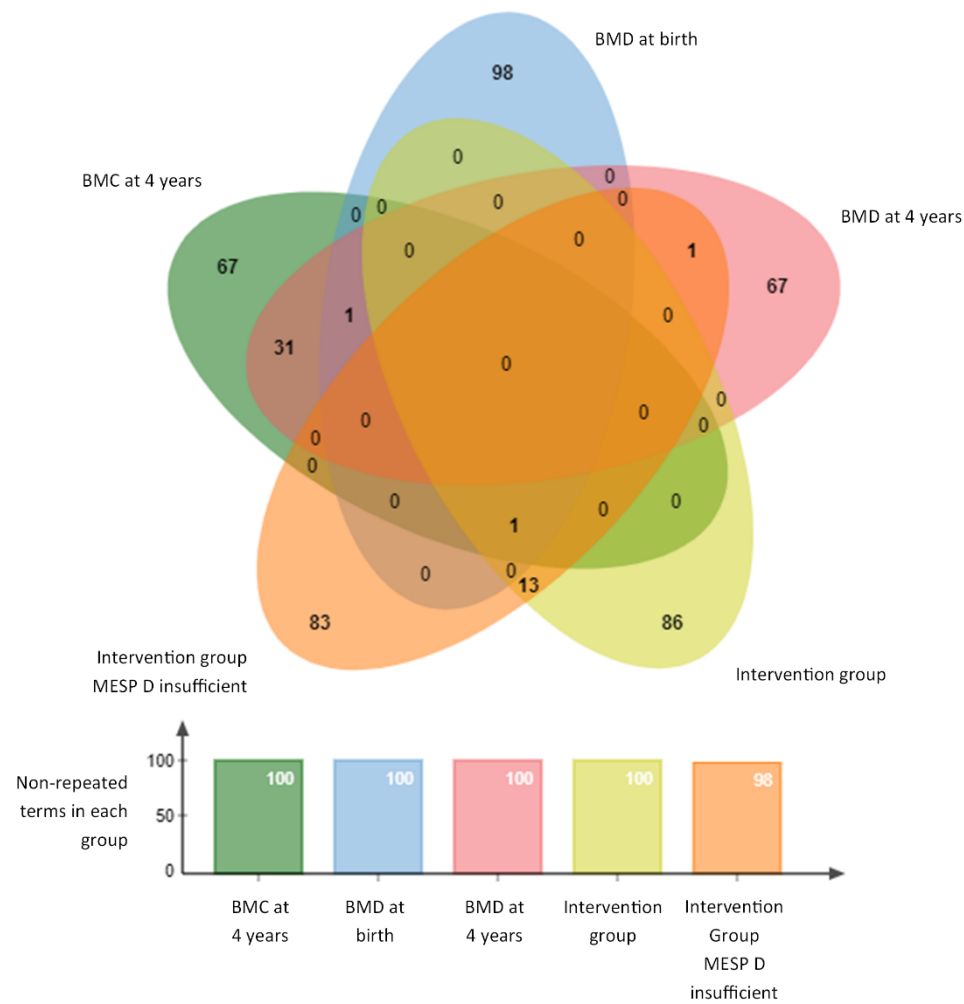


Figure 55. A a) venn diagram depicting the overlap between the top 100 dmCpGs associated with BMC at four years of age and BMD at four years of age and the b) associated identity of each overlapping dmCpG

A comparison of the top 100 dmCpGs when ranked by FDR was compared between differential methylation analyses investigating the relationship between umbilical cord tissue DNA methylation and measures of BMC and BMD at four years of age in the MAVIDOS RCT. A a) venn diagram depicts the overlap between dmCpGs taken from each analysis, and b) the overlapping dmCpG probe name, or the gene name if located within a known gene.



b

Analyses	dmCpG Overlap	Gene(s)
BMC 4 years, BMD birth, Intervention, Intervention MESP D insufficient	1	PTPRN2
BMD 4 years, Intervention MESP D insufficient	1	BRAF

Figure 56. The dmCpG overlap between BMC, BMD and intervention related analyses within the MAVIDOS RCT
A a) 5-way venn diagram assessing the overlap between the top 100 dmCpGs when ranked by FDR was compared between differential methylation analyses investigating the relationship between umbilical cord tissue DNA methylation and BMC at four years of age, BMD at both birth and four years of age, intervention group and intervention MESP D insufficient group in the MAVIDOS RCT and a corresponding bar graph of the number of unique dmCpGs within each group. Also b) a table indicates the identity of the total number and identity of dmCpGs overlapping each set of analyses. Analysis identified PTPRN to be common between Intervention, Intervention MESP D insufficient, BMC 4 years and BMD at birth. BRAF was found to be to be common between Intervention MESP D insufficient and BMD 4 years analyses.

Table 34. Results of differential methylation analysis interrogating umbilical cord tissue DNA for associations with BMC at four years, BMD at birth and BMD at four years of age adjusted and non-adjusted for intervention group

Cgs associated with a known gene represented by their respective associated gene symbol.

Bone measure	Model	Adjusted for intervention	Sample number	Lambda	FDR					Top 10 dmCpGs FDR <0.25
					<0.25	<0.20	<0.15	<0.10	<0.05	
BMC	4 years	✗	147	1.03	173	76	68	11	6	C14orf23, cg02973930, CSMD3, TMCC3, POU6F1, CD82, FER1L5, DAB2IP, cg16702610, cg25106913
		✓	144	1.03	182	93	21	6	4	C14orf23, cg02973930, cg25106913, CSMD3, cg00189927, STIM1, DAB2IP, cg02167856, RAB37, LRRC28
BMD	Birth	✗	274	1.07	29	23	14	6	6	CTBP2, NEUROD4, cg22209601, TOP3A, VAV2, cg07524558, cg15020206, SLFN12, CST5, C15orf27
		✓	269	1.05	15	8	7	7	3	CTBP2, cg07524558, NEUROD4, TOP3A, cg22209601, C15orf27, VAV2, cg15348679, ZNF282, COL11A2
	4 years	✗	147	1.10	559	279	170	60	22	CSMD3, cg26257545, PTPRM, FERMT2, cg25193525, GABRB3, cg16702610, RAB37, TIGAR, HIVEP1
		✓	144	1.18	2861	1381	610	256	36	CSMD3, cg02925006, cg26257545, RAB37, cg04143252, KIF1A, PCDH9, FBXO38, WDFY3, HOOK3

5.3 Discussion

5.3.1 Summary

Within the SWS study it has previously been shown that perinatal differential methylation of the *RXRα* promoter region is associated with BMC at four years of age and previously within this study we have identified other significant dmCpGs associated with both BMD at birth and eight years of age in the SWS cohort (section 3.2.3). Therefore here, within the MAVIDOS RCT we aimed to further this work by conducting a genome-wide scan in a separate cohort and tissue to ascertain i) whether further epigenetic signatures changes were associated with BMC or BMD at birth or four years of age, ii) whether there are common differential methylation changes between SWS and MAVIDOS RCT birth tissues and iii) whether these changes are similar to those seen to be induced by vitamin D supplementation within the MAVIDOS RCT.

Within this study we saw significant differential methylation changes associated with BMC at four years of age and BMD at both birth and four years of age. Although, we saw no associations whatsoever associated with BMC at birth, suggesting a lack of power in the study and a potential need for replication in studies with a greater number of participants. However, as we saw with the differential methylation analyses with respect to BMD in the SWS cohort (section 3.2.3), the differential methylation changes seen in each analysis were almost entirely distinct from those in other age groups as indicated by an overlap of only a single dmCpG between BMD at birth and BMD at four years of age (Figure 51). This may be due to the nature of early life as a period of rapid growth, where progression through different developmental stages can influence bone development through different the activation of different mechanistic pathways. An example of this progression can be seen between the ages of birth and four years of age where children become ambulatory, which will increase mechanical loading on the skeleton, a major regulator of skeletal bone mass and geometry (694). In contrast a comparison between the dmCpGs associated between BMC and BMD at four years of age showed reasonable overlap of 32 dmCpGs (Figure 55), however as BMD is a metric of BMC per unit area so this may be expected. However, when comparing bone measures within age groups across both SWS and MAVIDOS RCT studies we find overlap of only one dmCpG at four years of age. This perhaps reflects the largely cell-type specific nature of differential methylation and the reportedly mostly distinct methylomes of umbilical cord blood and umbilical cord tissue DNA samples (649,693). When attempting to ascertain the overlap between measure of postnatal BMC or BMD and intervention analyses within the MAVIDOS RCT we saw differential methylation of multiple CpGs within the *PTPRN2* gene between all analyses excluding BMD at four years of age indicating a potential mechanistic link or point of intersection of the factors.

Further functional analysis of the dmCpGs associated with BMC at four years of age showed enrichment for pathways associated with signal transduction and transmission processes (Figure

47) and transcription factor enrichment showed enrichment for four transcription factors binding site motifs (Table 29). No differentially methylated regions were identified. Whereas investigation of the functional nature of the dmCpGs associated with BMD at birth and four years of age showed no pathway enrichment of the dmCpGs associated with BMD at birth, but the dmCpGs associated with BMD at four years were found to be broadly enriched for signalling and transcriptional processes (Figure 53). Transcription factor enrichment identified significant enrichment of three and two transcription factor binding site motifs within the dmCpGs associated with BMD at birth and four years of age respectively. DMR analysis found no regions of differential methylation.

5.3.2 Top hits

The most significantly differentially methylated CpGs associated with BMC at four years of age included cg21542248 in the body of the chromosome 14 open reading frame 23 (*C14orf23*) gene, cg02973930 an intergenic CpG, cg24384472 in the body of the CUB and sushi domain-containing protein 3 (*CSMD3*) gene, cg02405318 in the 5'UTR of the Transmembrane And Coiled-Coil Domain Family 3 (*TMCC3*) gene, cg20098255 in the body of the POU Class 6 Homeobox 1 (*POU6F1*) gene and cg25723331 in the 5'UTR of the *CD82* gene. The functions of *C14orf23*, cg02973930 and *TMCC3* are as of yet uncharacterised and *CSMD3* and *POU6F1* are currently understood to function in neural or cancer-related roles respectively, however *CD82* has been suggested to play a role in the growth of bone.

CD82 is part of the family of 33 tetraspanin transmembrane proteins that function as membrane organisers, acting to facilitate membrane signalling, membrane fusion, actin dynamics, cell-cell matrix adhesion, immune surveillance and motility (855,856). Murine *CD82* Knockout models have also shown its specific and essential role in dendritic cell migration, angiogenesis and platelet function (857–859). *CD82* has also been shown to have an important role in osteoclast function and strongly upregulated during osteoclastogenesis (860,861). A study by *Bergsma et al.* (2017) showed deletion of *CD82* in a mouse model led to both defective osteoclast and osteoblast activity, an increase in bone marrow adipocytes and bones which are smaller in diameter, with no apparent change in bone density suggesting a concomitant decrease in BMC (862). Within this context of this study we saw methylation of cg25723331 within the 5'UTR of the *CD82* gene to be negatively associated with BMC at four years of age. A decrease methylation in the promoter region of a gene typically corresponds with an increase in transcriptional activity, suggesting an increase in transcription of the gene with increasing BMC, however this would need to be validated. There was also no overlap of this dmCpG with the analysis of BMD at four years of age, similar to the observations of *Bergsma et al.* (2017) (862). If the potential change in the transcriptional activity of this gene as a result of differential methylation were to be validated, this would suggest a dmCpG which may act as a potential marker for BMC alone at four years of age.

The authors also saw an increase in the number of adipocytes in CD82 null mice, proposed to be not as the result of a switch in lineage potential of bone marrow mesenchymal stromal cells (BM-MSCs), but the accumulation of an upstream regulator. This indicates further effects beyond that of osteoblastic and osteoclastic dysregulation which could affect bone growth and a further need to explore the mechanistic pathway of this factor.

The differential methylation analysis interrogating the association between cord tissue DNA methylation and BMD at birth found the significant differential methylation of cg05138702 within the 5'UTR of C-Terminal Binding Protein 2 (*CTBP2*) gene. The CTBPs are a family of transcriptional co-repressors made up of the highly homologous CTBP1 and CTBP2. Both CTBP1 and CTBP2 have been identified as having important roles in development and oncogenesis, although CTBP2 alone has been implicated in the maintaining the balance between pluripotency and differentiation in embryonic stem cells, key for retinoic acid signalling and in regulating the adipogenic differentiation of human mesenchymal stromal cells (MSCs) (863,864). A study by *Wang et al.* (2015) found that during the adipogenic differentiation of MSCs the inhibition of CTBP2 led to an increased accumulation of lipid droplets, greater triglyceride levels and increases in the expression of adipogenic marker genes such as fatty acid-binding protein 4 and lipoprotein lipase. However, levels of the master regulators of adipogenesis PPAR γ and CCAAT/enhancer binding protein α (C/EBP α) did not change, indicating inhibition of CTBP2 reduces its interaction with C/EBP α and so reduces its suppressive effects (863). This is similar to the action of retinoic acid of which CTBP2 is a key coactivator (865). Both *in vitro* and *in vivo* studies have demonstrated the importance of retinoic acid, the active metabolite of vitamin A as a key regulator of adipose tissue metabolism and obesity (866–868). Retinoic acid has recently been shown to inhibit the adipogenic differentiation potential of BM-MSCs to an adipocytic lineage through the upregulation of Fos-related antigen 1 (Fra-1) signalling, which was also proposed to act either on C/EBP α , PPAR γ 2 or both (869). Similarly, *Ayala-Summano et al.* (2016) showed supplementation pre-adipocytic cell line with retinoic acid during differentiation blocked adipogenesis through inhibition C/EBP β phosphorylation at Thr188, a critical step for the progression of the adipogenic program (870). This suggests any differential methylation of *CTBP2* and potential change in its transcriptional activity should also be considered for its potential effects on vitamin A activation.

Furthermore, CTBP2 has also been observed to mediate the repression of p16^{INK4A}. Previous work within the SWS cohort identified methylation in the promoter of the long non-coding RNA ANRIL and childhood adiposity at 6 years of age. The *CDKN2A* gene encodes ANRIL and the two cell cycle inhibitors p16^{INK4a} and p16^{ARF}. ANRIL acts to negatively regulate the activity of p16^{INK4a}, which along with p16^{ARF} have been shown to play a direct role in regulating adipocyte number, function and senescence (582). CTBP2 has also been observed to mediate the repression of p16^{INK4A}, therefore may be an extended part of this regulatory framework (871). This combination of work suggests an intrinsic role for CTBP2 in the regulation of adipogenesis. In this study we found a

methylation change at cg05138702 within the 5'UTR of the *CTBP2* gene that demonstrated a negative association with increasing BMD at birth (Figure 49). A reduced methylation typically corresponds to a reduction in the repressive effects of methylation in the promoter region, increasing expression. If any change were to be corroborated by validation of the expression state of this gene, this relationship could suggest a decreased methylation allows for a greater expression of this anti-adipogenic factor, potentially leading to a decrease in adipogenesis and an increase in osteogenesis due to the reciprocal nature of these processes (584).

The differential methylation analysis assessing the relationship between cord tissue DNA methylation and BMD at four years indicated the significant (FDR<0.05) differential methylation of 22 CpGs. The most significant dmCpGs included cg24384472, in the body *CSMD3* gene, cg26257545 an intergenic CpG and cg13030503 in the body of the Protein Tyrosine Phosphatase Receptor Type M (*PTPRM*) gene. The potential repercussions of differential methylation of a CpG site within the *CSMD3* gene and differential methylation of the intergenic cg26257545 will require further exploration, but *PTPRM* has been identified as influential in both the differentiation of MSCs and influential in skeletal development.

Cellular signalling is regulated by the post-translational phosphorylation and de-phosphorylation of amino acid residues such as serine, threonine and tyrosine. At tyrosine residues this process is regulated by a balance between the activity of the phosphorylating protein-tyrosine kinases and de-phosphorylating protein-tyrosine phosphatases (PTPs). PTPs are involved in a broad range of processes including proliferation, differentiation and cell-cell adhesion but have also been established as key for processes during embryonic development such gastrulation, somitogenesis, angiogenesis, stem cell self-renewal and differentiation, including the adipogenic or osteogenic differentiation of MSCs (872). Several studies have identified PTPs such as Protein Tyrosine Phosphatase Non-Receptor Type 13, Protein Tyrosine Phosphatase Receptor Type Q and leukocyte common antigen-related (LAR) tyrosine phosphatase as being important in adipogenesis (873–875). Similarly, a study by *Kim et al.* (2011) has shown the importance of receptor protein tyrosine phosphatase μ (RPTP μ) in adipogenesis, encoded by the *PTPRM* gene, in the body of which cg13030503 was seen as differentially methylated in the analyses associated with both BMC and BMD at four years of age (Figure 55) (876). The expression of RPTP μ has been seen to increase during adipogenic differentiation of both MSCs and a pre-adipocytic cell-line, the depletion of endogenous RPTP μ has been shown to inhibit adipogenic differentiation, whereas overexpression has been demonstrated to enhance adipogenesis (873–876). Curiously, despite adipogenesis and osteogenesis typically occurring in a reciprocal manner, a study by *Kim et al.* (2011) also showed no suppression of osteogenesis when RPTP μ was overexpressed in a pre-osteoblastic cell line (876). A reduction in methylation of cg13030503 in the body of the *PTPRM* gene suggests an increase in BMC or BMD could potentially correlate with the reduced expression of the *PTPRM* gene, reducing instances of adipogenic differentiation but initiation of osteogenesis

would be required by other stimuli. However, the role of gene body methylation is not entirely clear and so would require further work.

5.3.3 MAPK pathway enrichment

The differential methylation analyses for both BMC and BMD at four years of age showed significant enrichment for signal transmission and transduction processes. Whilst no specific signalling pathways were enriched for BMC at four years of age, BMD at four years of age indicated enrichment for protein serine/threonine kinase signalling activity. One of the largest families of serine/threonine kinases is the mitogen-activated protein kinases (MAPKs).

MAPKs regulate a diverse range of cellular processes including cell proliferation, differentiation, motility and survival but significantly for the purposes of this study, they also regulate the development of bone (877). Deletion of kinases upstream of the extracellular related kinase (ERK) MAPK pathway in osteoprogenitor cells results in severe osteopenia in and cleidocranial dysplasia in mouse models, similar to humans with impaired RUNX2 function (878). Additionally, MAPK has been found to induce wider epigenetic changes in osteoprogenitor cell types. A study by *Li et al.* (2017) found during the differentiation of a pre-osteoblastic cell line ERK/MAP kinase-mediated phosphorylation of RUNX2 is necessary for the chromatin remodelling required for osteogenic differentiation. Phosphorylation of RUNX2 was shown to lead to increased chromatin binding of P-ERK, P-RUNX2, p300, RNA polymerase II. Additionally, gene-activation associated marks such as H3K9 and H4K5 acetylation and H3K4 di-methylation increased and repressive marks such as H3K9 mono- di- and tri- methylation decreased (879). The methylation changes seen here may be an indication of a potential methylation-driven contribution to the upregulation of MAP-kinase which in turn favours BMD at four years old.

5.3.4 Transcription factor enrichment

Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age found enrichment for four transcription factors (Table 29). The most significantly enriched (NES=5.12) was the *FOSL1* gene which encodes the Fos-related antigen 1 (Fra-1) transcription factor. Fra-1 is a member of the Fos family of proteins, that along with the Jun proteins heterodimerise to form the dimeric transcription factor activator protein-1 (AP-1) complex (880). The AP-1 transcription factor complex has been implicated in a number of processes such as differentiation, proliferation and apoptosis (881). Components of the AP-1 complex have also been found to be differentially expressed over the course of osteogenic differentiation *in vitro*, expressed at sites of active bone formation *in vivo* and their expression induced by transforming growth factor β (TGF β), $1\alpha,25$ -dihydroxyvitamin D3 ($1,25(\text{OH})_2\text{D}$) and parathyroid hormone (PTH), all potent influences on the regulation of osteoblast proliferation and development (882). Overexpression of Fra-1 in transgenic mice by *Jochum et al.* (2000) lead to an osteosclerotic, or high bone density caused by an increase in the number of mature osteoblasts.

Parallel work *in vitro* showed enhanced osteoblast differentiation and osteoclastogenesis but no increase in proliferation after knockdown of Fra-1, suggesting the transcription specifically enhances bone formation (882). Conversely Fra-1 knockout in mice has been shown to compromise bone matrix formation and lead to osteopenia, which is characterised by a low bone mass (883). Additionally, as mentioned previously retinoic acid, the active metabolite of vitamin A is considered key regulator of adipose tissue metabolism and obesity (434–436) seen to inhibit the adipogenic differentiation of BM-MSCs through the upregulation of Fra-1 signalling, proposed to act either on C/EBP α , PPAR γ 2 or both (437). Although whether Fra-1 enhances bone formation through inhibition of adipogenesis or promotion of osteogenic processes remains to be seen. Interestingly, a study by *Rahimzadeh et al.* (2020) examined the methylation of the promoter region of *Fra-1* and other genes over the course of the osteoblastic differentiation of adipose-derived MSCs and saw no change in the hypomethylated promoter region (884). In the context of this study, if the enrichment of the Fra-1 transcription factor binding site in the dmCpGs associated with BMC at four years of age corresponds with an increase in Fra-1 activity, it is therefore unlikely to be driven by alterations in methylation state within the gene itself and more likely driven by the actions of an upstream regulator or other process. Genes enriched by the Fra-1 motif included Wingless-related integration site 11 (*Wnt11*), a factor important for MSC osteogenesis and MAF BZIP Transcription Factor (*Maf*), a key factor in mediating the switch between adipogenic or osteogenic differentiation of MSCs (appendix 9.4.5.1.2). Both of these genes would be a compelling starting point to explore the interactions between the enriched transcription factor and whether that played a role in the differentiation of MSCs.

Transcription factor motif enrichment of the dmCpGs associated with BMD at birth found enrichment for three motifs, the most significant of which was the Specificity Protein 1 (SP1) transcription factor (NES=4.42) (Table 32). SP1 belongs to the Sp/Kruppel-like family of transcription factors which have been seen to play a part in the regulation of a wide range of cellular processes including cell growth and differentiation, apoptosis, immune responses, response to DNA damage and chromatin remodelling. Moreover, SP1 transcriptional activity can be modified by post-translational modifications, including acetylation and methylation to affect its DNA binding activity, transactivation activity or protein level (885–887). The Sp1 transcription factor has been seen to play a key role in the transcriptional regulation of *PPAR α* promoter activity and increase gene expression, which has been seen to positively regulate bone growth (888–890). *Yu et al.* (2016) identified in both SaOS-2 and human foetal osteoblast (hFOB) cells the capacity for the SP1 transcription factor to enhance osteoblastic differentiation and mineralisation through transactivation of the Frizzled Class Receptor 1 (FZD1) promoter which partly mediates the activity of the Wnt signalling pathway (891). Intriguingly, one of the dmCpGs enriched for the SP1 transcription factor was collagen type XI α 2 (appendix 9.4.6.1.2). Type XI collagen is essential for skeltomorphogenesis and was identified as being regulated by SP1 activity

in SaOS-2 cells, raising the potential of a similar process occurring in the birth tissues within this study (892).

Sex determining region Y-box 30 (SOX30) was identified as the most significantly enriched (NES=4.37) transcription factor motif in the top 100 dmCpGs associated with the analysis of the relationship between umbilical cord tissue methylation and BMD at four years of age (Table 32). SOX30 is thought to play a role in early embryonic development, but its exact role or function is not yet entirely clear (893). In addition, TEA Domain Transcription Factor 1 (TEAD1) (NES=4.02-4.16) in mesodermal specification during development (894). It would not be abnormal to see enrichment for early developmental transcription factors as both MSC and haematopoietic stem cells may be a fraction of our tissue type interrogated by epigenome-wide association study (EWAS).

5.3.5 *Overlap between analyses*

5.3.5.1 *Vitamin D-adjusted models*

Alongside these analyses we aimed to assess whether receiving a 1000 IU/day vitamin D supplement during pregnancy may potentially be associated with postnatal measures of either BMC or BMD in at birth or four years of age in the neonates. We achieved this by adjusting each differential methylation analysis regression model for intervention group (Table 34). Comparison of each model to its respective intervention group adjusted model found an average overlap of 28.5% between the top 100 dmCpGs associated with each analysis. This suggests these may be a subset of CpGs whose differential methylation may also be possibly influenced vitamin D-associated pathways as a result of maternal vitamin D supplementation status. A useful marker would have been to look for the differential methylation of CpGs within the promoter region of the *RXRα* gene as seen in previous investigations, however these sites are not present on the array. Therefore, these sites could also be functionally unrelated biomarkers and so would require further investigation.

5.3.5.2 *SWS and MAVIDOS RCT studies*

Whilst not the primary aim of this study, by interrogating both the MAVIDOS RCT and SWS dataset for associations between birth tissue DNA methylation and postnatal BMC or BMD in offspring we aimed to potentially identify commonly differentially methylated CpGs across tissues. However in a comparison of the top 100 dmCpGs associated with BMC at four years of age, BMD at birth and four years of age in both the MAVIDOS RCT and SWS analyses we found only one dmCpG overlapping in the BMC at four years of age analysis. The dmCpG was located in the body of the HECT C2 and WW Domain Containing E3 Ubiquitin Protein Ligase 1 (*HECW1*) gene. Whilst this gene does not any direct link to skeletal development, it has been suggested to be involved in both Wnt and G-protein coupled receptor signalling, both crucial for the development

of bone (895–897). This could provide a base for further investigation, however it provides an indication that although both extracted from the umbilical cord, these tissues both have a unique methylome with respect to postnatal measures of either BMC or BMD.

5.3.5.3 *MAVIDOS RCT intervention group and postnatal BMC or BMD*

A comparison of the dmCpGs associated with the differential methylation analyses of umbilical cord tissue DNA with respect to BMC at four years of age, BMD at birth, intervention group and intervention group MESP D insufficient found a number of differential methylation changes within the body of the *PTPRN2* gene. *PTPRN2* encodes the insulinoma-associated protein-2 β (IA-2 β), also known as phogrin, which is a major type 1 diabetes mellitus autoantigen that also functions in secretory vesicle release (898). Both IA-2 β and its close homologue IA-2 have been implicated in the secretion of insulin, reproductive hormones and neurotransmitters, such that double knockout of these factors in mice results abnormal glucose tolerance, impaired insulin secretion and reduced insulin content in beta cells, female infertility, behavioural changes and loss of circadian rhythm (898–905). However, *PTPRN2* has also been seen to encode the miRNA miR-153 (906). In contrast to IA-2 β , the intronic miR-153 has been identified as a negative regulator of insulin and dopamine secretion. Although counter-intuitively, cells stimulated with glucose have been seen to upregulate both IA-2 β and miR-153 expression (906,907), suggesting a potential feedback loop system. However, a recent study failed to find evidence that human miR-153 has a classical TSS, but instead contains a CpG island in which demethylation of two sites increased miR-153 expression, suggesting miR-153 expression can be regulated independently of IA-2 β expression in a tissue-specific manner (908–910).

In the context of our study we have seen differential methylation at one CpG site associated with intervention and intervention MESP D insufficient analyses. We also saw differential methylation of two more CpG sites associated with analyses of BMC at four years of age and BMD at birth. This raises a number of possibilities for the potential effects of differential methylation of this gene. Vitamin D has been linked to improved glucose homeostasis and maternal vitamin D supplementation during pregnancy has been linked to a reduction in insulin resistance (830,837). Differential methylation which favours an increase in IA-2 β expression could therefore favour improved glucose homeostasis. Additionally, a study by *Cao et al.* (2015) indicated in human BM-MSCs that miRNA-153 suppresses osteogenic differentiation by targeting bone morphogenetic protein receptor type II (BMP2) (911), therefore differential methylation which suppresses the expression of this miRNA could potentially be beneficial to bone development. They may also act synergistically to enhance osteogenic differentiation, facilitate alternative splicing to generate one of the three currently three known alternative transcripts for this gene (912), or have no effect at all. Nonetheless this highlights the need to further work to understand the transcriptional and functional effects of differential methylation within this gene.

5.3.6 Future work

Previously within the SWS study we have seen perinatal differential methylation changes within the *RXRα* promoter region associated with BMC at four years of age. These markers are not on this EPIC array, so we cannot validate these findings in this cohort, although here, we found additional potential biomarkers of BMC at four years of age and BMD at birth and four years of age. However, to establish these dmCpGs as prospective biomarkers of BMC or BMD we would first need far greater replication of these findings, within umbilical cord tissue, in a number of cohorts. In addition, no associations were found with BMC at birth. This may be the summative effect of a lack of power in the study and a relatively high inter-individual variability that can occur when using cord blood in a neonatal EWAS when compared to cord tissue, diluting the potential epigenetic signatures present associated with postnatal BMC or BMD, further corroborating the need for replication of this work in cohorts of a greater size (140).

Furthermore, we have differential methylation changes in genes which may have biological, functional implications for skeletal development. Some of the differential methylation changes seen may have the capacity to enhance pro-osteogenic processes that may also repress pro-adipogenic processes and vice versa. This assumption is based upon previous work that has identified adipogenesis and osteogenesis as reciprocal processes where the suppression of osteogenic or adipogenic differentiation processes in MSCs often leads to the upregulation the opposing process (584). We have also identified common differential methylation changes between both bone-related and maternal vitamin D supplementation-related analyses in the MAVIDOS RCT. To establish the biological relevance of these differential methylation changes it would therefore be appropriate to establish in a cell type such as BM-MSCs whether i) the expression level of these genes during osteogenic differentiation and ii) if knockout of these genes using RNA interference would indeed either enhance or repress the expression of osteogenic or adipogenic genes. Additionally, whilst we cannot yet experimentally alter the methylation state of a single CpG we could potentially generate a mutant construct, omitting dmCpGs from their associated gene to assess the role of its absence on expression and therefore provide some insight into its function in context.

The analysis of the overlap between the top 100 dmCpGs associated with differential methylation analyses between BMC at four years of age and BMD and birth and four years of age found an overlap of one CpG. As mentioned previously, DNA methylation is commonly, but not always cell-type specific (458). DNA methylation can also be altered or primed by the tissue from which it was taken (913–915). Whilst common overlap between both the MAVIDOS RCT and SWS studies may have been favourable for a cohesive biological interpretation of any associations with respect to postnatal BMC or BMD, further studies in a studying a tissue type more closely biologically linked

to skeletal development such as bone marrow or BM-MSCs may allow for the potential validation of these hits. Although the utility of these dmCpGs cannot be discounted altogether.

5.3.7 Limitations

The aim of this study was to identify further differential methylation changes present in umbilical cord tissue DNA at birth which may potentially act as prospective biomarkers of bone measures in postnatal life. Indeed, we identified epigenetic marks associated with both BMC and BMD at four years of age, but also associated with BMD at birth. Whilst the marks at four years of age may be useful to suggest the potential growth trajectory of a neonate, the clinical utility of biomarkers at birth would not. This suggests the dmCpGs associated with BMD at birth will need to be investigated for its biology, but this may be difficult to discern the functional implications of these changes without further work. Umbilical cord tissue is a heterogeneous mixture of cell types, therefore the EPIC array will have interrogated the methylation state of a mixture of DNA from a number of cell types. With respect to a biomarker this isn't of concern if the changes are reproducible across a number of samples in multiple cohorts. However with respect to understanding the functional implications of this change, further functional work would be required to establish whether this methylation change is occurring in UC-MSCs, to establish functional implication of differential methylation changes in the progenitor of osteoblast, BM-MSCs and eventually to reproduce in an *in vivo* model.

Whilst differential methylation changes were seen in umbilical cord tissue associated with BMC at four years of age and BMD at birth and four years of age, no epigenetic signatures were seen associated with BMC at birth. This lack of signal may be accurate and associations may simply not exist at that age, however it may also reflect of a lack of power in the study and a reported high inter-individual variability in umbilical cord tissue DNA methylation when compared to the DNA of tissues such as umbilical cord blood (361). When comparing the methylation of DNA extracted from the umbilical cord tissue and umbilical cord blood of 295 neonates, *Lin et al.* (2017) found that the highest inter-individual variability in methylation was seen in umbilical cord tissue, a finding further corroborated by a follow-up study where the methylome of both tissue types was assessed when fractioned by cell type (458,649). This means an epigenetic signature present for BMC at birth may have been weakened by inter-individual differences in methylation inherent in the tissue, however this also may be an indication umbilical cord tissue DNA simply presents a more accurate reflection of the biological differences between individuals that occurs as a result of environmental epigenetic influences *in-utero* that may not be captured in umbilical cord blood DNA. However, this issue is ameliorated somewhat by adjustment for cell-type composition in our regression models, which reduces inter-individual differences in methylation introduced by changes in the proportion of cell-type between each umbilical cord tissue or umbilical cord blood

sample. But ultimately replication in further, larger cohorts would be required to establish any of these factors as a reason for the lack of any epigenetic signature seen for BMC at birth.

Finally, the practical clinical utility of a biomarker derived from umbilical cord tissue may be arguably more labour-intensive process when compared to umbilical cord blood. However which is ultimately more useful would depend on the fidelity of the prediction as a biomarker.

5.3.8 Conclusions

Similar to previous work conducted in the SWS study we have found differential methylation changes within a region of the same gene associated with BMC at four years of age, BMD at birth and induced by maternal vitamin D supplementation in the intervention and intervention MESP D insufficient analyses. This presents an opportunity to further this work by assessing the transcriptional effects of differential methylation on this gene and any potential linkage between maternal vitamin D supplementation and skeletal development. Within this study we have also shown differential methylation changes in umbilical cord tissue associated with BMC at four years of age and BMD at both birth and four years of age. If replicable, the markers associated bone measures at four years of age could act as an indication for the possible trajectory of bone growth early in life. However, the utility of the differential methylation change associated with BMD at birth may only be its biological implications. However interpreting the biological implications of these differential methylation changes would need to be done in the context of further functional validation within *in vitro* models of UC-MSCs or cell-type precursor to osteoblasts, BM-MSCs. Despite this, the epigenetic signatures produced from each analysis which have indicated dmCpGs that may have either potential implications for pro-adipogenic or pro-osteogenic development. Furthermore, in the differential methylation of *CD82* we saw a potential biomarker which associated with BMC alone at four years of age in line with the repression of this gene as seen in the literature. Within the study we also saw differential methylation of *CTBP2* associated with BMD at birth and Fra-1 transcription factor enrichment associated BMC at four years of age, both suggested to be part of a mechanistic pathway for the action of vitamin A in MSCs. Vitamin A has been seen to potentially work with vitamin D to influence BMD (814), suggesting this interplay may be worth investigating in the future.

6 Chapter 6 - Methylation changes *in vitro* upon treatment of umbilical cord mesenchymal stromal cells with vitamin D

6.1 Introduction

In previous chapters we have identified DNA methylation changes at birth in both umbilical cord blood and tissue associated with bone outcomes in later childhood (section 3.2, section 5.2). Furthermore, we have seen changes in umbilical cord tissue after maternal supplementation with cholecalciferol (section 4.2). However, the functional significance of these changes is unknown. DNA methylation is a largely tissue-specific (693) so whether methylation changes in either umbilical cord blood or cord tissue reflect similar methylation changes in MSCs is unknown. However, as the largest proportion of umbilical cord tissue the Wharton's Jelly has been identified as a comparatively rich source of mesenchymal stromal cells (MSCs) (431,916–918), so methylation changes in umbilical cord tissue may indeed reflect the epigenetic changes seen in MSCs with potential functional consequences for MSC function and differentiation potential. Alternatively, the DNA methylation changes observed in umbilical cord tissue and blood may reflect a bystander effect of changes during development or vitamin D supplementation. In order to gain further insights into how environmental or dietary factors may impact DNA methylation, here, the effect of vitamin D treatment on the DNA methylation of MSCs *in vitro* was assessed together with the effect of long term vitamin D supplementation on MSC cells from umbilical cord tissue from infants born to mothers in the Southampton Pregnancy Intervention for the Next Generation (SPRING) randomised controlled trial (RCT) (626).

6.1.1 Aims and hypothesis

The hypothesis of this work was that the differential methylation changes seen in umbilical cord tissue DNA as a result of long-term maternal cholecalciferol supplementation over the course of pregnancy in the Maternal Vitamin D Osteoporosis Study (MAVIDOS) randomised controlled trial (RCT) were driven by the mesenchymal stromal fraction of cells within the Wharton's jelly of the umbilical cord tissue.

To address the hypothesis and whether the methylation changes are unique to long-term supplementation or can recapitulated *in vitro* with short-term vitamin D supplementation the aims of this chapter are to:

- i) Utilise non-study primary umbilical cord mesenchymal stromal cells obtained from American Type Culture Collections (ATCC UC-MSCs) to assess the effect of short-term 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D) treatment on the methylation and differentiation potential of the cells *in vitro*.

- ii) Isolate and enrich UC-MSCs from umbilical cord tissue samples from the SPRING RCT to determine whether long-term supplementation of UC-MSCs alters the DNA methylation and additionally the differentiation potential of UC-MSCs *in vitro*.

6.2 Results

6.2.1 Effect of short-term vitamin D treatment on ATCC UC-MSCs

To determine how short-term $1,25(\text{OH})_2\text{D}$ treatment of UC-MSCs may affect the methylome of the offspring, we aimed to treat the UC-MSCs with $1,25(\text{OH})_2\text{D}$ for 24 hours and assess the effect on the methylome using the Illumina Infinium MethylationEPIC BeadChip (EPIC) array to interrogate DNA extracted from the UC-MSCs. However, firstly it was necessary to establish a reproducible method to indicate the activation of vitamin D-responsive pathways for any further *in vitro* experimentation.

6.2.1.1 What concentration of $1,25(\text{OH})_2\text{D}$ treatment is appropriate to activate vitamin D responsive pathways in ATCC UC-MSCs?

The typical human physiological concentration of $1,25(\text{OH})_2\text{D}$ is 50-150pM, which is dramatically different from the concentration conventionally used to induce responses *in vitro* where 10nM and above are routinely used (919). Therefore, to identify a suitable concentration of $1,25(\text{OH})_2\text{D}$ with which to treat UC-MSCs *in vitro*, we first generated a vector construct in which we fused the DNA sequence of a well-characterised vitamin D responsive element (VDRE), the genomic target site typically found in the promoter region of $1,25(\text{OH})_2\text{D}$ -regulated genes. UC-MSCs were treated at a range of vitamin D concentrations and the effect on i) VDRE-reporter constructs and ii) the expression of vitamin D responsive genes assessed.

6.2.1.1.1 Generating the VDRE-reporter gene constructs

VDRE sequences characterised from both the osteocalcin (OC) and CYP24A1 (CYP) genes were used to generate VDRE-reporter gene constructs. Both osteocalcin (OC) and CYP24A1 (CYP) play integral roles in osteoblast formation and function. CYP24A1 acts to regulate $1,25(\text{OH})_2\text{D}$ activity through the 25-hydroxylation to form the inactive $1,24,25(\text{OH})_3\text{D}$ (117–122) while OC is a protein with a high affinity for calcium and hydroxyapatite expressed by osteoblasts during differentiation to facilitate the mineralisation of bone.

OC was selected to generate a VDRE-reporter gene construct due to containing one of the most comprehensively and consistently characterised active VDRE sites (673,920–923), the inclusion of which would therefore increase the likelihood of generating a functional, vitamin D inducible construct. CYP24A1 was selected for its direct and reciprocal relationship with $1,25(\text{OH})_2\text{D}$ as the primary enzyme which mediates its degradation, suggesting a dose dependant and potentially rapid response to $1,25(\text{OH})_2\text{D}$ treatment *in vitro*. In addition, the physiologically active metabolite of vitamin D, $1,25(\text{OH})_2\text{D}$ has been reported in many studies to robustly induce the expression of both CYP24A1 and OC through the formation of a $1,25(\text{OH})_2\text{D}$ -vitamin D receptor (VDR)-retinoid x receptor (RXR) complex, which subsequently binds to VDRE sequences present in the promoters of these genes (924,925).

A VDRE sequence from vitamin D responsive genes such as *CYP24A1* or *OC* could therefore be used to generate a VDRE-responsive reporter gene construct to test a suitable concentration of vitamin D to use for subsequent experiments. 1,25(OH)₂D treatment of cells transfected with this construct may then provide a suitable system to identify 1,25(OH)₂D mediated activation of vitamin D responsive pathways.

To construct a VDRE-responsive reporter gene construct, oligonucleotides containing 3x identical VDREs in series from the *OC* and *CYP* genes, known vitamin D responsive genes, were fused upstream of a minimal promoter within the luciferase reporter gene vector pGL4.23. After cloning and restriction mapping all potential positive clones containing 3x VDREs of either *OC* or *CYP* (Figure 14) were sent to GATC Biotech for sequencing. Sequencing of putative positive clones with the pGL3-RV3 primer produced a sequence that matched the proposed template sequence of both the pGL4.23-*OC* VDRE (pGL4-*OC*) and pGL4.23-*CYP* VDRE (pGL4-*CYP*) vector constructs (full sequencing results in appendix 9.8).

6.2.1.1.2 *Vitamin D elicits a luminescence response in SaOS-2 cells, but not ATCC UC-MSC cells when transfected with a VDRE reporter construct*

To determine the concentration of 1,25(OH)₂D necessary for the induction of the vitamin D pathway, SaOS-2 cells and UC-MSCs were transfected with the empty control plasmid pGL4 and the pGL4-*OC*, pGL4-*CYP* vector constructs. An internal control *Renilla* luciferase plasmid was also transfected alongside to allow normalisation of data which may be caused by differences in cell culture viability or transfection efficiency.

Initially the vector-VDRE constructs were transfected into SaOS-2 cells, a robust, mature osteosarcoma cell line. These cells were selected to establish proof-of-principle for the vector-VDRE constructs due to evidence of 1,25(OH)₂D-induced increases in both *OC* and *CYP24A1* expression within these cells *in vitro* (926–928), findings mirrored by experimental work within this chapter for both *OC* (Figure 58a) and *CYP24A1* (Figure 59a) respectively. This establishes not only that these cells express the machinery necessary for vitamin D signalling but may also have the capacity to induce the expression of our vector-VDRE constructs.

The SaOS-2 was transfected with pGL4-*OC*, pGL4-*CYP* and pGL4 constructs and treated with 1,25(OH)₂D (10nm) or vehicle for 24 hours (Figure 57). Vehicle treatment of SaOS-2 cells transfected with either pGL4-*OC* (Figure 57a) or pGL4-*CYP* (Figure 57b) VDRE-containing vectors elicited no significant change in luminescence when compared to SaOS-2 cells transfected with a non-VDRE containing control vector (pGL4). However, when treated with 1,25(OH)₂D (10nm), there was a significant increase in the expression of pGL4-*OC* ($p < 0.0001$) when compared to vehicle-treated, pGL4-*OC* transfected SaOS-2 cells (Figure 57a). Interestingly the treatment of pGL4-*CYP* transfected SaOS-2 cells with 1,25(OH)₂D did not lead to a significant difference in the level of pGL4-*CYP* activity compared to the vehicle-treated plasmid.

Primary ATCC UC-MSCs were also transfected with the pGL4-OC, pGL4-CYP and control vectors in the absence or presence of $1,25(\text{OH})_2\text{D}$ (10nM) (Figure 57). These are cells extracted from the birth tissues of a 'normal pregnancy' and comprehensively characterised for both cell surface marker expression and tri-lineage differentiation potential (appendix 9.10). Whilst the parameters for the definition of an MSC culture remains in contention (459,462–464,929), these cells may provide a reference control for what may be considered the typical state of the methylome, transcriptome and the phenotypic characteristics of UC-MSCs from a 'normal pregnancy' against which we can compare UC-MSC cells isolated and enriched within this chapter. There was no significant difference in the level of expression of OC or CYP when compared to the control pGL4 vector in the absence of $1,25(\text{OH})_2\text{D}$. The addition of $1,25(\text{OH})_2\text{D}$ (10nM) also did not affect the activity of either the pGL4-OC or pGL4-CYP plasmids.

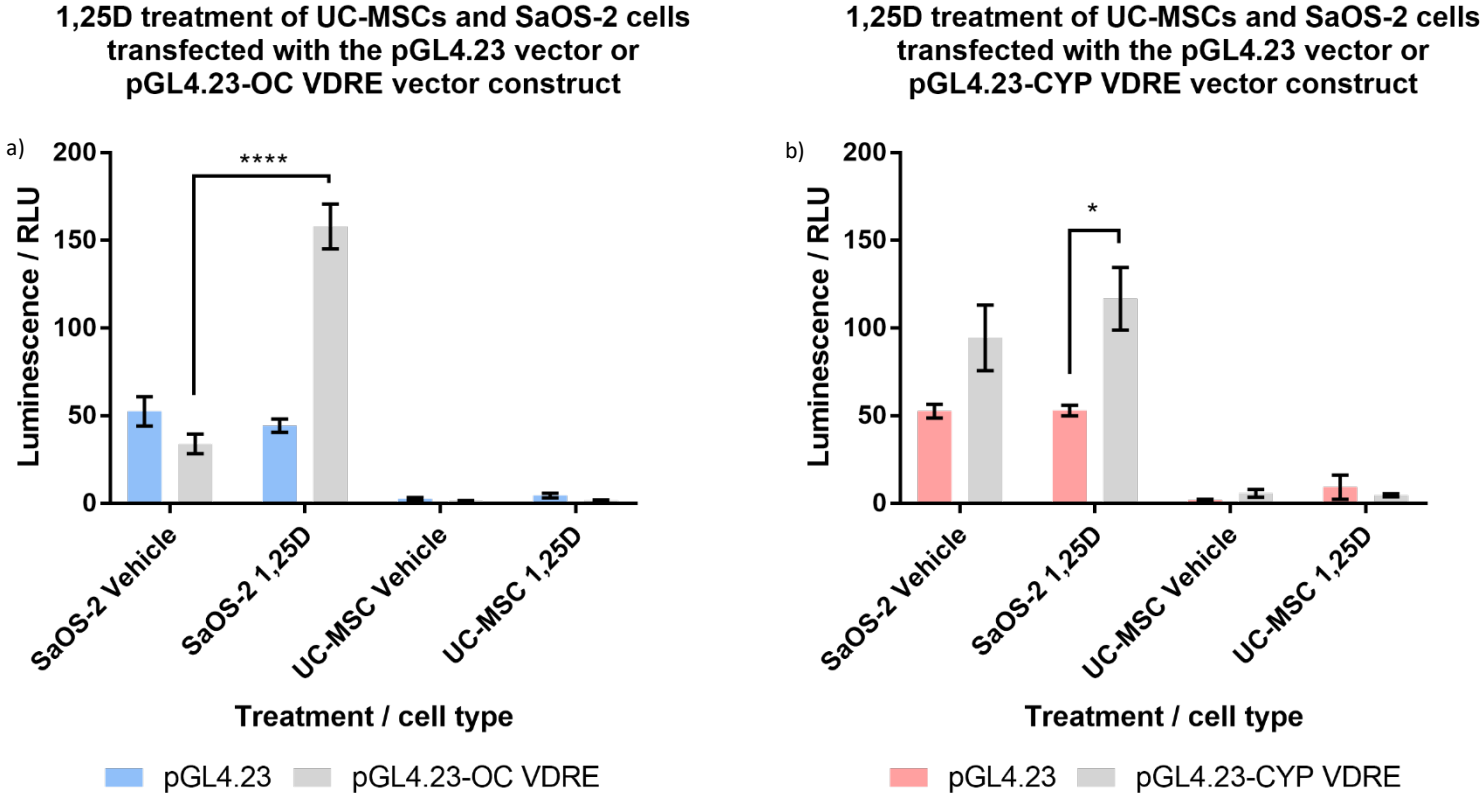


Figure 57. The normalised luminescence response from pGL4.23 plasmid with an a) osteocalcin promoter-derived or b) CYP24A1 promoter-derived VDRE insert when transfected into either SaOS-2 cells or ATCC UC-MSC and treated with 1,25(OH)₂D

The bar charts show the relative, normalised luminescence expressed by a pGL4.23 vector construct containing an insert of three vitamin D responsive elements characterised from the a) promoter of the osteocalcin gene or b) the CYP24A1 gene promoter post-10nm 1,25(OH)₂D treatment when transfected into SaOS-2 cells and ATCC UC-MSCs. Cells were treated with 10nm 1,25(OH)₂D or a vehicle control for 24 hours. Three-way ANOVA was used to assess significance of type and time of treatment and the presence of a plasmid with VDRE insert. Results shown as mean ±SEM *p<0.05, ****p<0.0001.

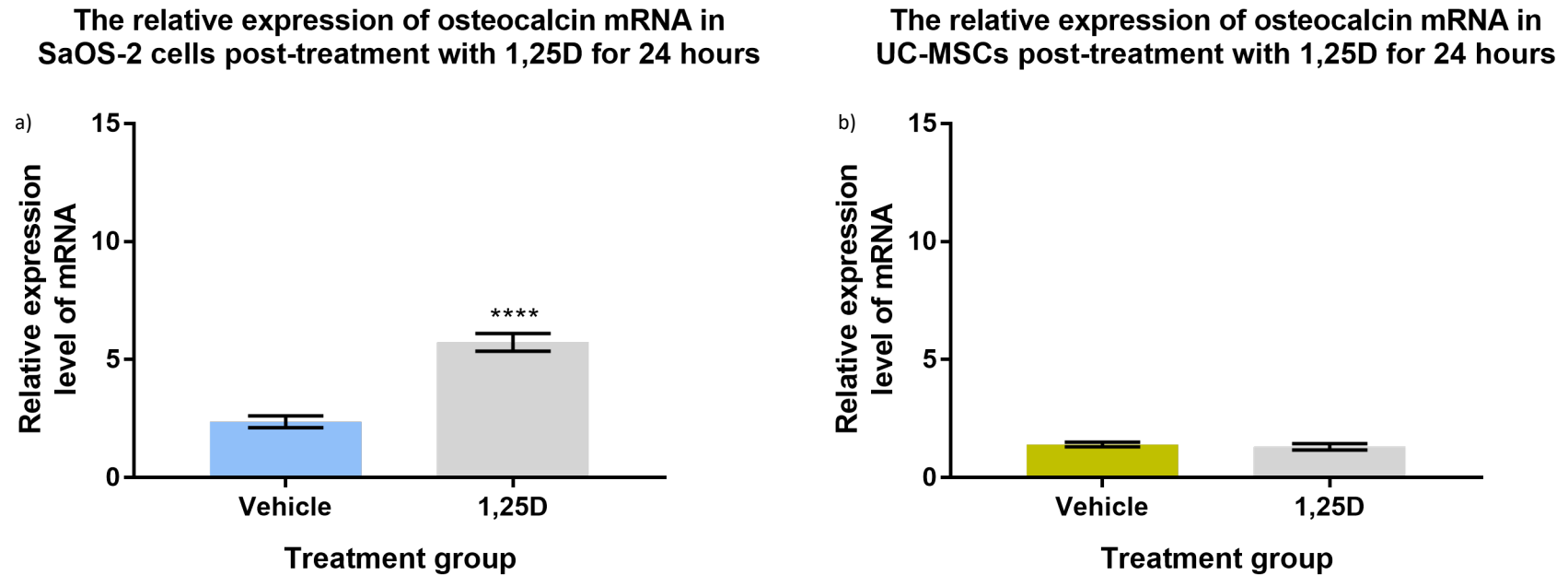


Figure 58. The relative expression of osteocalcin in a) SaOS-2 cells and b) ATCC UC-MSCs post treatment with 1,25(OH)₂D for 24 hours

Bar charts show the relative fold-change in expression of osteocalcin mRNA in ATCC UC-MSCs in response to treatment with 1,25(OH)₂D for 24 hours. Significance was assessed using an unpaired, two-tailed t-test with Welch's correction. Results shown as mean $\pm$ SEM ****p<0.0001.

6.2.1.1.3 Effect of 1,25(OH)₂D treatment on the expression of vitamin D responsive genes in ATCC UC-MSCs

As there was no induction of the OC or CYP VDRE reporter constructs with 1,25(OH)₂D 10nM in UC-MSCs, we examined the effect of 1,25(OH)₂D on the endogenous expression of OC and CYP24A1 transcripts in SaOS-2 cells and UC-MSCs with 1,25(OH)₂D. To examine this the expression of CYP24A1 and OC mRNA were assessed post-1,25(OH)₂D treatment at a range of concentrations in UC-MSC and SaOS-2 cells in which these genes are expressed.

The treatment of SaOS-2 cells for 24 hours with 1,25(OH)₂D (100nm) elicited a significant increase in CYP24A1 mRNA expression ($p<0.0001$) (Figure 59a) and OC mRNA expression ($p<0.0001$) (Figure 58a). UC-MSCs cells were then treated with a range of 1,25(OH)₂D concentrations and the effect on OC and CYP24A1 expression examined. The treatment of UC-MSCs with 1,25(OH)₂D for 24 hours elicited a significant increase in CYP24A1 expression with 1,25(OH)₂D media concentrations of 10nm ($p<0.001$), 50nm ($p<0.0001$) and 100nm ($p<0.001$) (Figure 59b). However, there was no effect at these 1,25(OH)₂D treatment concentrations on the expression of OC in UC-MSC cells (Figure 58b).

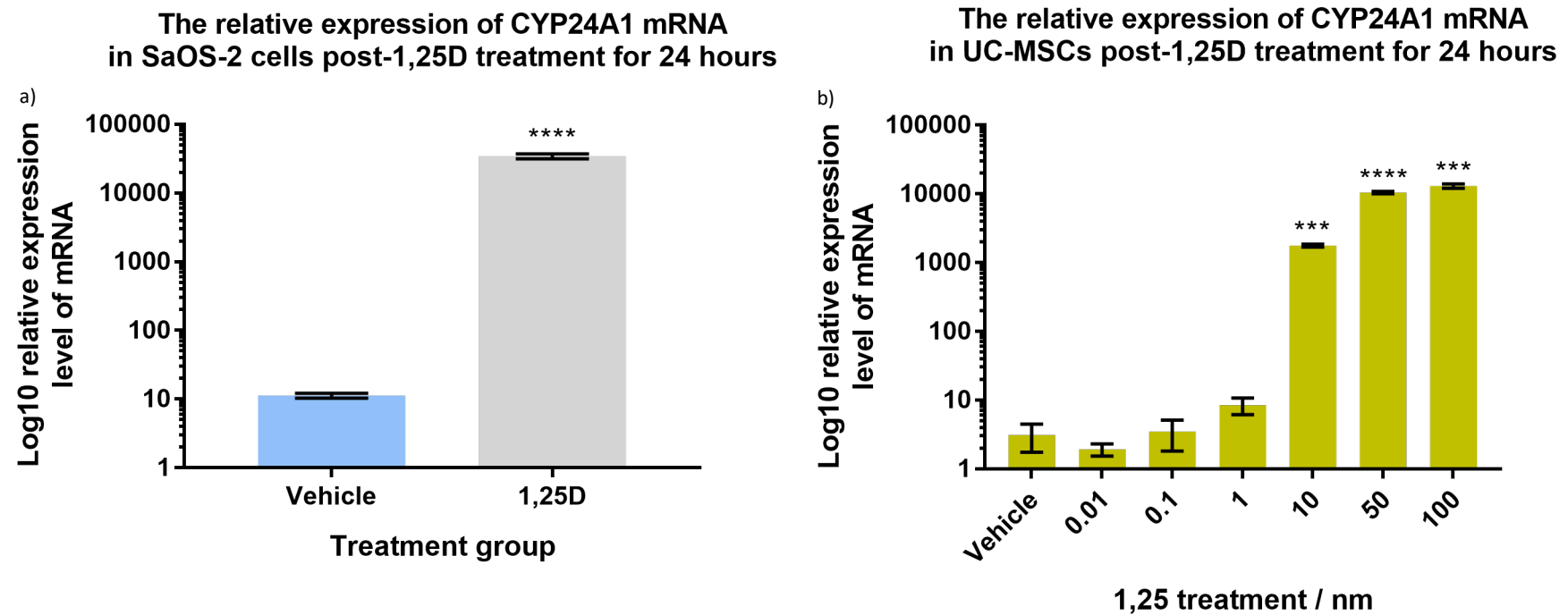


Figure 59. The relative expression of CYP24A1 mRNA in a) SaOS-2 cells and b) ATCC UC-MSCs in response to 24h treatment with 1,25(OH)₂D

Bar charts to show the relative expression level of CYP24A1 mRNA in a) SaOS-2 cells and b) ATCC UC-MSCs in response to treatment with a) 100nm 1,25(OH)₂D and b) a range of 1,25(OH)₂D concentrations for 24 hours. Significance was assessed using a two-tailed unpaired t-test with Welch's correction with all analysis relative to vehicle. Results shown as mean $\pm$ SEM

p<0.001, *p<0.0001.

6.2.1.2 *Effect of short-term vitamin D treatment on ATCC UC-MSCs on DNA methylation in vitro*

6.2.1.2.1 *Pre-processing EPIC array samples*

Vitamin D has been reported to influence DNA methylation on both directly or indirectly (629,745). To determine whether short-term 1,25(OH)₂D treatment can alter DNA methylation patterns, UC-MSC cells were treated with 1,25(OH)₂D (10nm), the lowest concentration of 1,25(OH)₂D to show a robust induction of CYP24A1 mRNA expression after 24 hours of treatment (Figure 59b). DNA was extracted from these cells (n=12) as in section 2.3.3. Tabulated quantitative nanodrop data from extracted DNA (appendix 9.5.2) and gel electrophoresis (Figure 60) demonstrated the quality of the samples was such that 1.5µg of sample was sufficient for EPIC 850K array analysis which allows for the interrogation of 850,000 methylation sites across the genome. Beadchip plans for EPIC array interrogation of the DNA were generated to include both inter-chip and intra-chip 0% and 100% methylation controls and a randomised distribution of samples from both treatment groups across the chips (appendix 9.6). Samples were dispatched for array analysis as described in section 2.3.2.2.

6.2.1.2.2 *EPIC array quality control (QC)*

6.2.1.2.2.1 Beta values QC

Prior to differential methylation analysis, beta values generated for each sample were normalised as in section 3.2.2.1 and a series of QC steps undertaken (Figure 13).

Post-normalisation, beta values were used to generate a principal component analysis (PCA) plot to indicate potential outliers or abnormal clustering of samples based on either beadchip, position of the sample on the beadchip, treatment group (vehicle or 10 nm 1,25(OH)₂D) and a singular value decomposition (SVD) plot to indicate the contribution of each variable to variance in the dataset. The PCA plot indicated samples were splitting based on the first principal component indicating a potential beadchip or batch effect, a finding corroborated by the accompanying SVD plot (Figure 61). The PCA plot also identified two potential outliers which was confirmed by MADscore calculation, indicating both samples had a score of <-5. Both samples were excluded from further analysis leaving 10 samples in the dataset. Additionally, due to indications of a batch effect, beta values were batch-corrected using the ComBat function of the surrogate variable analysis (SVA) package (v3.20.0).

6.2.1.2.2.2 Probes QC

Probes were excluded from further analysis based on detection value and bead count criteria as described in section 3.2.2.2. Further probes were removed based on their single nucleotide polymorphism (SNP) content, ability to cross-hybridise, non-CpG relatedness or location on sex chromosomes as identified in Zhou W *et al.* (2017), Pidsley *et al.* (2016) and Nordlund *et al.* (2013) (645–647) (Table 35).

6.2.1.2.2.3 Establishing model covariates and model QC

As a small-scale cell culture-based project for which QC has indicated no apparent technical sources of influence such as beadchip or position effects on the methylation state of the cellular DNA (Figure 62), the regression model required for differential methylation analysis only included the outcome (methylation) and the predictor (treatment group). The quality of the differential methylation analysis model was assessed by the inflation factor (λ), a quantile-quantile plot (qqplot) and a p -value histogram. Any inflation or bias ($\lambda > 1.2$) was adjusted for using the bacon package (v.1.12.0) and newly generated p -values were adjusted for multiple testing (650). A model with a qqplot exhibiting a largely normal distribution and a p -value histogram with a sloping curve was taken forward for further analysis.

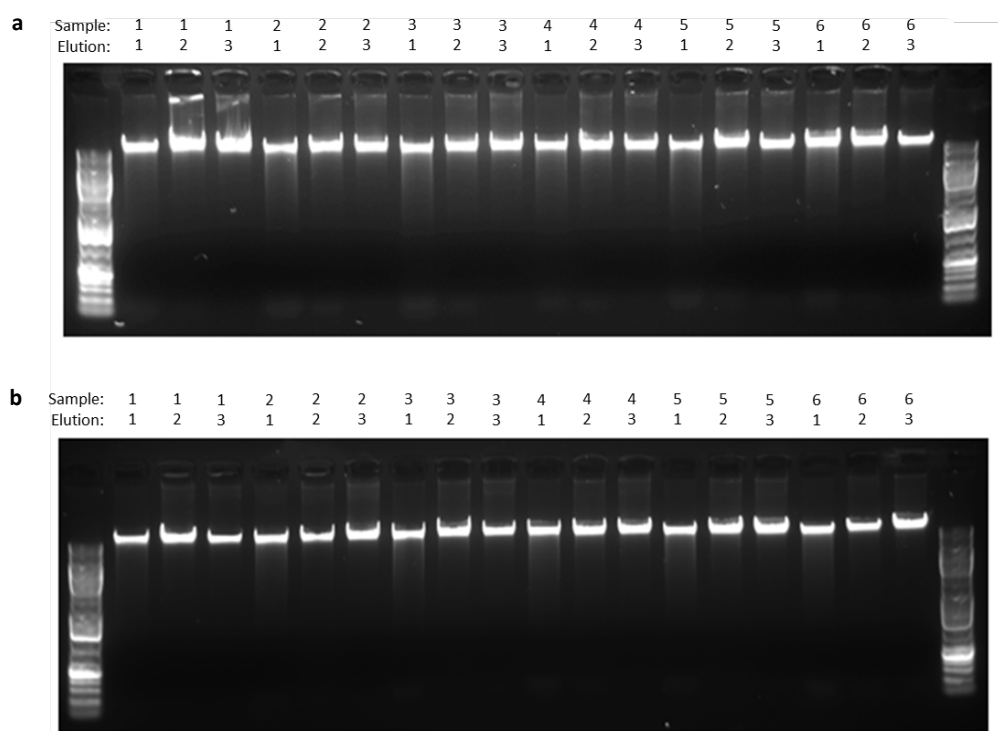


Figure 60. Agarose electrophoresis gels of DNA extracted from ATCC UC-MSC cells (n=12) post-treatment *in vitro* with either a) vehicle (n=6) or b) 1,25(OH)₂D (n=6) for 24 hours for the purpose of EPIC array interrogation
 ATCC UC-MSC cells were passaged to produce 12 cultures which were treated *in vitro* with either a) vehicle (n=6) or b) 1,25(OH)₂D (n=6) at 50nm for 24 hours, trypsinised and pelleted as in section 2.5.2, excess media removed and DNA extracted using Sigma GeneElute™ Mammalian Genomic DNA Miniprep Kit (section 2.3.3.1). DNA was extracted in three elutions, quantified using a nanodrop spectrophotometer and 100 ng assessed using electrophoresis as described in section 2.3.4.2.

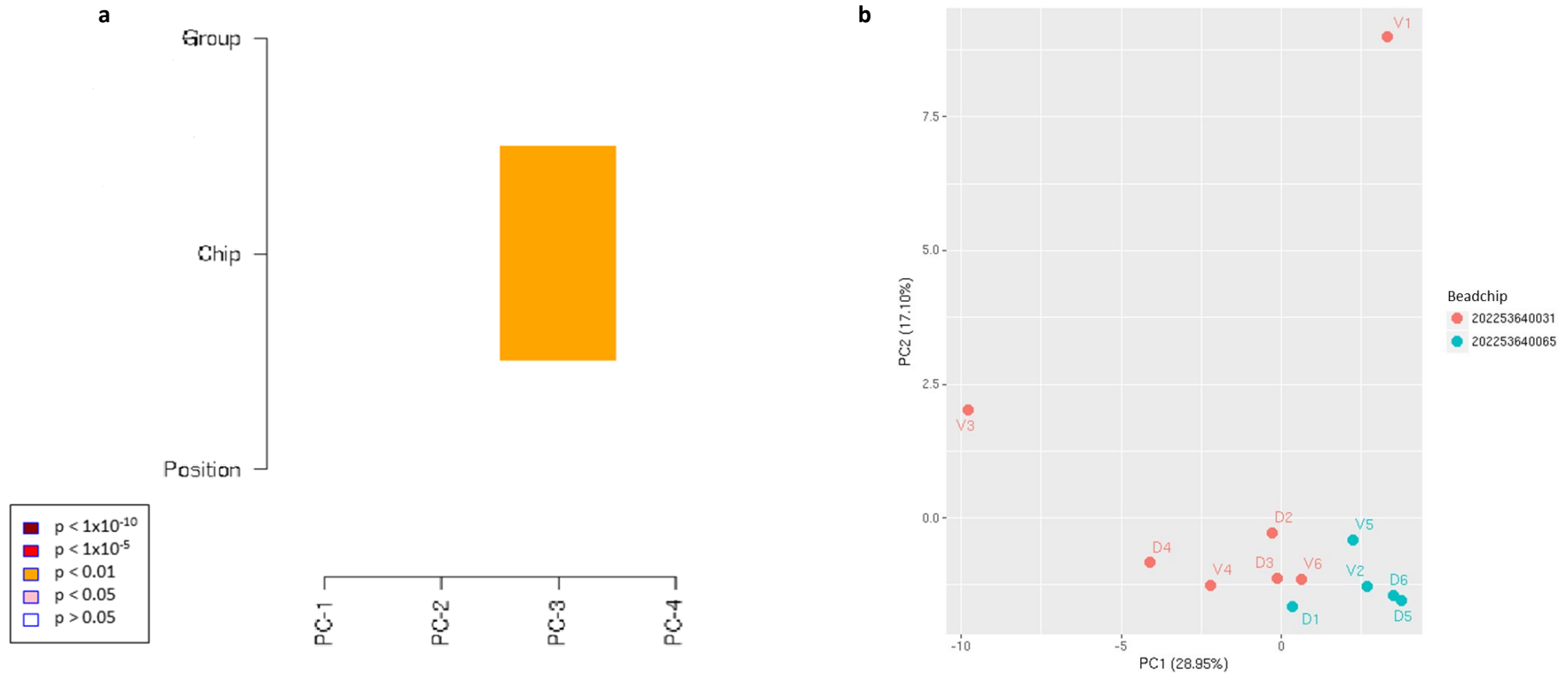


Figure 61. A a) singular value decomposition plot and b) principal component plot for the post-normalisation methylation data generated from the EPIC array interrogation of ATCC UC-MSC DNA treated short-term with 1,25(OH)₂D

The EPIC array interrogation of DNA extracted from ATCC UC-MSCs treated short-term with 1,25(OH)₂D generated methylation data for each sample which post-QC was subjected to a singular value decomposition (SVD) analysis. SVD analysis allowed for the assessment of each phenotypic characteristic as a potentially significant source of variance in the methylation dataset. A a) post normalisation SVD plot beta values associated with UC-MSCs treated with vehicle or 1,25(OH)₂D for 24 hours indicated a potential beadchip effect and b) a plot of the first two principal components indicated a split on the 1st principal component indicating a potential beadchip effect and two potential sample outliers.

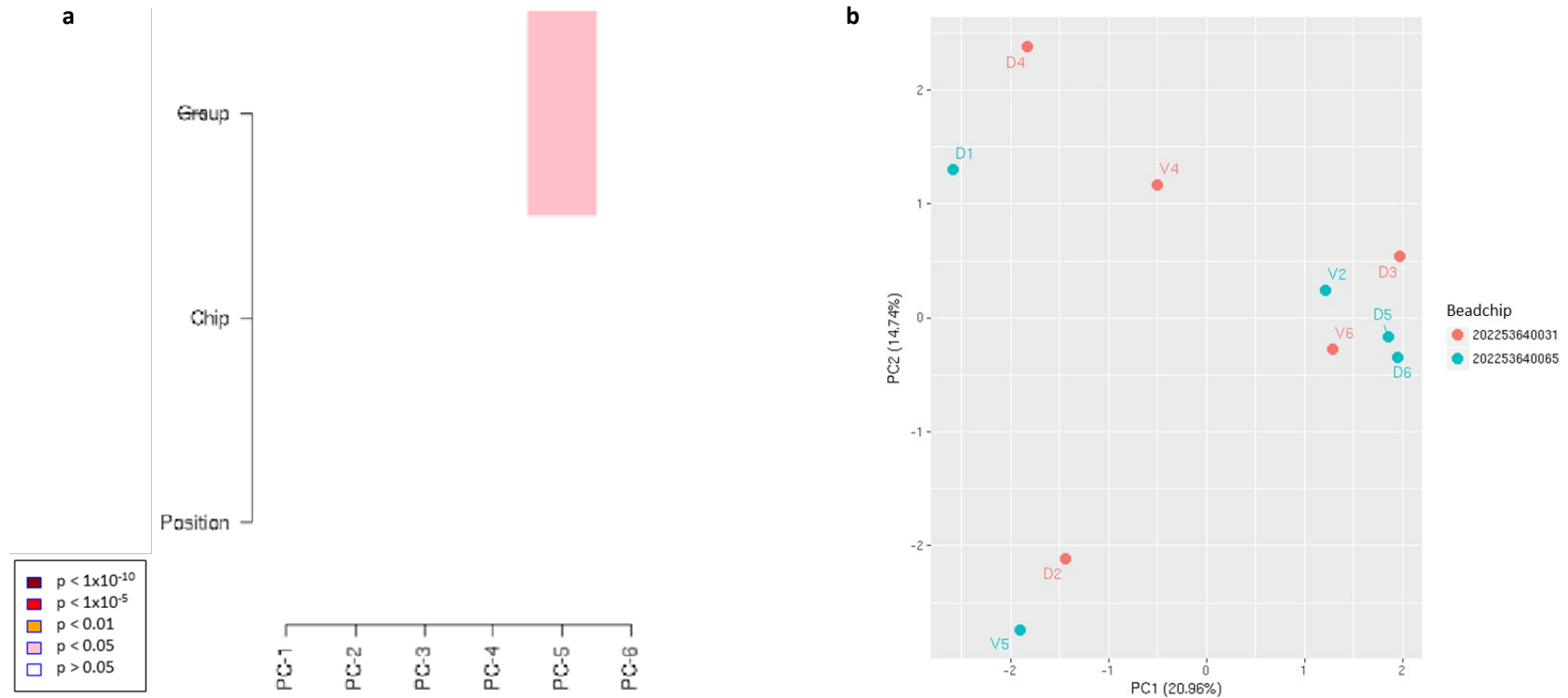


Figure 62. A post-normalisation, batch corrected and outlier sample removal a) singular value decomposition plot and b) principal component plot for the post-normalisation methylation data generated from the EPIC array interrogation of ATCC UC-MSC DNA treated short-term with 1,25(OH)2D

The EPIC array interrogation of DNA extracted from ATCC UC-MSCs treated short-term with 1,25(OH)2D generated methylation data for each sample which post-QC was subjected to a singular value decomposition (SVD) analysis. SVD analysis allowed for the assessment of each phenotypic characteristic as a potentially significant source of variance in the methylation dataset. A post normalisation a) SVD plot indicating variance introduced by treatment group and b) PC plot indicating no significant beadchip effect or potential outliers.

Table 35. The number of probes excluded from further analysis of treatment group during probe QC based on exclusion criteria described in section 3.2.2.2

Exclusion criteria	Number of probes excluded
Detection value >0.01	625
Bead count <3 in 5% of samples	12117
SNP probes	93049
Cross-hybridising probes	43302
Non-CpG probes	2893
Sex chromosome	17512
Probes remaining post-QC	737729
Post removal of probes with range < 5%	275826

6.2.1.2.3 *Differentially methylated CpGs*

A differential methylation analysis was carried out to identify the effect of short-term 10nm 1,25(OH)₂D on DNA methylation in UC-MSCs. Interrogating the association between the DNA methylation of UC-MSCs treated with either a vehicle or 10nm 1,25(OH)₂D for 24 hours found 78 differentially methylated cytosine-phosphate-guanine dinucleotides (dmCpGs) with a false discovery rate (FDR)<0.05 (Table 36). The two most significant dmCpGs were cg11347955 (FDR= 2.56e⁻⁵) and cg17523024 (FDR=1.30e⁻⁴), both intergenic cytosine-phosphate-guanine dinucleotides (CpGs) which showed a decrease in methylation within the vitamin D treated group. The most significant dmCpG associated with a gene was cg21185075, located in the three prime untranslated region (3'UTR) of the myosin light chain kinase 3 (*MYLK3*) gene (FDR= 1.63e⁻⁴), which showed an increase in methylation within the vitamin D treated group (Figure 63). *MYLK3* is a cardiac-specific kinase which promotes sarcomere formation in cardiomyocytes and increases cardiomyocyte contractility (930).

6.2.1.2.4 *Genomic locations of dmCpGs*

The location of the top 100 dmCpGs associated with the vitamin D treated UC-MSCs was ascertained relative to both CpG islands and genomic structure. The dmCpGs were most often found in open sea regions (78% of associated dmCpGs), 21% were located within CpG shores or shelves, whereas only 1% were identified within a CpG island (Figure 64a). Relative to genomic structure 55% were found within a gene body, 26% up to 1500 bases upstream of a transcriptional start site (TSS), 16% in a five prime untranslated region (5'UTR) or exon boundary and 3% in a 3'UTR (Figure 64b).

6.2.1.2.5 *Pathway enrichment analysis*

6.2.1.2.5.1 *Cytoscape pathway analysis*

Initial clustering of the top 100 dmCpGs associated with the DNA methylation of UC-MSCs treated with vehicle or 10nm 1,25(OH)₂D for 24 hours found three distinct clusters of genes (Table 37). gene ontology (GO) term enrichment analysis of the cluster indicated enrichment for a number of pathways, including most significantly the generation of precursor metabolites and energy (GO:6091, FDR=7.65e⁻⁴), energy derivation by oxidation of organic compounds (GO:15980, FDR=1.19e⁻²) and glucose metabolic processes (Figure 65).

6.2.1.2.6 *Overlap with previous vitamin D-related analyses*

An assessment of common differential methylation changes occurring in the MAVIDOS RCT intervention (section 4.2.4), Southampton Women's Survey (SWS) maternal late-stage pregnancy vitamin D (MLSP D) (section 4.2.5) and the short-term 1,25(OH)₂D treatment of ATCC UC-MSCs analyses was made by comparing the top 100 dmCpGs from each analysis when ranked by

significance. A comparison between analyses found no common differential methylation changes occurring in the top 100 dmCpGs from each analysis.

Table 36. The results of differential methylation analysis interrogating the association between ATCC UC-MSC DNA methylation of treatment with either a vehicle or 1,25(OH)₂D for 24 hours
The differential methylation analysis investigating the association between ATCC UC-MSC DNA methylation and treatment with either a vehicle or 1,25(OH)₂D for 24 hours found 78 significant dmCpGs (n=10). Some under-inflation was observed $\lambda=0.748$. DmCpGs associated with a known gene are represented by their respective associated gene symbol.

Model	Sample number	Lambda	FDR					Top 10 dmCpGs (FDR <0.25)
			<0.25	<0.20	<0.15	<0.10	<0.05	
Treatment group	10	0.748	291	225	190	133	78	cg11347955, cg17523024, MYLK3, XXYLT1, cg24119516, cg08821715, cg11974480, cg23087546, FER, cg04772776

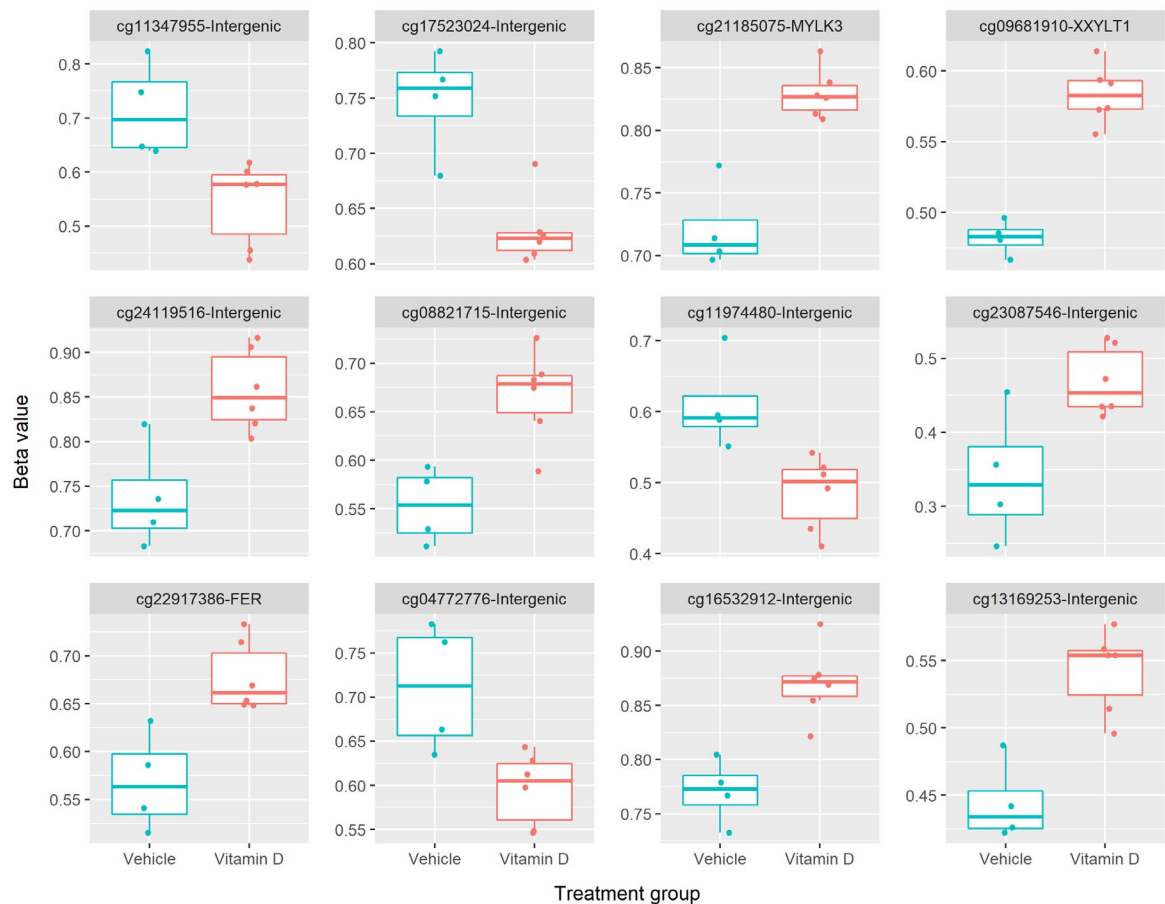


Figure 63. The association between methylation and treatment group for 12 of the 78 dmCpGs significantly (FDR <0.05) associated with treatment group for ATCC UC-MSC cells treated with either a vehicle or 1,25(OH)₂D for 24 hours

The differential methylation analysis assessing the association between the methylation of 10 samples of ATCC UC-MSC DNA and treatment group found significant (FDR <0.05) differential methylation of 78 CpGs, 12 of which are pictured. Of the 12 pictured, nine were mapped to an intergenic region and 3 were mapped to known genes. The methylation of four dmCpGs was lower in the 1,25(OH)₂D-treated group whereas in 8 dmCpGs the methylation was higher.

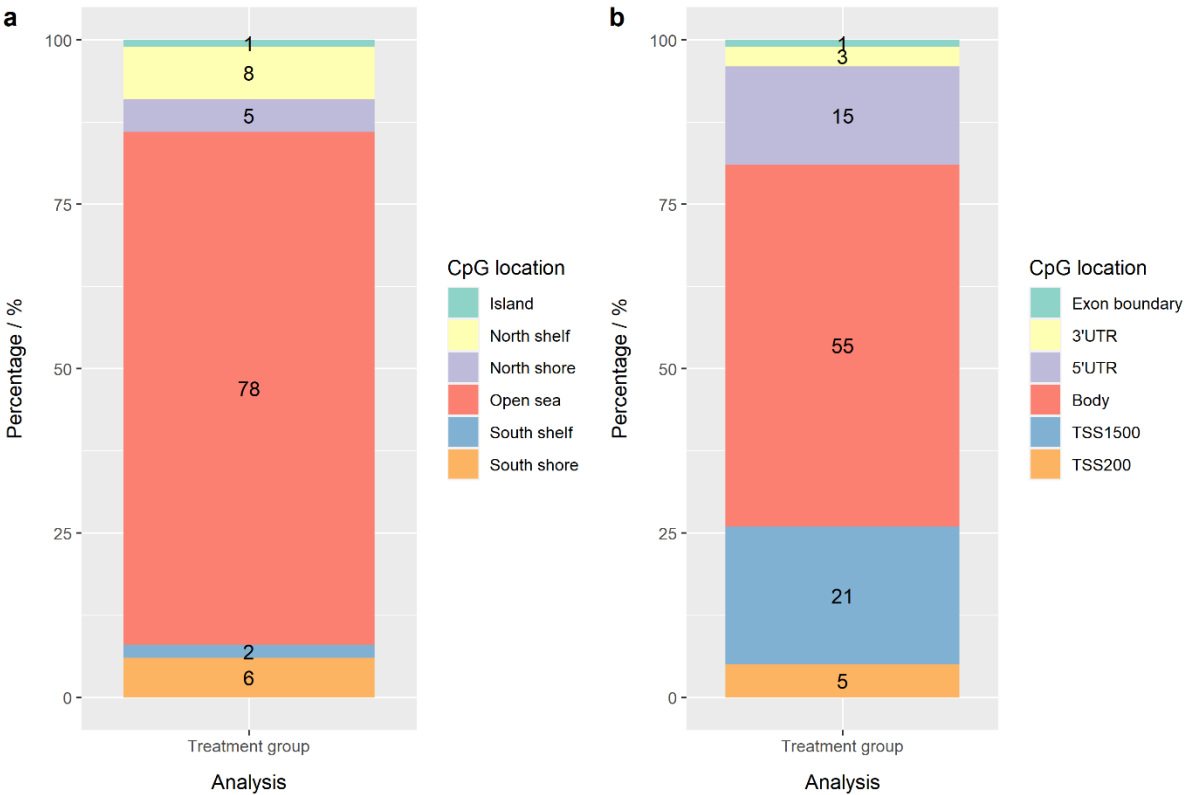


Figure 64. The location of the top 100 dmCpGs associated with treatment group analysis relative to a) CpG islands and b) genomic structure
The top 100 dmCpGs associated with treatment group, relative to CpG islands were found largely within open sea regions and relative to genomic structure, most commonly within gene bodies. CpG shores, shelves and open sea regions are defined in appendix 9.3.

Table 37. Gene clusters produced during the pathway analysis of the top 100 dmCpGs associated with treatment group analysis

Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with treatment group produced three distinct clusters of dmCpGs. However only clusters 2 and 3 saw significant enrichment of pathways with the involvement of three or more dmCpGs.

Model	Cluster	Genes	Nodes	Edges	Score	Pathways FDR <0.05	Gene count ≥3
Treatment group	1/3	ANKRD11, BCL9, FER, KIDINS220, TGFB2, CSNK2A1, CBFA2T3, ANKRD28, TWIST1, EDAR, PPP1R12A, MYO10, ANKS1B	13	45	5.429	0	0
	2/3	CSNK1G3, DDC, TMED10, DTNA, NDUFA5, TEX10, NDUFA10, CADPS, ARID5B, TFPI, NCOR2, PPP1CC, MDFIC, KIF26B, PFKM, PFKP	16	34	3.529	59	12
	3/3	RRM1, WDR74, WWP1, MYO5A, PUS7, USH2A, PTPRE, IFNG, LAS1L, TMX1, PPP1R8, CNNM2, AGFG1	13	26	3	32	1

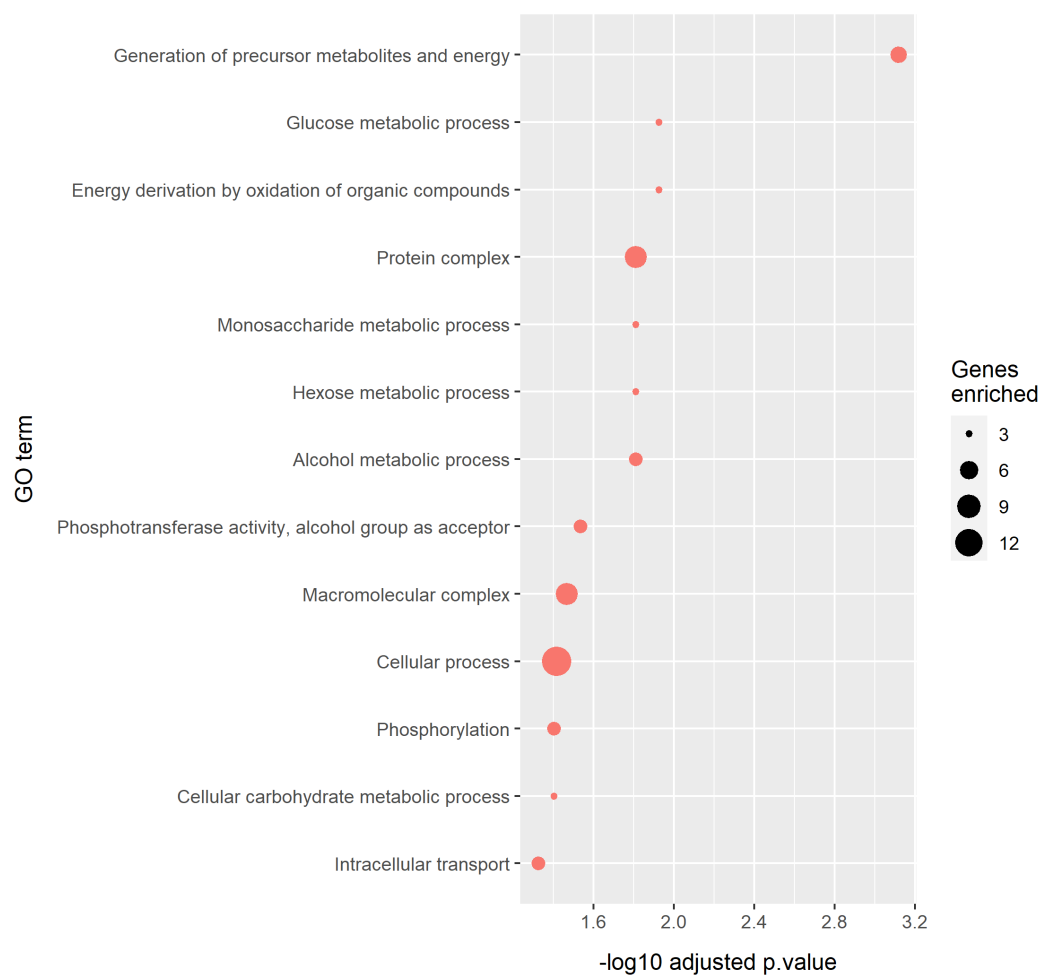


Figure 65. GO terms significantly enriched for the top 100 dmCpGs associated treatment group
Gene ontology pathways shown to be significantly enriched in the dmCpG clusters produced from the clustering of the top 100 dmCpGs associated with treatment group. A larger point size and -log10 adjusted *p*-value indicates a greater significance and number of dmCpGs enriched for the pathway.

6.2.2 *Effect of long-term vitamin D supplementation on UC-MSCs obtained from SPRING study primary umbilical cord tissue*

Whilst in previous chapters we have identified differential methylation changes at birth in both umbilical cord tissue and blood, these tissues are a heterogeneous mixture of cells. Therefore, this would make interpreting the functional significance of these changes difficult. So, to gain further insight into how long-term vitamin D supplementation may impact DNA methylation in MSCs alone, we aimed to isolate and enrich UC-MSCs from umbilical cord tissue taken from the SPRING RCT. Both the SPRING RCT and MAVIDOS RCT mothers received a 1000 IU/day vitamin D or placebo, however within the SPRING RCT half of each group were also given healthy conversation skills support, aimed at improving the quality of the pregnant mothers' diet. Whilst these studies were not identical, this would allow us to identify potential similarities in the epigenetic profile of both the cells and tissue which we could therefore suggest may potentially be originating within the UC-MSC fraction of the tissue as a result of maternal vitamin D supplementation. As umbilical tissue has been suggested to have an epigenetic profile similar to that of the mesoderm, this may allow us to infer the potential consequences of maternal vitamin D supplementation on skeletal growth.

However, initially we were required to establish and optimise a reproducible method for the classification, isolation and enrichment of mesenchymal stromal cells from primary umbilical cord tissue. This process could then be used going forward for the continued collection and culture of these primary cells.

6.2.2.1 *Optimisation of the characterisation, isolation and enrichment of primary UC-MSCs*

Primary UC-MSCs collected were extracted from umbilical cord tissue obtained from participants of the SPRING study. The umbilical cord is a heterogeneous mix of a number of cell types with varying characteristics. The extraction process as described in section 2.6.3.1 will inevitably dilute a proportion of the primary culture with non-UC-MSCs that will influence further assays if the culture is not enriched for UC-MSCs alone. Therefore the method of culture, identification and purification of all become important considerations for generating a homogenous culture UC-MSCs.

6.2.2.1.1 *Using cell-surface markers to optimise the enrichment of UC-MSCs*

The umbilical cord is a tissue made up of a number of cell types, therefore the positive sorting of cells using a cell-specific marker is essential to grow a culture consisting of only UC-MSCs. CD90 was selected because as well as being recommended by the International Society for Cell and gene Therapy (ISCT) as a positive marker for MSCs (appendix 9.1), it was also found to be a marker of UC-MSCs by a number of other studies (446,931). Cells isolated from the umbilical cord in this

study were further classified by the expression of CD29 and CD44, both expressed by UC-MSCs (446). The presence of both markers in our UC-MSC cultures was assessed via flow cytometry.

Cultures were labelled with UC-MSC X, where X is a number denoting the order in which each of the isolated UC-MSCs cultures were collected and cultured. To assess whether our isolated UC-MSC cultures express these markers, UC-MSC culture 1 was resuscitated, separated into four populations and stained with either no antibody, CD29, CD44, or both CD29 and CD44 antibodies. When integrated into one dot-plot diagram, staining showed four distinct populations of cells with each population demonstrating a >95% staining for their respective antibodies (Figure 66).

To assess the effect of CD90 cell sorting, three UC-MSC cultures were isolated into four populations and stained with both CD29 and CD44 antibodies, CD29 and CD44 isotype controls, propidium iodide and unstained controls. Isotype controls are fluorochrome-conjugated primary antibodies lacking specificity to any epitope on the surface of the target cell. However the type and class of primary antibody is identical to the one used for initial staining, therefore any positive isotype control staining would be non-specific and the antibody used for staining may need to be reassessed. Isotype control antibody staining of UC-MSC 2, 3 and 4 with the appropriate CD29 and CD44 isotype control (Figure 69) demonstrated a distinct similarity to unstained cells demonstrating no non-specific staining. Staining with conventional antibodies demonstrated that >95% of each cell population stained for the CD29 and CD44 antibodies prior to the CD90 selective sorting process. When positively sorted for the presence of the CD90 cell-surface marker (Figure 67) cultures UC-MSC 2 and 4 showed an increase in the proportion of the cell population staining with both CD29 and CD44 antibodies. However culture UC-MSC 3 showed a small reduction in staining. Propidium iodide assay of UC-MSC 3, a measure of cytotoxicity (932,933) (Figure 68) only showed a small increase in cell death when compared to the unsorted cells after sorting cells by the presence of the CD90 marker.

6.2.2.1.2 *Is either a plastic or gelatin cell culture surface more optimum for UC-MSC growth?*

A characteristic of MSCs as defined by the ISCT (appendix 9.1) is an ability of the cells to grow on plastic (446). However, coating cell culture surfaces with gelatin can improve the attachment and rate of proliferation of primary adherent UC-MSCs whilst allowing multipotency to be maintained (934,935). Culture of UC-MSC 1 on both plastic and gelatin and subsequent flow cytometry indicated 95.7% of the gelatin-grown cells (Figure 70aii) and 85.9% of plastic-grown unstained cells (Figure 70bii) stained for both the CD29 and CD44 cell-surface markers, suggesting a less homogenous population when the cells were cultured on plastic. Additionally, unstained UC-MSCs grown on plastic (Figure 70bi) demonstrated a higher degree of autofluorescence when compared to gelatin grown cells (Figure 70ai).

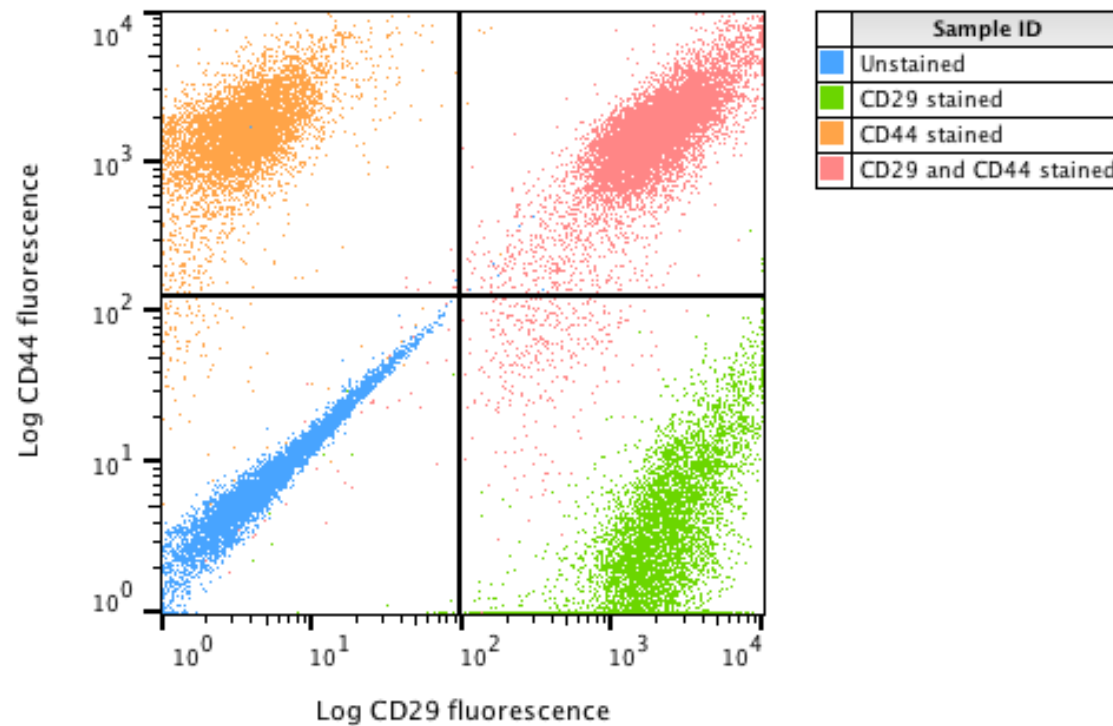


Figure 66. MSC 1 isolated UC-MSCs either unstained, stained with CD29, CD44 or both CD29 and CD44 antibodies

A dot-plot showing relative expression of the CD29 and CD44 cell surface markers on four separate populations of UC-MSC 1 isolated UC-MSCs. Cells were unstained, or stained with either the CD29-PE, CD44-FITC, or both the CD29-PE and CD44-FITC antibodies and the resulting dot plots overlaid to create the above figure. Gating strategy see section 2.6.4.2.

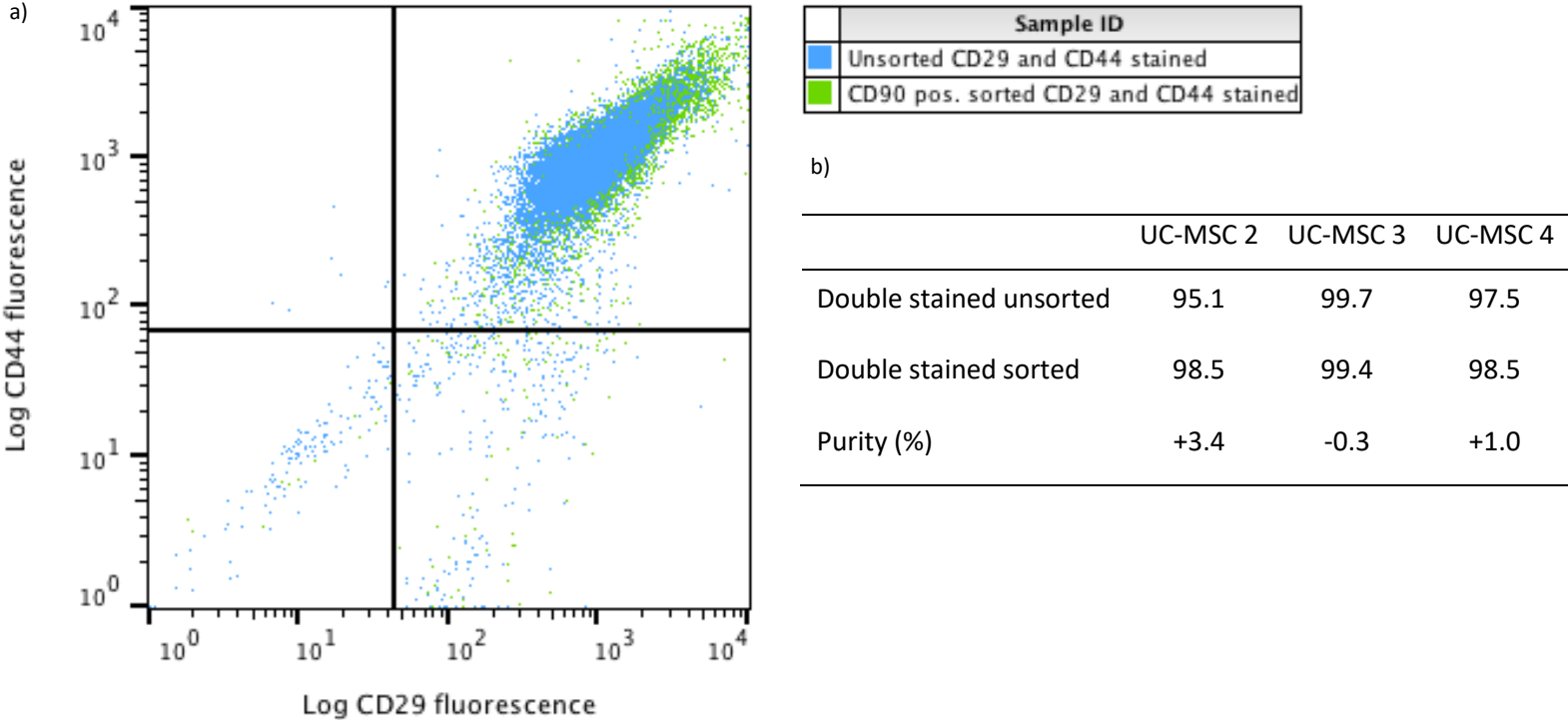


Figure 67. The proportion of cells expressing the CD29 and CD44 markers when either CD90 sorted or unsorted
a) A representative dot plot showing the degree of staining for the CD29 and CD44 cell surface markers by unsorted and CD90 sorted MSC 2 isolated UC-MSCs. Cells were either CD90 sorted or unsorted, both stained with CD29-PE and CD44-FITC antibodies, assayed and overlaid to produce the above figure. b) The percentage of cells from three isolated UC-MSC cultures CD90 sorted and unsorted and stained for the CD29 and CD44 cell surface markers. Percentage purity was calculated by subtracting sorted population from unsorted population of the same culture.

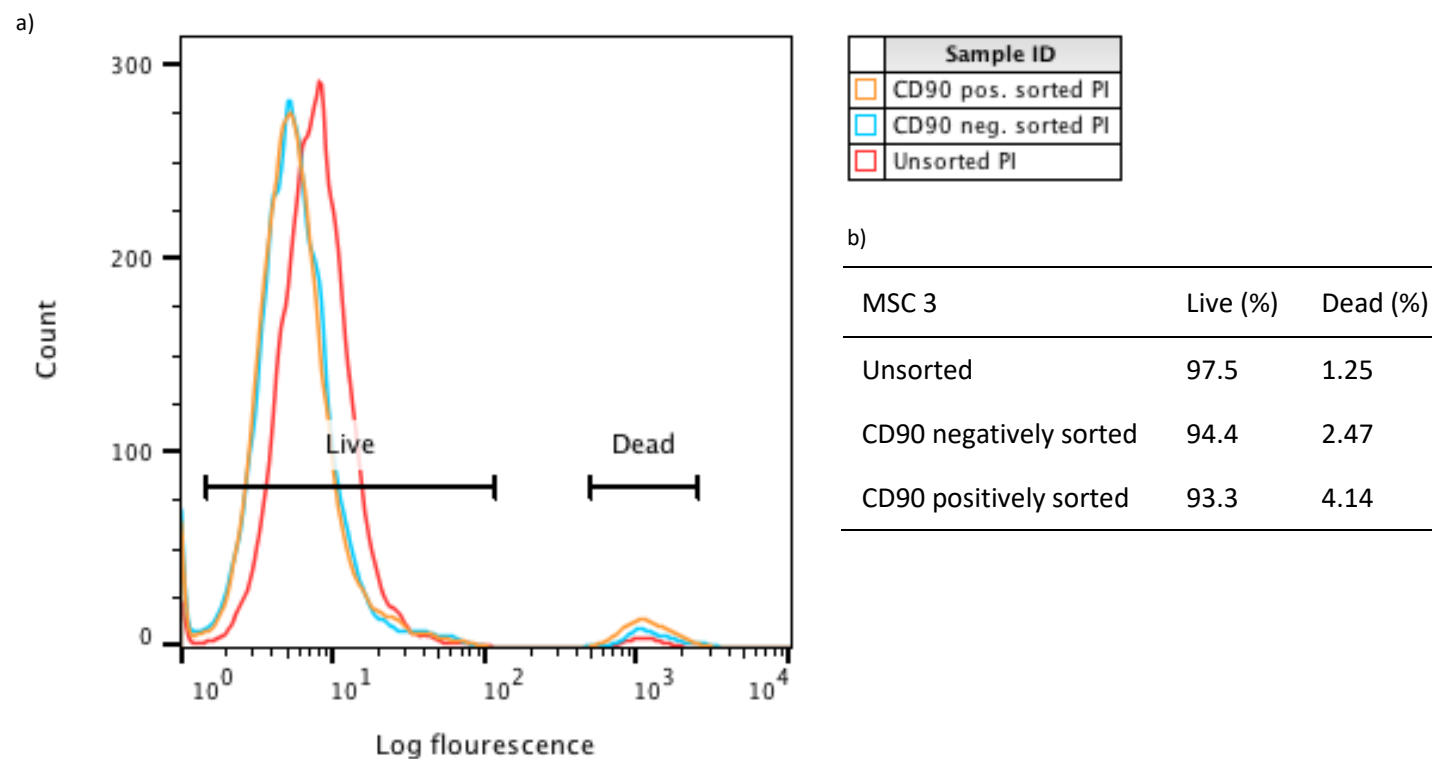


Figure 68. The proportion of cell death associated with CD90 sorting of UC-MSC 3 isolated UC-MSCs

a) A histogram to show the proportional cell death associated with process of sorting culture UC-MSC 3 according to the presence of the CD90 cell surface marker. CD90 positively sorted, CD90 negatively sorted (absence of the CD90 marker) or unsorted cells from the sorting process were stained with PI, assayed and overlaid to produce the above figure. b) The percentage of live and dead cells associated with CD90 sorting MSC 3 unsorted, positively and negatively sorted cells as assessed by PI staining and flow cytometry analysis.

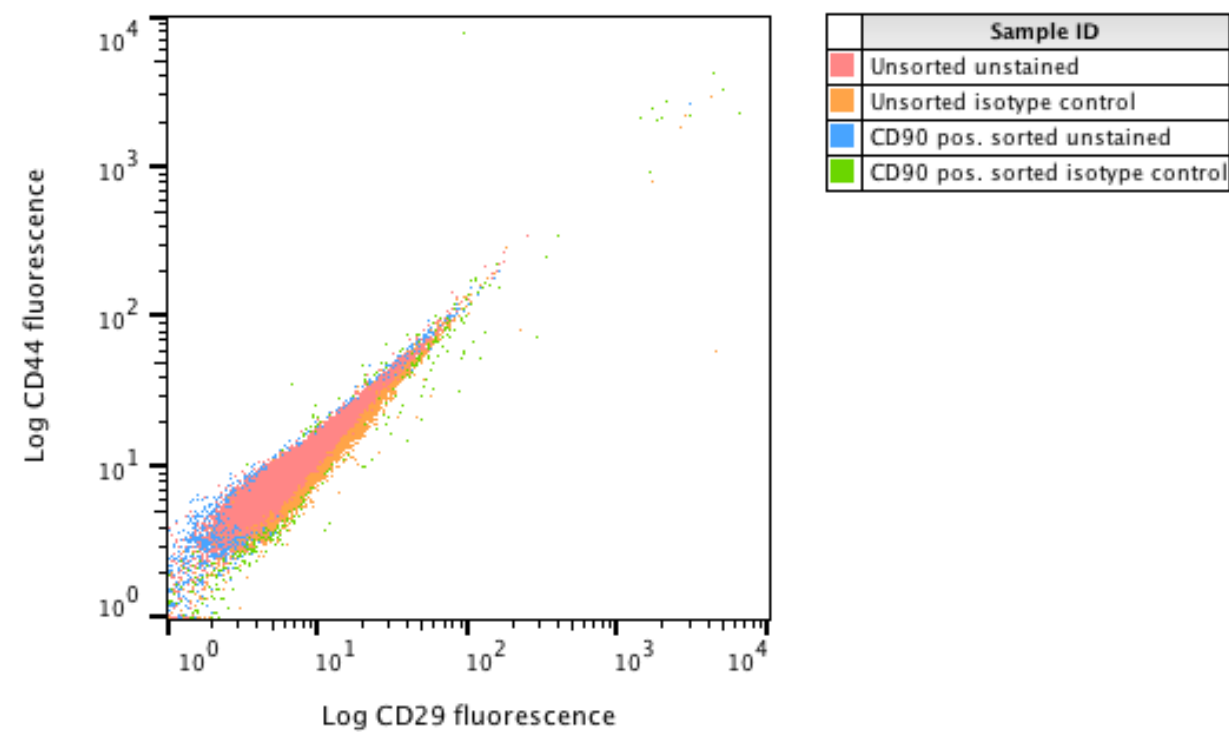


Figure 69. The isotype control staining of three CD90 sorted and unsorted isolated UC-MSC cultures to determine whether antibodies stain non-specifically
Dot-plot diagrams to show unsorted, unstained and CD90 positively sorted cultures a) UC-MSC 2 b) UC-MSC 3 and c) UC-MSC 4 stained either with or without the appropriate isotype control antibody.

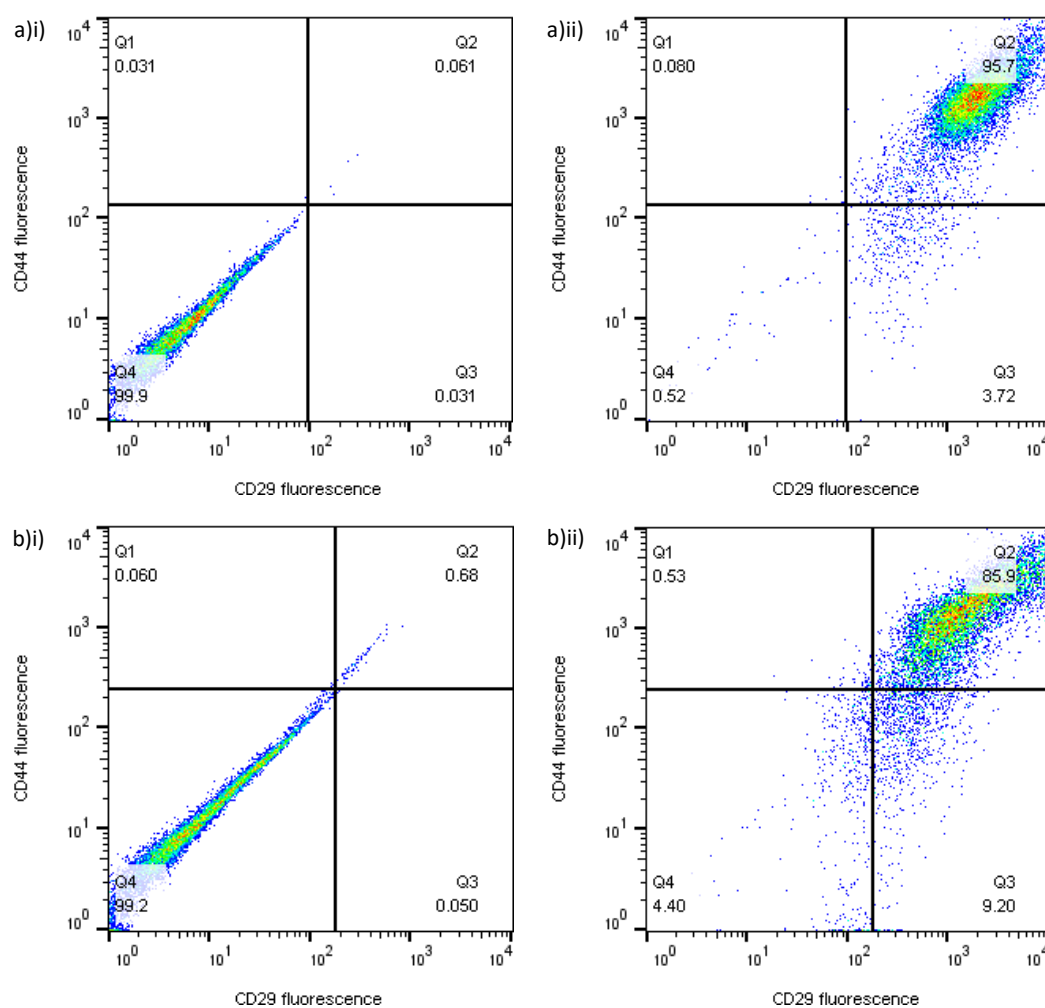


Figure 70. The characterisation of cells either cultured on plastic or 0.1% gelatin coated 100mm cell culture dishes

Heat-map dot-plot diagrams of culture MSC 1 isolated UC-MSCs grown until 70-80% confluent on either a) 0.1% gelatin coated 100mm cell culture dishes i) unstained or ii) stained with CD29-PE and CD44-FITC antibodies or b) untreated plastic 100mm cell culture dishes i) unstained or ii) stained with CD29-PE and CD44-FITC antibodies. Gating strategy see section 2.6.4.2.

6.3 Discussion

6.3.1 Effects of short-term vitamin D treatment on ATCC UC-MSCs

6.3.1.1 CYP24A1 mRNA is suitable for identifying 1,25(OH)₂D mediated induction of vitamin D related pathways in SaOS-2 and UC-MSCs

In order to investigate the effects of 1,25(OH)₂D on UC-MSCs it was necessary to determine a treatment concentration of 1,25(OH)₂D that would activate vitamin D-responsive pathways in these cells. The transfection and 1,25(OH)₂D (10 nm) treatment of the vector constructs generated in this study had the potential to induce a luciferase response in SaOS-2 cells and UC-MSCs, indicating the activation of vitamin D responsive pathways.

Within the context of this and previous studies, supraphysiological concentrations of 1,25(OH)₂D have been required to elicit the stimulation of vitamin D responsive pathways or processes (Figure 59b) (919). Whilst the reason for this is currently not entirely clear, it has been suggested that *in vivo* only a minor portion of free or unbound 1,25(OH)₂D enters the cell through simple diffusion and the majority is transported into the cell via membrane proteins such as caveolin, cubilin, and megalin bound to carrier proteins such as vitamin D binding protein or albumin (936). Within *in vitro* culture these proteins are going to be absent, suggesting the supraphysiological concentrations of 1,25(OH)₂D required to stimulate vitamin D responsive pathways *in vitro* may be due to a lesser transport of vitamin D into the cell and so a muted response when compared to an *in vivo* system. This may also be exacerbated a short half-life of free 1,25(OH)₂D which has been suggested to be approximately 15 hours *in vivo* (937,938).

6.3.1.1.1 Osteocalcin response to 1,25(OH)₂D treatment

In the context of this study we have shown that the transfection and treatment of SaOS-2-cells with pGL4-OC and 1,25(OH)₂D (10nm) respectively induced a significant increase in luciferase expression when compared to vehicle treated cells (Figure 57a), alongside an increase in OC mRNA expression upon induction with 1,25(OH)₂D (Figure 58a). However 1,25(OH)₂D treatment of PGL4-OC transfected UC-MSCs did not elicit the same response (Figure 57a). This may have been due to inefficient transfection of the vector constructs into UC-MSCs when compared to SaOS-2 cells, however in both the SaOS-2 and UC-MSC cell types the level of the Renilla normalisation control was similar, indicating that the lack of a response seen from treatment of the transfected UC-MSCs is unlikely to reflect a lower transfection rate in the cultures. Alternatively, a study by Shen *et al.* (2002) showed that 1,25(OH)₂D treatment only acts to enhance OC expression after an increase in basal transcription is initiated by bone specific factors, where the level of responsiveness to 1,25(OH)₂D is inversely related to the expression level of OC (939). The increase in OC expression has been shown to occur around day 21 in osteodifferentiating bone marrow mesenchymal stromal cells (BM-MSCs) (940) and as late as day 40 in osteodifferentiating UC-

MSCs (941), largely due to its role in facilitating mineralisation later in bone development. Therefore SaOS-2 cells which are of a mature osteoblast phenotype are likely to have undergone this increase in OC expression, whereas UC-MSCs will have not, which may explain the lack of response to $1,25(\text{OH})_2\text{D}$ in the UC-MSC cells. Therefore $1,25(\text{OH})_2\text{D}$ mediated upregulation of OC mRNA expression or pGL4-OC activation may not be a suitable indicator for identifying $1,25(\text{OH})_2\text{D}$ mediated induction of vitamin D responsive pathways in UC-MSCs.

6.3.1.1.2 *CYP24A1 response to $1,25(\text{OH})_2\text{D}$ treatment*

RT-qPCR showed a strong induction of expression of CYP24A1 mRNA upon treatment of SaOS-2 cells with $1,25(\text{OH})_2\text{D}$ (Figure 59) indicating CYP24A1 expression is inducible in SaOS-2 cells. Similarly, transfection of SaOS-2 cells with the pGL4-CYP plasmid and short-term treatment with 10nm $1,25(\text{OH})_2\text{D}$ significantly increased luciferase expression ($p < 0.05$) when compared to the pGL4.23 vector alone (Figure 57b). However whilst not significant, transfection of SaOS-2 cells with the pGL4-CYP plasmid and treatment with vehicle also showed a similar increase in luciferase expression when compared to the pGL4.23 vector alone. This may be due to the structure of CYP24A1 gene and associated VDRE sites. The pGL4-CYP plasmid contained a VDRE sequence characterised from the distal region of the human CYP24A1 promoter located -293/-273 nucleotides from the TSS (674). However this sequence alone may not be sufficient for vitamin D induced activation of this gene. Chromatin immunoprecipitation microarray hybridisation (ChIP-chip) and chromatin immunoprecipitation sequencing (ChIP-seq) analysis of VDR/RXR binding sites in the osteoblast genome have found that genes are frequently regulated by multiple enhancers and the elements are often located in clusters (942). Indeed, *Kumar et al.* (2010) define a promoter region between nucleotides -470/-390 in the human CYP24A1 gene suggested to be required for the $1,25(\text{OH})_2\text{D}$ mediated induction of expression (943). Also, *Meyer et al.* (2010) characterised at least four potential enhancers 50-69kb downstream of the human CYP24A1 gene which also were suggested to contribute to the $1,25(\text{OH})_2\text{D}$ induced upregulation of CYP24A1 (944). Therefore the pGL4-CYP plasmid may lack portions of the CYP24A1 gene required for $1,25(\text{OH})_2\text{D}$ -mediated induction, whereas the SaOS-2 genome will contain the full sequence required for the $1,25(\text{OH})_2\text{D}$ -mediated induction of CYP24A1 expression which can therefore occur unencumbered. Similarly transfection of UC-MSCs with the pGL4-CYP construct and subsequent $1,25(\text{OH})_2\text{D}$ treatment elicited induced no change in luciferase expression, however CYP24A1 mRNA was strongly induced when treated with 10nm $1,25(\text{OH})_2\text{D}$, suggesting the pGL4-CYP construct was not $1,25(\text{OH})_2\text{D}$ inducible in either cell type. This in summary suggests $1,25(\text{OH})_2\text{D}$ mediated upregulation CYP24A1 mRNA expression at 10nm or above (Figure 59b) would make a suitable tool for identifying $1,25(\text{OH})_2\text{D}$ induction in UC-MSCs, but transfection with the construct would not.

6.3.1.2 Differentially methylated CpGs are associated with short-term 1,25(OH)₂D treatment in ATCC UC-MSCs *in vitro*

6.3.1.2.1 Summary

Previous work in the MAVIDOS RCT has shown vitamin D supplementation over the course of pregnancy leads to significant differential methylation in umbilical cord tissue DNA (section 4.2.4). Here, through comparison to SPRING RCT UC-MSCs (green dotted box, Figure 71) we aimed to address aim I of this chapter by assessing whether methylation changes seen previously in the MAVIDOS RCT were unique to long-term maternal vitamin D supplementation or could be recapitulated *in vitro* with short-term 1,25(OH)₂D treatment of ATCC UC-MSCs. If indeed these changes were replicable this could i) have implications for the length of time supplementation may be required over the course of pregnancy to elicit these changes and ii) provide some indication that changes were happening in the UC-MSC fraction of the tissue. However, establishing whether methylation changes were in the UC-MSC fraction of the tissue could be conducted in a more robust manner by a direct comparison between the methylation state of SPRING RCT umbilical cord tissue and SPRING RCT UC-MSC DNA (purple dotted box, Figure 71).

Due to an unforeseeable university-wide lack of access to lab facilities for an extended period of time it was not possible to establish a comparison with isolated and enriched SPRING RCT UC-MSCs as originally intended (green dotted box, Figure 71). Although, we did establish that short-term 1,25(OH)₂D treatment of ATCC UC-MSCs *in vitro* lead to a number of significant and potentially pro-osteogenic differential methylation changes. MSCs are multipotent cells with the capacity to differentiate along osteogenic, adipogenic and chondrogenic pathways. These differential methylation changes in potentially pro-osteogenic genes may be expected as the action of 1,25(OH)₂D on MSCs derived from various sources has typically been found as anti-proliferative, pro-differentiation and pro-osteogenic (375,376,945–948). In contrast, the influence of 1,25(OH)₂D on the adipogenic potential of MSCs has been suggested to depend on the stage of differentiation, acting to inhibit commitment and the early stages of pre-adipocyte proliferation and differentiation, but enhancing the later stages of differentiation and maturation (378,382–386). *In vivo*, 1,25(OH)₂D has also been suggested to be involved in the maturation of chondrocytes and possibly in the calcification of growth cartilage (949). *In vitro* 1,25(OH)₂D has been seen to enhance the migration and differentiation of rat BM-MSCs in a chondrogenic medium (950). This highlights the importance of assessing the differentiation potential of these cells, the results of which can then be used alongside our differential methylation results to assess whether the action 1,25(OH)₂D in this context is indeed pro-osteogenic. Differential methylation changes seen with the short-term 1,25(OH)₂D treatment of the ATCC UC-MSC cells did not overlap with the changes seen in previous MAVIDOS RCT intervention and SWS MLSP D status differential methylation analyses, however this may have been expected due to the significantly different

experimental conditions and cell and tissue-specific nature of methylation (458,649,693). However, dmCpGs associated with the short-term $1,25(\text{OH})_2\text{D}$ treatment of ATCC UC-MSCs did show enrichment for a number of cellular respiration and glycolysis-related pathways, reflecting significant differential methylation seen in glycolysis-related genes in previous SWS MLSP D-related analyses (section 4.2.5).

6.3.1.2.2 Top hits

Differential methylation analysis of DNA extracted from UC-MSCs treated with either vehicle or $1,25(\text{OH})_2\text{D}$ over 24 hours *in vitro* indicated significant differential methylation ($\text{FDR} < 0.05$) at 78 CpG sites. The two most significant dmCpGs associated with the short-term treatment of UC-MSCs with vitamin D were cg11347955 ($\text{FDR} = 2.56 \times 10^{-5}$) and cg17523024 ($\text{FDR} = 1.30 \times 10^{-4}$). As intergenic dmCpGs these sites are not mapped to any specific gene, therefore any functional effects of differential methylation at these sites will take further investigation to ascertain. However further to these dmCpGs there were a number of differentially methylated sites specifically relation to the function of bone which were of particular interest. The CpG cg24719910 was found to be differentially methylated within the body of the Transforming Growth Factor Beta Receptor 2 (*TGF β -R2*) gene ($\text{FDR} = 0.0133$), where methylation was higher in the vitamin D treated group (appendix 9.4.7.1.3).

The transforming growth factor-beta (*TGF β*) superfamily is a group of cytokines including *TGF β s*, activin, bone morphogenic proteins (BMPs), inhibins and others which play a crucial role in embryonic development, tissue homeostasis and the pathogenesis of various diseases. This family of 33 ligands is split into subfamilies based on structure and function, two of which are *TGF β s* and BMPs. Significantly, whilst there are some conflicting findings, *TGF β* signalling is broadly suggested to promote the early proliferation and differentiation of osteoprogenitors such as MSCs, promote matrix production but inhibit later differentiation and matrix mineralisation (276–279). This achieved through the modulation of the expression and activity of key transcription factors required for the differentiation of these cells into each of their respective multipotential lineages. *TGF β* , of which three forms exist (*TGF β 1*, *TGF β 2* and *TGF β 3*) are receptor ligands for three isoforms of the *TGF β* transmembrane receptor, *TGF β R1*, *TGF β R2* or *TGF β R3*. Binding of one of the three ligands to a receptor leads to the transduction of the signal through a canonical, smad-dependant or non-canonical, smad-independent pathway. Canonical signalling classically occurs through binding to *TGF β R2* (951), a receptor which has been seen as active in the early limb bud mesenchyme, the deletion of which in murine models has been shown to result in severe skeletal defects and embryonic lethality (280). Furthermore, *Abou-Ezzi et al.* (2019) highlighted targeted deletion of *TGF β R2* within MSCs in developing foetal mice resulted in a reduction in the number of mature osteoblasts and an increase in bone marrow adipocytes, whereas postnatal deletion had no effect, findings reinforced by *Peters et al.* (2017) (282,952).

This indicates the potent role of TGF β in both permitting osteoblastic differentiation and inhibiting adipogenic differentiation. In this study we saw an increase in methylation within the body of the *TGF β R2* gene, which has been suggested to positively influence transcriptional activity of a gene, however this is in contention (762–771). This, pending both replication and confirmation of the effects differential methylation at this CpG site may have on the expression of the *TGF β R2* gene suggests treatment of these cells with 1,25(OH) $_2$ D may enhance the osteogenic capacity of these cells through enhanced expression of *TGF β R2* and so the promote the early proliferation and commitment of MSCs to an osteogenic pathway through the TGF β canonical signalling pathway.

In addition, the CpG cg27334919 was found as differentially methylated in the 1500 bases upstream of the Twist Family BHLH Transcription Factor (*TWIST1*) gene TSS (FDR= 0.0381), indicating a greater overall methylation in the vitamin D group (appendix 9.4.7.1.3). *TWIST1* is a factor known to be necessary for development of the mesoderm but downregulated during foetal endochondral and intramembranous bone formation. Indeed, overexpression of *TWIST1* in BM-MSCs both *in vivo* and *in vitro* has been shown to decrease their osteogenic differentiation potential and subsequently enhance their adipogenic differentiation potential (953,954). Similarly, in the developing mouse limb bud mesenchyme *Reinhold et al.* (2006) showed Wnt-induced expression of *TWIST1* strongly inhibited chondrocyte gene expression, whereas knockdown increased expression of the chondrocyte-specific genes aggrecan and type II collagen (955). Further work by *Gu et al.* (2012) have suggested this may be through the activity of *TWIST1* in directly inhibiting the transcriptional activity of SRY-box transcription factor 9 (*SOX9*), the master regulator of chondrogenesis (956). Furthermore, *TWIST1* has also been suggested to a significant role in regulating cellular senescence. Cellular senescence is typically where a cell will stop proliferating and undergo phenotypic alterations as a response to various stresses such as DNA damage, oxidative stress or oncogene activation. This process has been suggested to act as a guard, or ‘fail-safe’ against the un-restricted proliferation of potentially damaged or cancerous cells (957–959). The *Ink4A/Arf* locus, a region of the genome encoding both p14Arf and p16^{INK4a} is central to cellular senescence, signalling via p53 and retinoblastoma to inhibit proliferation. In BM-MSCs, *Cakouros et al.* (2012) found *TWIST1* not only to enhance the life span of these cells, but do so through the induction of the histone methyltransferase enhancer Of Zeste Homolog 2 (*EZH2*), leading to an increase in H3K27me3 along the *Ink4A/Arf* locus, repressing the transcription of both *p16*, *p14* and so the senescence of the BM-MSCs (960).

An increase in the methylation within the promoter region of a gene as seen in *TWIST1* is generally understood to negatively influence transcriptional activity, however the influence of this differential methylation on the expression of the gene would need to be confirmed experimentally. If a negative influence of promoter methylation on the expression of *TWIST1* was confirmed experimentally, this suggests 1,25(OH) $_2$ D treatment of these cells may lead to a

reduction in expression of TWIST1, reducing the proliferative capacity of these cells and enhancing the capacity of these cells to undergo either osteo- or chondrogenic differentiation, reinforcing the view of vitamin D as an anti-proliferative, pro-differentiation agent (375,376,945–948).

6.3.1.2.3 *Pathway enrichment*

Pathway enrichment analysis of the top 100 dmCpGs associated with the short-term vitamin D treatment of UC-MSCs showed enrichment for pathways related to cellular respiration, such as generation of precursor metabolites and energy derivation by oxidation of organic compounds. Additionally, we saw enrichment of glycolysis-specific pathways such as hexose, monosaccharide and glucose metabolic processes (Figure 65).

Vitamin D has been seen to act in a number of target tissues and cell types to upregulate the expression of the insulin-responsive glucose transporter type 4 (GLUT4) and its translocation to the cell surface to increase glucose uptake (961–964). Additionally, alongside the upregulation of GLUT4, there also may be indications vitamin D supplementation enhances glycolytic activity. A study by *Manna et al.* (2012) showed in a pre-adipocytic cell line that vitamin D treatment induced upregulation of GLUT4 expression and its translocation to the cell surface, but treatment also increased glucose utilisation (961). Whilst we did not see differential methylation of GLUT4, within the context of our own study we saw enrichment for glucose metabolism in the dmCpGs associated with short-term treatment with vitamin D, also indicating potentially enhanced glycolytic activity in these cells. Whilst vitamin D has not been seen to induce differentiation of MSCs, an increase in glycolytic activity within MSCs could act to enhance their osteogenic differentiation potential. Wnt3a-induced osteoblastic differentiation of BM-MSCs has been seen to require the upregulation of key glycolytic enzymes to enhance aerobic glycolysis and permit differentiation, such that inhibition of these enzymes impairs osteogenesis *in vitro* (965). In addition, work conducted with committed BM-MSCs and pre-osteoblastic or pre-adipogenic cell types has indicated osteoblastic differentiation favours glycolysis and adipogenic differentiation favours oxidative phosphorylation (966–968). However, MSCs in their undifferentiated state also largely rely on glycolysis for energy production, meaning a 1,25(OH)₂D-induced increase in glucose metabolism may also simply facilitate the metabolic activity of the MSCs in their basal state (969–971).

6.3.1.2.4 *Overlap with previous analyses*

To assess whether there was any overlap in the differential methylation changes seen between the short-term 1,25(OH)₂D treated ATCC UC-MSCs, MAVIDOS RCT intervention (section 4.2.4) and SWS MLSP D (section 4.2.5) differential methylation analyses, a comparison was made between the top 100 dmCpGs when ranked by statistical significance from each analysis (FDR). Previous investigation into dmCpGs commonly differentially methylated between the MAVIDOS RCT

intervention and SWS MLSP D analyses found no overlap (section 4.2.6). As previously discussed (section 4.3.2.1), this may have been largely due to the methylomes of umbilical cord tissue and umbilical cord blood being largely distinct from one another, highlighting the predominantly cell type and tissue-specific nature of methylation (458,649,693). Consequently, it may not have been unexpected to see no commonality in differential methylation changes between UC tissue of the MAVIDOS RCT, umbilical cord blood of the SWS study and ATCC UC-MSCs. Therefore, in addressing aim 1 of this chapter, this assessment of overlap between these analyses has suggested the changes seen in MAVIDOS RCT umbilical cord tissue as a result of long-term vitamin D supplementation over the course of pregnancy are not able to be recapitulated with short-term treatment of ATCC UC-MSCs *in vitro*. However, aim 1 would be better addressed by a more robust comparison between UC-MSCs isolated and enriched from SPRING RCT umbilical cord tissue and the short-term 1,25(OH)₂D treated ATCC UCMSC (as detailed in Figure 71).

6.3.2 *Effect of long-term vitamin D supplementation on UC-MSCs obtained from SPRING study primary umbilical cord tissue*

6.3.2.1 *Optimising the characterisation, isolation and enrichment of primary UC-MSCs*

The umbilical cord contains a number of different cell types (931), many of which can be successfully cultured *in vitro*, an issue when trying to isolate and grow a culture of UC-MSCs alone. In this study the primary culture was enriched to ensure results obtained from any further assays were reflective of a homogenous culture of UC-MSCs only. The characteristics which define an MSC culture according to the ISCT are a i) ≥95% positivity for three specified markers, ≤2% negativity for five specified markers (full list of markers see appendix 9.1), ii) the ability to differentiate into adipocytes, osteoblasts and chondrocytes and iii) the ability to attach to and grow on plastic (462). These guidelines provide a minimum guideline for characterisation of MSCs, however they also present issues. Cultures produced following these guidelines have typically been found to produce heterogeneous, non-clonal cultures with different multipotential properties based on donor site, isolation protocol and passage (459,462–464,929). Therefore optimisation for the isolation and enrichment of MSCs typically differs between studies.

6.3.2.1.1 *i) Cluster of differentiation (CD) marker expression*

Characterising MSC cells by the expression of CD markers presented some challenges in producing homogenous MSC cultures. CD markers suggested by the ISCT have been seen to be absent on some MSC populations (412,972,973) and their expression can be affected by the transfer from *in vivo* to *in vitro* culture (974). Among many, ISCT markers such as CD105 and STRO-1 have been suggested as ambiguous markers for UC-MSCs. This ambiguity has led to the exploration of a large range of potentially more definitive markers, currently 19 positive and 20 negative potential markers are currently cited in literature for the identification of UC-MSCs (459). In literature and

within this study we found CD29, CD44 and CD90 are strongly and unambiguously expressed by UC-MSCs isolated from the Wharton's jelly tissue of the umbilical cord (412,457,468). We have standardised and refined the UC-MSC isolation and enrichment process such that we have been able to isolate, label and discriminate between labelled cells without any indirect staining effects (Figure 66, Figure 69). We have also been able to enrich the cultures to increase or maintain the proportion of cells staining for our chosen markers above 95% (Figure 67b) whilst maintaining viability (Figure 68b). However fibroblasts, a potential contaminating cell type have also been shown to express the CD29, CD44 and CD90 markers (975,976). Fortunately the cluster of differentiation 146 (CD146) marker has shown the ability to discriminate between these cell types. A study by *Amable et al.* (2014) demonstrated that in a UC-MSC culture, 96.7% of cells were found to express CD146 (977), a marker found to be most strongly expressed in the Wharton's jelly (412) from which UC-MSCs are typically isolated, and either weakly or not expressed by fibroblasts (978). This marker could be easily detected by flow cytometry and or integrated into our enrichment process used within this study. UC-MSCs have also been shown to be unaffected by the presence of fibroblasts (979), so a low proportion of fibroblasts in our culture would not affect our future cultures or assay.

6.3.2.1.2 ii) *Growth on gelatin and plastic*

A characteristic of MSCs as defined by the ISCT (appendix 9.1) is an ability of the cells to grow on plastic (187). However, coating cell culture surfaces with gelatin can improve the attachment and rate of proliferation of primary adherent UC-MSCs whilst allowing multipotency to be maintained (631,632).

ISCT guidelines state MSC cells need to be capable of growth on plastic. However, there are a range of cell culture substrates which offer to enhance the attachment, proliferation and survival of this cell type including fibronectin, collagen, gelatin and other proprietary formulations. The cell culture substrates also can be costly, complex to prepare and have the potential to bias the differentiation potential of the MSCs (980–982). Gelatin however offered a cost-effective, simple preparation procedure and has previously been shown to enhance the attachment and proliferation of BM-MSCs when compared to untreated cell culture surfaces without altering surface marker expression or differentiation potential (935). Therefore UC-MSCs in this study were assessed for their marker expression on gelatin when compared to plastic (Figure 70). Gelatin grown cultures Figure 70 aii) showed a greater concentration of cells with similar CD29 and CD44 fluorescence characteristics when compared to plastic-grown cultures, suggesting growing UC-MSCs on a gelatin surface is conducive for the growth of a more homogenous cell type. Alongside a reduction in the number of cells staining for the CD29 and CD44 markers, growing on plastic also had the effect of increasing the number of autofluorescing cells in the unstained population (Figure 70 bi). Autofluorescence is due to an accumulation of lipofuscin, the

residue of lysosomal degradation typically characteristic of dead cells (983,984). This indicated plastic as a cell culture surface is less conducive to UC-MSC attachment and growth, corroborating previous findings that gelatin enhances attachment of MSC cells. Overall our findings suggested a gelatin is more preferable for the growth of a viable, homogenous UC-MSC culture. Although it has been suggested the presence of gelatin could influence MSC differentiation towards an osteoblastic phenotype (985). This could be explored during the optimisation of the differentiation potential assay and if it has an effect it could be ameliorated by increasing cell number on gelatin, but conducting assays on un-coated cell culture dishes and plates.

6.3.2.1.3 *iii) Tri-lineage differentiation*

Whilst our cells have not yet been differentiated into all three cell types as suggested by ISCT standards, our standardised isolation and enrichment process has produced a homogenous culture of cells with the characteristics of UC-MSC cells. However there have also been suggestions that fibroblasts can differentiate into all three cell types making them a potential contaminant for differentiation potential assays. Although a variation in capability has been demonstrated between each fibroblastic cell-type (986–988) and not confirmed in umbilical cord-derived fibroblasts.

6.3.3 *Further work*

Within this study we have seen a number of significant differential methylation changes associated with the short-term vitamin D treatment of UC-MSCs. This highlights the dynamic nature of methylation and suggests the methylation changes associated with long-term supplementation with vitamin D may indeed be significantly different from those short-term treated. Further analysis suggested the changes occurring in 1,25(OH)₂D treated UC-MSCs may potentially act to enhance glucose uptake and metabolism, a process which may favour either the enhanced proliferation of MSCs or afford a greater osteogenic differentiation potential to the cells. We could therefore replicate this study in UC-MSCs in greater numbers, perform glucose uptake tests, measure metabolic activity with the Agilent Seahorse XF analyser and assess osteogenic differentiation potential. If indeed 1,25(OH)₂D treatment enhances glucose uptake and metabolism, this could be replicated in BM-MSCs to move towards understanding the potential effects on development *in vivo*. Furthermore, a time course experiment could be conducted to establish the potential changes in the methylome or metabolic activity associated with the 1,25(OH)₂D treatment of UC-MSCs over a period of greater than 24 hours or potentially during the course of differentiation.

6.3.3.1 *Effects of short-term vitamin D treatment*

The differential methylation analysis of UC-MSC DNA after short-term treatment with vitamin D indicated a number of significant, potentially pro-osteogenic differential methylation changes. To

build on this work we could move forward with the aim to understand the functional biological implications of these changes, which will require further validation of these findings. Our next steps would therefore initially be to replicate this experiment in a far greater number of UC-MSC cultures to ensure a robust statistical analysis and use these cultures to assess the potential transcriptional changes associated with the differential methylation changes in this study. This replication would most likely therefore take the form of a further epigenome-wide association study (EWAS) with ribonucleic acid sequencing (RNA-seq) conducted in parallel. Although to understand the potential biological implications of these methylation changes on osteogenic differentiation potential, we would ideally need to replicate this study in cells which ultimately form osteoblasts *in vivo*, BM-MSCs. MSCs have been suggested to retain a biased differentiation potential plasticity or 'memory' of their tissue of origin (913–915,989). This suggests that whilst suggested as having a methylome similar to that of tissues of a mesodermal origin, UC-MSCs may not necessarily act exactly as BM-MSCs in further *in vitro* functional work. However, primary BM-MSCs are more difficult to obtain, harvest and grow at a slower rate, suggesting any replication may potentially have to be of a smaller-scale. Furthermore, ultimately *in vivo* studies would be required as osteogenic differentiation of MSCs *in vitro* mimics that of intramembranous ossification *in vivo*, lacking an osteochondral progenitor as in endochondral ossification which is the process by which the axial and appendicular skeleton develops (13).

6.3.3.2 *Effects of long-term vitamin D supplementation*

6.3.3.2.1 *ISCT*

The ISCT has clarified in a past (442) and more recent position statement (443) the distinction between mesenchymal stromal cells and mesenchymal stem cells. The group clarify that the former refers to a stem cell population with demonstrable progenitor cell functionality of self-renewal and differentiation, whereas the latter refers to a larger, bulk population with notable secretory, immunomodulatory and homing properties. The current criteria provided by the ISCT (appendix 9.1) defines an *in vitro*, heterogeneous population of multipotent mesenchymal stromal cells that may include fibroblasts, myofibroblasts and a small population of stem cells (438,443–445). To be defined as a mesenchymal stem cell, there needs to be rigorous evidence for stemness that can be supported by both *in vitro* and *in vivo* data. It is suggested that the *in vivo* and *in vitro* characterisation of the stromal cells is undertaken using a matrix of functional assays including quantitative RNA analysis of select genes, flow cytometry of cell surface markers and protein analysis of the MSC secretome (456). This is all work that is possible in the future with the cells isolated within this study that would allow for a retrospective re-interpretation of results, if any is needed. However due to both time and money constraints, this will not be possible within the context of this study. In addition, tri-lineage differentiation will be optimised and carried out

in the future (appendix 9.10) at an early passage as possible, along with comparative characterisation of SPRING UC-MSCs and UC-MSCs obtained from ATCC.

6.3.3.2.2 *Further characterisation and enrichment of UC-MSCs*

Whilst within this work we have established a protocol for the isolation and enrichment of UCMSCs, the inclusion of CD146 in our enrichment process would allow for a better discrimination between fibroblast and UC-MSC cells and therefore a more effective enrichment of our UC-MSC cultures. We could also attempt to add further positive selection markers such as CD73 to the enrichment process, however the addition of these markers to the enrichment process would need to be evaluated for the overall effect this may have on cell viability.

In addition, with access to a more advanced flow cytometer, or through the optimisation of immunohistochemistry protocols, we could further characterise our cultures for the presence of certain cell surface markers. Whilst the capacity for ISCT-defined MSC cell-surface markers to produce a homogenous culture of MSC cells is in contention (412,972,973), these markers do allow for identification of potentially contaminating cell types such as CD34 expressing haematopoietic cells and CD45 expressing endothelial markers (446). These techniques could therefore allow us to further assess the integrity of our cultures and whether any further processing or enrichment may be required.

6.3.3.3 *Previously planned experimentation*

Due to unforeseeable circumstances and subsequent lack of access university-wide to lab facilities, experimentation previously planned for this chapter could not be completed and would therefore become work to be completed in the future. A schematic of previously planned and potential further experimentation is detailed in Figure 71.

6.3.3.3.1 *SPRING RCT UC-MSC and MAVIDOS RCT UC-tissue EPIC arrays*

Once we had further optimised the UC-MSC isolation and enrichment process, to address the hypothesis of this chapter and aim 2 directly, we aimed to conduct a genome-wide screen in UC-MSC DNA and UC for differential methylation changes associated with maternal vitamin D or placebo supplementation in the SPRING RCT. This would allow changes within UC tissue and UC-MSC cells to be compared directly, permit comparison with the results from the short-term treatment of ATCC UC-MSCs with 1,25(OH)₂D and allow for a comparison with the MAVIDOS RCT intervention analysis where umbilical cord tissue was interrogated with respect to maternal vitamin D supplementation (section 4.2.4). This would allow us to identify common methylation changes between these analyses and significantly whether methylation changes occurring as a result of maternal vitamin D supplementation in MAVIDOS RCT UC tissue are indeed potentially occurring in the MSC fraction of the tissue. In addition, this analysis could be made more robust

by conducting RNA-seq in parallel which would allow us to make an assessment of the transcriptional activity of the genes in which differential methylation changes have occurred.

This approach may potentially provide a clearer picture of the effects of maternal supplementation in birth tissues. The EPIC array analysis of the MAVIDOS RCT has the issue of being conducted on umbilical cord tissue DNA, therefore any differential methylation changes *in vivo* associated with UC-MSCs may therefore be diluted by the confluence of methylation changes in DNA from a number of other cell types. Thus, the comparison of methylation changes in UC-MSCs and UC tissue from the SPRING RCT will allow the direct comparison of methylation changes and by inference the contribution that UC-MSC DNA methylation makes to the signal observed in UC tissue. Moreover, it would have also allowed for a comparison with the changes that occur as a result of short-term 1,25(OH)₂D supplementation.

6.3.3.3.2 *In vitro* characterisation

The ATCC UC-MSC primary cell culture, which meets the current classification of UC-MSCs as defined by the ISCT (appendix 9.1, 9.10) provides a useful reference for what may be considered the typical phenotypic characteristics, methylation or transcriptional state of UC-MSCs against which the cells we have isolated and enriched can be compared.

Initially, to address aim 1 of this chapter, differential methylation changes identified from the EPIC array interrogation of short-term 1,25(OH)₂D treated ATCC UC-MSCs could be compared to potential methylation changes from the yet to be conducted EPIC array interrogation of SPRING RCT UC-MSC DNA. This would allow us to establish whether methylation changes as a result of long-term maternal cholecalciferol supplementation over the course of pregnancy are unique, or can be recapitulated *in vitro* with short-term treatment. If similar changes were found when compared to respective untreated or supplemented controls, this may inform the length of time supplementation would be required over the course of pregnancy. Any differentially methylated CpGs which overlap between these analyses could be suggested to be attributable to either an artefact of *in vitro* culture or a vitamin D-induced change.

In addition, phenotypic characterisation of the short-term 1,25(OH)₂D treated ATCC UC-MSCs and SPRING RCT UC-MSCs could be conducted. This would include the estimation of differentiation potential, colony forming unit fibroblast (CFU-F) capacity and proliferation capacity to assess the possible influence of long-term maternal cholecalciferol supplementation or short-term 1,25(OH)₂D treatment on these processes. These assays would be conducted alongside untreated, or vehicle treated ATCC UC-MSCs as a reference control.

6.3.3.3.3 *SPRING* UC tissue EPIC array

Finally, EPIC array and RNA-seq interrogation of SPRING RCT whole-tissue UC DNA could allow for us to further address aim 2 of the hypothesis of this dissertation by providing a similar instance of

an RCT study in which mothers were supplemented with cholecalciferol over the course of pregnancy. Comparison with our EPIC array interrogation of MAVIDOS RCT UC DNA and differential methylation analysis (section 4.2.4) could potentially provide corroboration of vitamin D induced changes in the birth tissue. Furthermore, this SPRING RCT umbilical cord tissue would provide an instance where methylation changes in both umbilical tissue and UC-MSCs within the tissue can be compared directly, allowing us to assess whether changes in whole-tissue are reflected in the UC-MSCs and vice-versa.

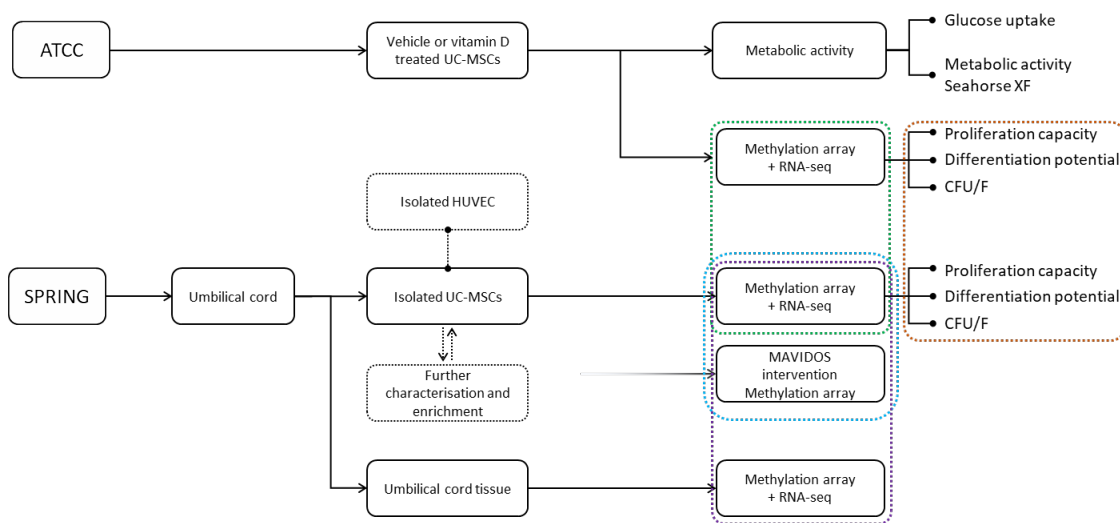


Figure 71. A schematic depicting both the previously planned and potential further experimentation unable to be completed

Due to unforeseeable circumstances and subsequent lack of access university-wide to lab facilities for an extended period of time, experimentation previously planned for this chapter could not be completed and so became work to be completed in the future. Initially the SPRING RCT UC-MSCs would be further enriched and characterised as detailed in 6.3.3.2.2, the DNA extracted and interrogated using methylation array and RNA-seq. To address the hypothesis of this chapter, comparisons would be made between SPRING RCT UC-MSC DNA methylation and the MAVIDOS RCT DNA methylation analyses to assess whether the maternal vitamin D supplementation induced methylation changes identified in section 4.2.4 may be occurring in the MSC fraction of the UC tissue (blue dotted box). This comparison could also be augmented to include HUVEC cells as a reference control, however the isolation and enrichment procedure would have to be optimised also. The methylation and transcriptomic state of SPRING RCT UC DNA and UC-MSC DNA would be compared to assess whether maternal vitamin D supplementation-induced changes in UC-MSCs are distinct from the changes in UC tissue as a whole, and comparisons could be made between SPRING RCT UC DNA and MAVIDOS RCT UC DNA to assess whether there are coherent methylation or transcriptomic changes between studies as a result of maternal vitamin D supplementation during pregnancy (purple dotted box). ATCC UC-MSCs would be compared to SPRING RCT UC-MSC DNA to assess whether potential methylation changes seen in SPRING RCT UC-MSCs are unique, or can be recapitulated *in vitro* with the short-term $1,25(\text{OH})_2\text{D}$ treatment of ATCC UC-MSCs (green dotted box). Finally, ATCC UC-MSC cells, along with long-term maternal vitamin D supplemented SPRING RCT UC-MSCs would provide a resource to assess for potential changes in various characteristics of these cells as a result of either vehicle or $1,25(\text{OH})_2\text{D}$ treatment *in vitro* (ATCC UC-MSCs) and long-term maternal vitamin D supplementation (SPRING RCT UC-MSCs) (orange dotted box).

6.3.4 Limitations

6.3.4.1 Effects of short-term vitamin D treatment

The most significant limitation of the EWAS assessing differential methylation changes associated with the short-term treatment of UC-MSCs was the number of cultures analysed. Replication with a greater number of cultures would allow for a more robust statistical analysis and confidence in the methylation changes seen. Additionally, whilst birth tissues have been suggested to be an appropriate surrogate for foetal tissues derived from the mesoderm for the purposes of an EWAS (458), any biological implications of differential methylation changes associated as a vitamin D treatment in terms of osteogenic differentiation would need to be verified in cells which would ultimately generate bone *in vivo*, BM-MSCs. As mentioned previously MSCs have been suggested to retain an epigenetic memory of their tissue which may bias their differentiation potential. Furthermore an *in vitro* BM-MSC model would ultimately need to be recapitulated *in vivo* as *in vitro* BM-MSC osteogenic differentiation will mirror intramembranous ossification, not endochondral ossification which is responsible for the growth of the axial and appendicular skeleton.

6.3.4.2 Effects of long-term vitamin D supplementation

Despite a refinement of the UC-MSC extraction process, we cannot be sure we are not selecting a clonal sub-population of cells with phenotypic characteristics that may differ from those isolated with different criteria from a different lab. This is a problem which is prevalent throughout MSC research which potentially could be ameliorated by comparing the phenotypic characteristics and transcriptome of our cells to our other cell types and the range of publicly available phenotypic and transcriptomic data available in the public domain.

There are a wide range of CD markers either identified by the ISCT as MSC-specific (446) or used by other groups to isolate or characterise MSC-like cultures (459). However, in the context of this study we were only able to use three cell-surface markers for isolation and characterisation of our UC-MSCs. This was due to i) the potential detrimental effect of a multi-marker enrichment process (detailed in section 2.6.4.4) on the viability of the cultures, ii) the expense of a flow-cytometry specific MSC antibody panel, iii) limitations of the FACSCalibur flow-cytometer as a two-laser machine, limiting the number of fluorophore-tagged antibodies that could be used in the characterisation of our cultures. Whilst this does not discount the markers used in this study as MSC-specific markers, inclusion of further markers (as detailed in section 6.3.3.2.2) in the isolation, enrichment and characterisation of the UC-MSCs cells would allow for a more confident assessment of our cultures as reflecting the methylation, transcriptional and phenotypic state of an MSC culture.

6.3.5 Conclusions

The vitamin D-mediated upregulation of CYP24A1 mRNA will provide a suitable indicator for the induction of vitamin D related pathways within either UC-MSCs or the SaOS-2 cell line for any future work *in vitro*. EPIC array analysis of UC-MSCs treated short-term with vitamin D has produced indications of potentially pro-osteogenic differential methylation changes, but interestingly also a potential influence of 1,25(OH)₂D on the metabolic activity of the cells. These methylation changes will also prove to be useful as a comparison to long-term supplemented SPRING RCT UC-MSCs as an indication of methylation changes which may be vitamin D driven.

With some modification, the process of isolating and characterising SPRING UC-MSC cells can be refined to produce a suitable population of mesenchymal stromal cells to adequately assess the effect of long-term vitamin D supplementation to the foetal methylome and cellular phenotypic characteristics such as proliferation rate, differentiation potential, CFU-F, transcriptome and phenotypic characteristics over the course of pregnancy. A subsequent EWAS of these cells will also provide a further insight into the potential effects of maternal vitamin D supplementation of UC-MSCs in umbilical cord tissue alone.

7 Chapter 7 - Is epigenetic age altered by maternal gestational vitamin D supplementation or vitamin D status and is it associated with bone mass?

7.1 Introduction

Studies have shown that around 2% of cytosine-phosphate-guanine dinucleotide (CpG) sites in the genome show significant changes in methylation with increasing age. Methylation clocks can use a selection of these CpGs, coupled with an algorithm to estimate the biological age of the tissue.

The first epigenetic clock developed by *Horvath* (2013) has been demonstrated to be highly accurate in doing so, showing a 96% correlation with chronological age and a mean difference of three years between estimated and actual ages across a range of cell-types and tissues (669).

Exhibiting a greater epigenetic age than chronological age, or age acceleration (AA) has been associated with a range of conditions including obesity, Alzheimer's disease and all-cause mortality (990–994). A number of additional clocks have been developed since the inception of the Horvath clock which differ in the platform used, the tissue used in training, the number of CpG sites in the algorithm, the overall accuracy and the outcome (995). A study by *Knight et al.* (2016) used methylation data obtained from the interrogation of neonatal umbilical cord blood and blood spots from 1434 neonates to train a methylation clock, which using elastic net regression found 148 CpGs that accurately estimated gestational age after birth (672).

Acceleration of the gestational age clock, gestational age acceleration (GAA) has been associated with increased birthweight and length, both of which are linked to altered developmental trajectories (996). This proposes the potential for an umbilical cord blood sample to be taken at birth, interrogated via a methylation array and the information supplied to the Knight methylation clock algorithm which based on 148 CpGs would generate estimates of gestational age and gestational age acceleration birth. This could not only provide potential to provide estimates of gestational age when ultrasound measures were not taken, or when the data of last menstrual period is unknown, but potentially identify the impact of an adverse *in-utero* environment on developmental trajectories of the offspring.

These associations between DNA methylation and disease state have highlighted the utility of methylation clocks to identify what health outcomes are associated with epigenetic ageing and what exposures can accentuate or attenuate this ageing phenomenon. Vitamin D supplementation has previously been shown to slow down AA in a group of young African American adults according to the Horvath clock and in a further study maternal vitamin D supplementation in a multi-ethnic population was identified to slow GAA in neonates according to the Knight clock. Whilst there is a general paucity of data related to bone biology and methylation clocks, it has been shown MSCs extracted from the femoral heads of patients of women undergoing hip replacement due to hip fractures have shown AA in the Horvath clock alone (997–

999). Here, we aimed to investigate whether changes in either epigenetic age, an estimation of chronological age calculated using the Horvath clock, or epigenetic gestational age, an estimation of gestational age calculated using the Knight clock were at birth associated with measures of BMC or BMD in childhood and whether maternal vitamin D supplementation affects AA or GAA.

7.1.1 *Aims and hypothesis*

The hypothesis of this work was that lower measures of both total body bone mineral content (BMC) and bone mineral density (BMD) in the Maternal Vitamin D Osteoporosis Study (MAVIDOS) randomised controlled trial (RCT) and Southampton Women's Survey (SWS) cohort would be associated with increased AA or GAA at birth. In addition, vitamin D supplementation in the MAVIDOS RCT and a greater vitamin D status in the SWS cohort would be associated with a decreased AA or GAA at birth.

Therefore, the aim of this work was to calculate both epigenetic age and epigenetic gestational age in both MAVIDOS RCT umbilical cord tissue DNA and SWS umbilical cord blood DNA. Using previously published methods we then aimed to estimate both AA and GAA and assess:

- i) The effects of maternal vitamin D supplementation on AA and GAA in the MAVIDOS RCT.
- ii) The relationship between AA and GAA with postnatal bone measures of BMC and BMD at the ages of birth and four years of age in the MAVIDOS RCT.
- iii) The effect maternal vitamin D status on AA and GAA in the SWS cohort.
- iv) The relationship between AA and GAA with postnatal measures of BMC and BMD at the ages of birth, four, six and eight years of age in the SWS cohort.

7.2 **Results**

7.2.1 *MAVIDOS RCT*

7.2.1.1 *Cohort characteristics*

The cohort characteristics of the MAVIDOS RCT participants used for the assessment of the association between both AA, GAA and measures of BMC or BMD at the ages of birth or four years of age or maternal vitamin D supplementation can be found in section 5.2.1 and split by intervention group in section 4.2.1.

7.2.1.2 *Epigenetic age estimation*

7.2.1.2.1 *Epigenetic age*

The epigenetic age of the umbilical cord tissue DNA extracted from the MAVIDOS RCT was calculated using the model as described by Horvath (2013) (669). The correlation between the

estimated and reported age was non-significant ($r^2=1.63e^{-4}$, $p=0.81$) and so no further acceleration analysis (AA) was conducted (Figure 72a).

7.2.1.2.2 Epigenetic gestational age

Epigenetic gestational age was calculated using the model as in *Knight et al.* (2016) (672). The correlation between the reported and estimated gestational age was very low ($r^2=0.024$, $p=0.00197$) albeit significant when compared to the correlation as reported by the original publication in the combined ($r=0.91$) or individual datasets ($0.52 < r < 0.65$) (672) (Figure 72b).

7.2.1.3 Age acceleration analysis

7.2.1.3.1 Gestational age acceleration

GAA was calculated for umbilical cord tissue DNA extracted from the MAVIDOS RCT by regressing estimated epigenetic gestational age values against gestational age, the residuals from which were used as an estimate of GAA. GAA values were then regressed against intervention group, BMC and BMD at the ages of birth and four years of age according to the multivariate model in Table 38. Regression diagnostics such a qqplot, Cook's distance, residuals and heteroscedasticity were used to check the model.

GAA was ($r^2=0.716$, p -value=0.017) inversely associated with BMC at birth (Figure 73a) however no association was found with BMC at four years of age, BMD at birth or four years of age. No significant association were found between GAA and intervention group. Due to the suggestion that sex can both influence estimations of age acceleration and modify the response to vitamin D supplementation in relation to serum 25-hydroxyvitamin D3 (25(OH)D) concentration, age acceleration was also estimated in males and females separately (1000,1001). In males, a significant association was no longer seen between GAA and BMC at birth, however a significant association remained for females ($r^2=0.731$, p -value=0.0245) (Figure 73b).

Table 38. The regression model used to assess the association between gestational age acceleration and maternal intervention group or offspring measures of BMD or BMD at birth and four years of age in the MAVIDOS RCT

Covariates	
GAA ~	Variable of interest, CD4T, CD8T, Endothelial, Epithelial, Granulocytes, Monocytes, Stromal, Position on beadchip, Sex, Smoking during pregnancy, respective DXA age (if a bone-related variable of interest)

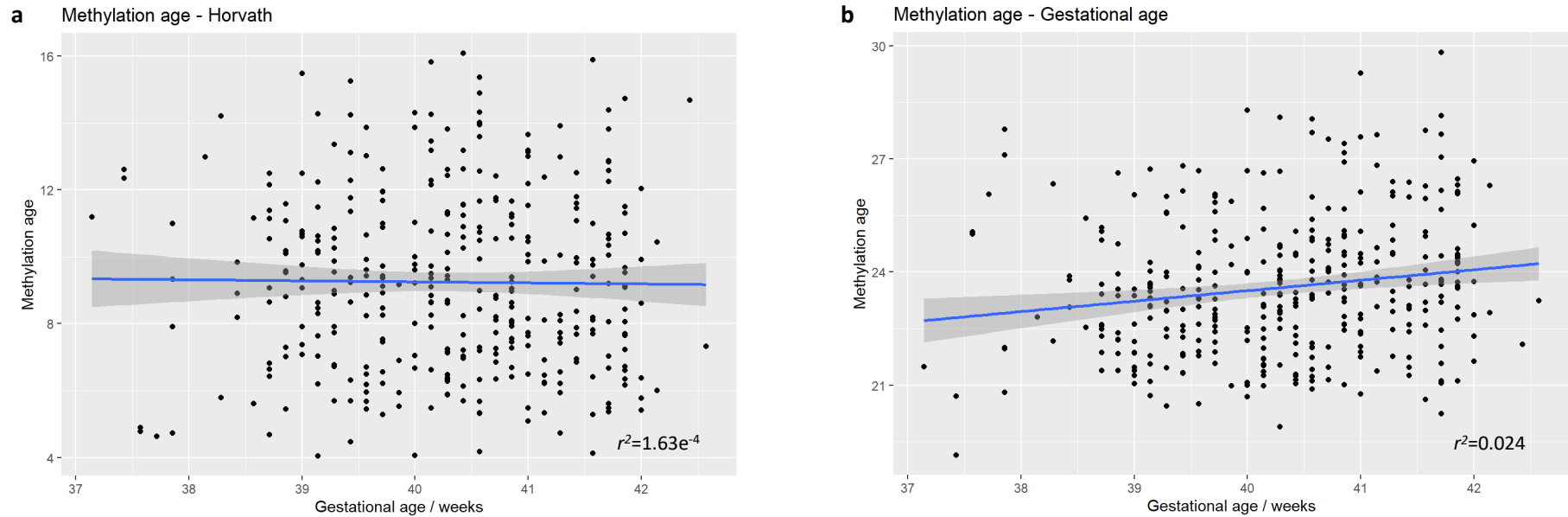


Figure 72. The association between a) epigenetic age and reported gestational age and b) gestational epigenetic age and reported gestational age in the MAVIDOS RCT
 The associations between a) reported and estimated chronological age as reported by the Horvath clock and b) reported and estimated gestational age reported by the Knight clock within the MAVIDOS RCT. Estimated epigenetic age and gestational epigenetic ages associated poorly with gestational age.

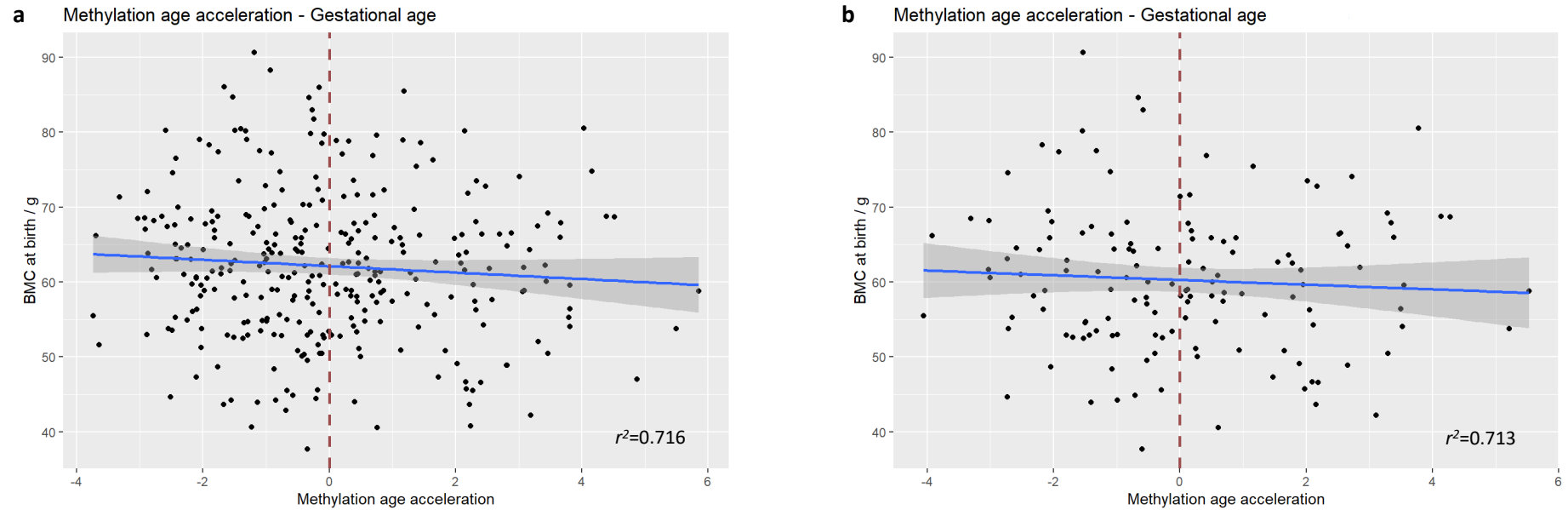


Figure 73. The association between a) gestational age acceleration and BMC at birth in both males and females and b) females alone in the MAVIDOS RCT

An assessment of the strength of the association between gestational age acceleration and BMC at birth in both sexes and females alone showed a strong association in both instances.

7.2.2 SWS study

7.2.2.1 Cohort characteristics

The characteristics of the SWS cohort used for the assessment of the association between both AA, GAA and measures of BMC or BMD at the ages of birth, four, six or eight years of age or maternal late-stage pregnancy vitamin D status can be found in section 3.2.1.

7.2.2.2 Epigenetic age estimation

7.2.2.2.1 Epigenetic age

We calculated the epigenetic age of umbilical cord blood extracted from the SWS cohort using the model as described in *Horvath* (2013) (669). The correlation between the estimated and reported epigenetic age was significant, albeit weak ($r=0.0243$, $p=5.72e^{-4}$) when compared to the correlation between the two variables in the original publication ($r=0.96$) (Figure 74a).

7.2.2.2.2 Epigenetic gestational age

We also used the model as described in *Knight et al.* (2016) to calculate an epigenetic estimation of gestational age of umbilical cord blood extracted from the SWS cohort (672). The correlation between the estimated and reported gestational ages was low ($r=0.318$, $p=8.76e^{-39}$) (Figure 74b). However, although not as high as the correlation seen in the original publication ($r=0.91$), this was closer to the correlations within the individual datasets used by *Knight et al.* (2016) in the original publication ($0.52 < r < 0.65$).

7.2.2.3 Age acceleration analyses for SWS cohort

7.2.2.3.1 Age acceleration

Epigenetic age acceleration values calculated from umbilical cord blood DNA extracted from the SWS cohort study was regressed against BMC and BMD at the ages of birth, four, six and eight years of age and maternal late-stage pregnancy vitamin D status according to multivariate model in Table 39. Regression diagnostics were used to check the model. No significant associations were found between AA and BMC or BMD at the ages of birth, four, six and eight years of age or maternal late-stage pregnancy vitamin D status.

7.2.2.3.2 Gestational age acceleration

Assessment of GAA found a significant ($r^2=0.211$, $p\text{-value}=0.039$), inverse association with BMD at four years of age (Figure 75a). Assessment of GAA in the SWS cohort umbilical cord blood DNA found no significant associations any other measures of BMC or BMD at the ages of birth, four, six or eight years of age or maternal late-stage pregnancy vitamin D status. GAA was estimated separately in both males and females due to suggestions sex may influence the estimation of GAA (1000,1001). Upon separation by sex the significant association with BMD at four years of age was

lost in the female group ($r^2=0.194$, p -value=0.587), but the significance of the association between GAA in the male group increased ($r^2=0.207$, p -value=0.0101) (Figure 75b).

Table 39. The regression model used to assess the association between epigenetic age or gestational age acceleration and maternal late-stage vitamin D status or offspring measures of BMD or BMD at birth, four, six and eight years of age in the SWS cohort

Covariates	
AA/GAA ~	Variable of interest, B cells, CD14, CD4T, CD8T, Granulocytes, Natural killer cells, Position on beadchip, Sex, Smoking during pregnancy, respective DXA age (if a bone-related variable of interest)

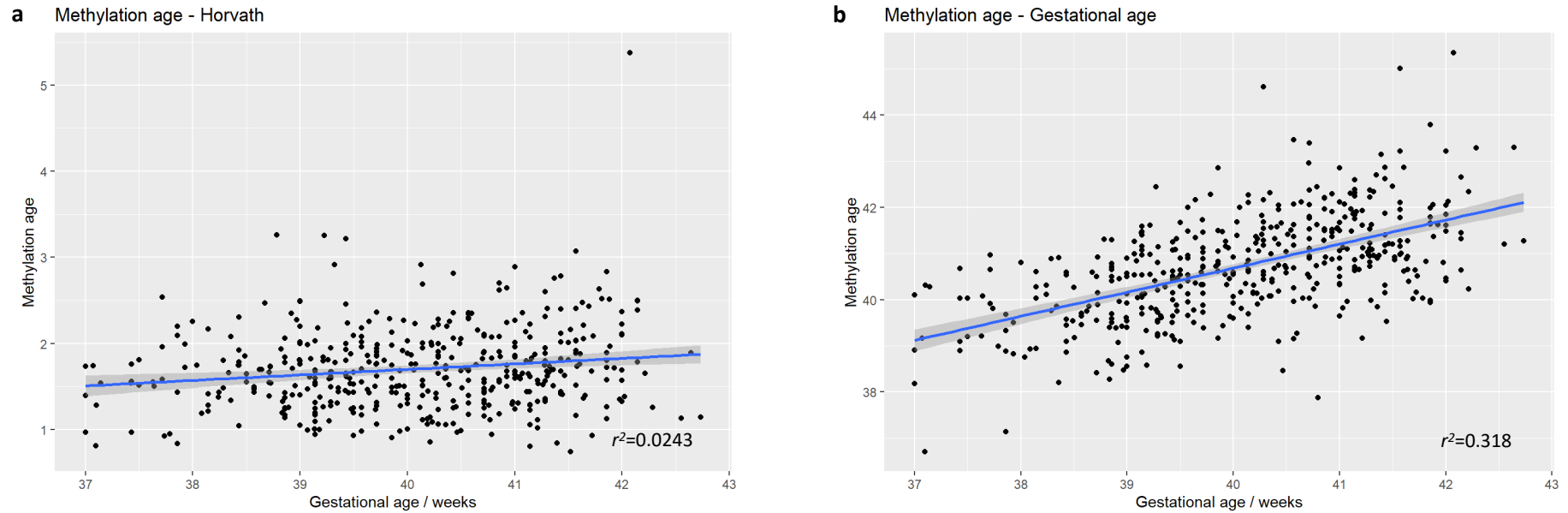


Figure 74. The association between a) epigenetic age and reported gestational age and b) gestational epigenetic age and reported gestational age in the SWS cohort

The associations between a) reported and estimated chronological age as reported by the Horvath clock and b) reported and estimated gestational age reported by the Knight clock within the SWS cohort found estimated epigenetic age to be poorly associated with gestational age and estimated gestational epigenetic ages reasonably associated with gestational age.

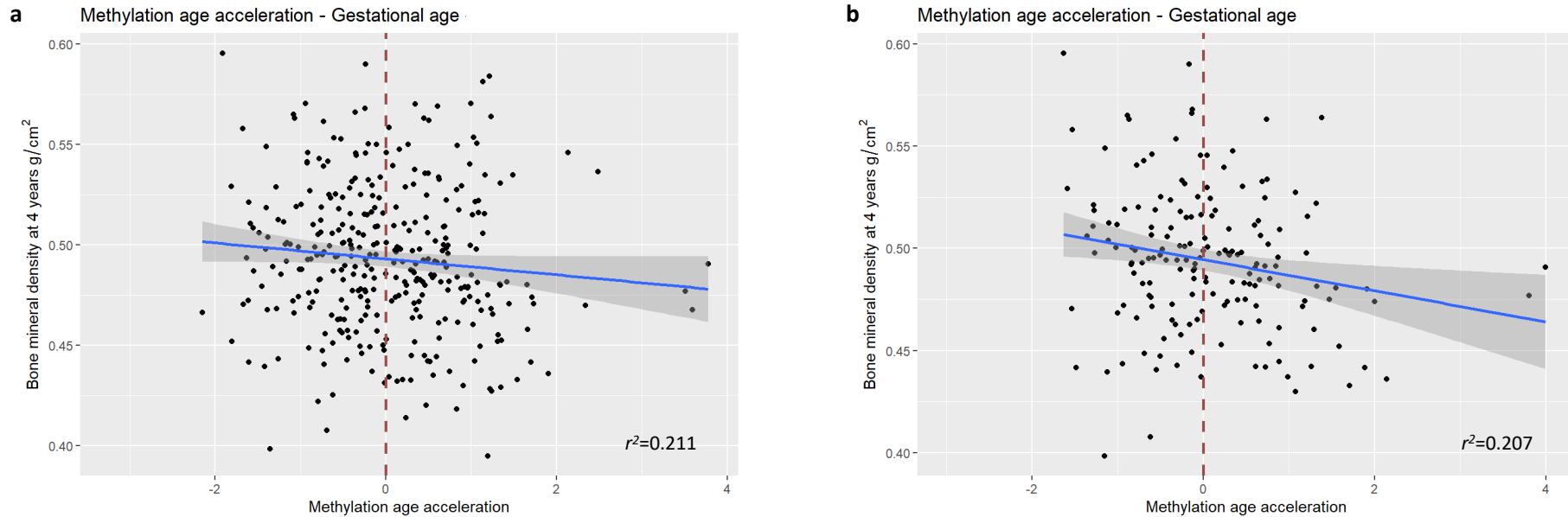


Figure 75. The association between a) gestational age acceleration and BMD at four years of age in both males and females and b) males alone in the SWS cohort

An assessment of the strength of the association between gestational age acceleration and BMD at four years in both sexes and males alone showed a reasonable association in both instances.

7.3 Discussion

7.3.1 Summary

Methylation clocks represent a burgeoning area of research with the potential to predict the risk of a range of conditions and all-cause mortality. Whilst the existence of few, expensive technologies to assess the methylation state of the whole methylome currently means the methylation of only 2% of CpGs been seen to change with age, utilisation of different combinations of these CpGs as methylation clocks has found close associations with both chronological age and a range of conditions, including Alzheimer's disease. Within this study we saw inverse associations between GAA and BMC at birth in the MAVIDOS RCT and between GAA and BMD at four years of age in the SWS study. However, we saw no associations between either AA or GAA and maternal vitamin D supplementation or vitamin D status in the MAVIDOS RCT or SWS study respectively. The estimations of epigenetic age from umbilical cord tissue DNA using the Horvath clock showed no association with gestational age in the MAVIDOS RCT but a significant, albeit a very weak association with gestational age in SWS umbilical cord blood DNA. The Horvath clock was trained on umbilical cord blood and blood spots, whereas the MAVIDOS RCT and SWS studies collected methylation data from umbilical cord tissue and blood alone, which may have affected the strength of the associations. Furthermore the association between epigenetic age and gestational age has previously been shown as near to zero when using the Horvath clock (1001), however this finding is presented in isolation and the Horvath clock was trained on tissues from individuals 0-100 years of age suggesting investigation was warranted. The breadth of research currently existing in this area makes it difficult to interpret the biological implications of our findings, however, the significant associations found may present potential biomarkers for the future.

7.3.1.1 The Horvath epigenetic age clock

A previous study by *Simpkin et al.* (2016) interrogated peripheral blood DNA taken from children and adolescents and using the Horvath clock showed associations between AA and maternal BMI, lower maternal selenium and cholesterol during pregnancy and higher birthweight of the offspring. The authors also showed associations in umbilical cord blood between AA and offspring born to mothers who had smoked during pregnancy or delivered by caesarean section (1001). These findings were technically within the capacity of the Horvath clock algorithm as it was trained on a large range of tissues taken from 0 to 100-year-old individuals, including tissues relevant to this study such as umbilical cord blood and human umbilical vein endothelial cells (HUVECs) (669). Additionally, although the Horvath clock is typically calculated with chronological age, birth tissues effectively have a chronological age of 0 so gestational age is typically used. However, *Knight et al.* (2016) identified in the study by *Simpkin et al.* (2016) that the correlation between epigenetic age according to the Horvath clock and gestational age was close to zero

(672,1001). This would corroborate our findings where within this study we found no significant association was between the epigenetic age of umbilical cord tissue DNA and gestational age within the MAVIDOS RCT but a significant, albeit weak association was found between epigenetic age of umbilical cord blood DNA and gestational age within SWS.

7.3.1.1.1 *The Knight epigenetic gestational age clock*

Knight et al. (2016) highlighted that a system which could accurately classify developmental time and maturity would be invaluable to predict neonatal risk, as differences in gestational age as little as one week have been shown to have a significant impact on neonatal morbidity, mortality and long-term outcomes. Currently gestational age (GA) is estimated using early obstetric ultrasound which is currently the gold standard of measurement or alternatively last menstrual period (LMP) or neonatal estimation. However, ultrasound it is not always available, LMP estimates can be influenced by uncertainty around LMP dates and neonatal estimation can be less precise than the other methods. *Knight et al.* (2016) therefore developed a clock which showed a strong correlation with ultrasound-based measures of GA and the capacity to predict GA where the absolute difference between the epigenetic gestational age and GA was 1.49 weeks. However, whilst AA has been associated with a number of negative outcomes according to the Horvath clock including obesity, lower physical and cognitive function, Alzheimer's disease, HIV, menopause and all-cause mortality (990–992,1002–1004), both GAA and gestational age deceleration (GAD) have been associated with potentially negative outcomes. This falls in line with current research which suggests the association between birthweight and later health outcomes such as cardiovascular risk, type 2 diabetes and term mortality in neonates follows a U-shaped trend where both a high and low birthweight may be associated with an increased risk for poor health outcomes in later life (1005–1007).

In the original publication by *Knight et al.* (2016) the authors describe GAA as associated with birthweight, birth length and head circumference of the child suggesting those with a higher GAA are more developmentally mature and by implication those with GAD developmentally immature (672). *Girchenko et al.* (2017) further built on this work and using the Knight clock found GAA to be associated with a number of maternal and offspring characteristics known to reflect the offspring's exposure to environmental adversity during the prenatal period. These included a number of pre-eclampsia and intrauterine growth restriction pre-pregnancy risk factors, lower 1-min Apgar but also female sex. This suggests GAA may also reflect epigenetic programming by early-life environmental exposures or pregnancy disorders which may compromise neonatal health status. Conversely, GAD was associated with insulin-treated gestational diabetes mellitus (GDM) in a previous pregnancy, Sjögren's syndrome and was also found in a separate study to be associated with antenatal depression (1008,1009). This suggested that beyond an indication of

developmental maturity, both GAA and GAD could be conceived as indicators of risk of future adverse outcomes.

7.3.1.1.2 GAA and skeletal development

Studies haven't yet assessed the association between GAA and postnatal measures of skeletal development, however a number of studies have identified a significant positive association between skeletal development and birthweight (516,517,519,1010,1011). Furthermore, negative associations have also been found between skeletal development and being of a lower developmental maturity at birth, such as being born pre-term, small for gestational age or determined to have experienced restricted growth *in-utero* (515,518,1012). Birthweight could therefore perhaps act as a surrogate to skeletal growth in this work. Other studies, including the original publication of the Knight clock indicate a positive association between GAA and birthweight (996,1008,1013). This is counter to what we have identified in this study where GAA shows a negative, albeit insignificant association with birthweight. However, although *Bright et al.* (2019) found an association between GAA and birthweight in line with previous studies, they also identified that by five years of age this trend began to reverse, such that by 10 years of age this association was completely inverted (672,996,1013). Furthermore, *Bright et al.* (2019) found those with a greater GAA may have a higher birthweight, but experience a slower rate of growth during childhood, perhaps driven by compensatory mechanisms as a result of higher birthweight. With respect to our study, this may suggest a lesser BMD at four years of age in the SWS study being associated with a greater acceleration and vice versa may be indication of compensatory mechanisms attempting to bring bone growth back into equilibrium. However, there may also be a factor influencing GAA that we do not yet know of, such as in *Khouja et al.* (2018) where associations were found between a higher maternal BMI and greater birth size with greater gestational age acceleration, suggesting maternal obesity is associated with more rapid development of the foetus *in-utero* (1013). However, this discrepancy could also be technical. We identified a low correlation between GA and estimated gestational age in the SWS cohort (Figure 74b) and an extremely low correlation in the MAVIDOS RCT (Figure 74a) when compared to the individual datasets in the original publication ($0.52 < r < 0.65$) (672). However, the Knight clock was trained on cord blood and blood spots, not umbilical cord tissue as in the MAVIDOS RCT or umbilical cord blood alone as in SWS, so this may be expected. Additionally, estimations of gestational age can be affected by differences in clinical estimations between studies.

Whilst we may not currently be able to fully appreciate the biological relevance of GAA or GAD in relation to skeletal development, these associations may still present potential utility as biomarkers. This is a developing field of research and more work would be required for methylation clocks to move towards being used as predictive biomarkers in a clinical setting. However, it could be foreseen that if replicated and validated in a large enough cohort, age

acceleration values could be calculated at birth and if they fall into a particular reference range, potentially provide a prospective estimation of BMD at four years of age for example.

7.3.1.1.3 GAA and Vitamin D

Within this study we saw no associations between GAA and maternal vitamin D supplementation in the MAVIDOS RCT or GAA and maternal late-stage pregnancy vitamin D status in SWS. This lack of significance with respect to maternal vitamin D supplementation in the MAVIDOS RCT could be due to a very weak association between GA and epigenetic GA (Figure 72b) from which GAA was calculated. Also, the Knight clock was trained on umbilical cord blood and blood spots, so a weaker association may be expected when conducted on umbilical cord tissue DNA extracted from the MAVIDOS RCT, or the associations may also simply not exist.

However, whilst we did not find any association between GAA and maternal vitamin D supplementation in the MAVIDOS RCT, a study by *Chen et al.* (2020) has shown an association between maternal vitamin D supplementation and a decreased GAA in offspring. However, in a multi-ethnic cohort consisting of Caucasian, Hispanic and African American participants, significant associations were seen between GAA and maternal vitamin D supplementation of the African Americans participants alone (999). There are a number of characteristics which can affect an individual's response to vitamin D supplementation in the form of a rise in serum 25(OH)D concentration, some of the most critical of which are baseline vitamin D and ethnicity (1014). The hydroxylation of vitamin D in the liver has been suggested to be a process which can be saturated, therefore the higher the initial serum 25(OH)D concentration, the more quickly a plateau will be reached upon supplementation with vitamin D and a lesser the 'benefit' conferred. Ethnicity is significant as sunlight is the major source of vitamin D in the northern latitude countries, but the melanin content of the skin will dictate the amount of sunlight that reach the lower layers of the skin where vitamin D synthesis takes place (1015). This has a significant enough effect that it has been suggested African American populations in North America may fail to achieve optimal serum 25(OH)D concentrations at any period during the year (1016). This suggests this subset of the study participants will have begun the study with the lowest baseline serum 25(OH)D concentration and indeed that was found to be the case, where the circulating concentration of 25(OH)D in African Americans was significantly lower than Hispanics and whites ($p = 0.001$) (1017). They will therefore have received the greatest 'benefit' from supplementation, suggesting that despite a poor correlation with GA, we may not have seen an association with GAA in the SWS study as a primarily Caucasian cohort. However a potential way to mirror this in the MAVIDOS RCT would be to assess the association between GAA and intervention group only in those born during the Winter months. This would be a subset of the study which were largely deficient in serum 25(OH)D in the placebo group and replete in the vitamin D supplemented group, however this will reduce numbers and the power of the analysis.

Chapter 7

In contrast to the MAVIDOS RCT we saw a much stronger association between GA and epigenetic GA for the SWS cohort (Figure 74b), but we also found no significant association between GAA and maternal late-stage pregnancy vitamin D (MLSP D) status. Whilst a stronger association than seen between GA and epigenetic GA in the MAVIDOS RCT, the association between the two factors in the SWS cohort was not as strong as the associations in the original publication of the clock, which may have affected the strength of this analysis.

7.3.2 Future work

Whilst this study did find associations between BMC at birth in the MAVIDOS RCT and BMD at four years of age in the SWS cohort, there were no associations found between GAA and maternal vitamin D supplementation in the MAVIDOS RCT or MLSP D in the SWS cohort. The strength of these associations, or the significance of other currently nonsignificant associations may potentially be strengthened by replication in a larger, more ethnically diverse cohort as the findings of *Chen et al.* (2020) suggest those with darker skin pigmentation have a more acute response to vitamin D supplementation in terms of slowing of the methylation clock (1017). However, this analysis could also be mirrored in the MAVIDOS RCT by assessing only those born in the Winter months as this is when we see the most profound insufficiency in the placebo group and sufficiency in the supplemented group, although this would also reduce numbers significantly and so potentially impact the power of the analysis.

7.3.3 Limitations

7.3.3.1 Horvath clock

No associations were found with between AA and gestational age with respect to either the MAVIDOS RCT or SWS study according to the Horvath clock. There are a number of limitations which may impact the analyses in this study. Methylation clocks, of which there are now many, differ in the platform used, the tissue used in training, the number of CpG sites in the algorithm, the overall accuracy and the outcome. The Horvath clock was developed on a wide range of tissues, making it one of the most versatile clocks in use. However, whilst the Horvath clock was trained on umbilical cord blood and HUVEC cells during development, this didn't include umbilical cord tissue, as collected by the MAVIDOS RCT (669). As we've seen both within the context of current scientific literature and this study, the epigenetic profile of umbilical cord tissue and umbilical cord blood are largely distinct (458), suggesting achieving a correlation between age and epigenetic age comparable to the original publication of the clock may always have been difficult.

In contrast, the SWS cohort collected umbilical cord blood, which should be suitable for analysis using the Horvath clock. We did indeed see a significant correlation between age and epigenetic age with respect to the SWS cohort ($p\text{-value} = 5.72e^{-4}$), however, the correlation itself was very weak ($r=0.0243$), a finding corroborated by another study by *Simpkin et al.* (2016) where the

correlation was also almost zero (1001). This may be due to the fact that despite the Horvath clock being trained on a dataset including birth tissues, these tissues were given an age of zero during training, meaning this clock cannot distinguish between gestational ages and therefore unlikely to produce meaningful results within this study (669).

7.3.3.2 *Knight clock*

Despite showing a significant association between GAA and BMC at birth in the MAVIDOS RCT the initial calculation of the correlation between GA and epigenetic GA was very low ($r=1.63e^{-4}$), meaning it's unlikely we may be able to trust the veracity of this finding. In the SWS cohort we saw a reasonable association between GA and epigenetic GA, however it was lower than the correlation reported in the original publication ($r=0.024$). The Knight clock, similar to Horvath clock was trained on umbilical cord blood, but with the addition of neonatal blood spots (672). This may be a perhaps be a potential reason for the significant, albeit very weak association seen in the MAVIDOS RCT as this study collected cord tissue and the stronger association seen in the SWS cohort which was umbilical cord blood alone. However, with respect to the SWS cohort there may also be unforeseen issues such as foetal cord blood contamination with maternal blood (1018), or technical issues that may not be apparent until more studies are conducted.

7.3.4 *Conclusion*

Whilst we did see associations between GAA and BMC at birth in the MAVIDOS RCT and BMD at four years of age in the SWS cohort, where both associations showed an acceleration in GAA with decreasing postnatal bone measures, the biological implications of these changes are not yet clear. An acceleration in GA has been interpreted as an indication of exposure to an adverse prenatal environment *in utero*, but also a sign of developmental maturity. As mentioned previously we could be therefore seeing the potential compensatory activity acting to try and bring a low bone BMD back into equilibrium, or this may be an indication that greater acceleration at birth is associated with a lower BMD at birth or four years of age. Nonetheless, as a new field of research, further work will be required to established for these associations to be understood fully. However, if these associations can be reproduced and validated in multiple, larger datasets, a reference range could be established for this methylation clock at birth to potentially act as a prospective biomarker for BMD at four years of age. In contribution to the overall hypothesis of this dissertation, this work provides evidence of an association between the methylation of birth tissues and postnatal bone measures, indicating the potential influence of the early life environment on both the methylome and postnatal bone development.

8 Chapter 8 - Final discussion

8.1 Summary of main findings

Studies in both animal and human models have suggested that environmental exposures in prenatal life may influence the trajectory of skeletal development in postnatal life through epigenetic mechanisms, consistent with the developmental origins of health and disease (DOHaD) hypothesis (524,529,530,641). As a factor critical to the development and mineralisation of the foetal skeleton it has been suggested that maternal vitamin D may be an exposure that through a period of foetal programming *in utero* may directly, or indirectly influence postnatal bone mineral content (BMC) or bone mineral density (BMD) in the offspring (1019). Within this study we aimed to identifying the epigenetic signatures in prenatal life potentially associated with postnatal skeletal development and the possible overlap with maternal vitamin D associated or induced changes. This may not only allow us to develop markers which could potentially predict the trajectory of bone growth in postnatal life, but lay the groundwork for an investigation into the biological significance of potential vitamin D associated or induced changes which may enhance skeletal development. This would not only potentially allow us to identify those with a high risk of developing a sub-optimal peak bone mass, but potentially intervene to facilitate bone formation before it develops into osteoporosis.

The aim of this study was therefore to use umbilical cord tissue and blood as surrogates of mesodermal tissue genetically derived from the neonate to establish whether i) DNA methylation in birth tissues is associated with BMC or BMD in postnatal development and ii) whether maternal vitamin D supplementation alters DNA methylation in genes within birth tissues which may be associated with improved BMC or BMD in postnatal development. To do so I outlined 5 aims:

Aim 1 was to identify DNA methylation signatures in umbilical cord blood DNA associated with offspring BMC or BMD at birth, four, six or eight years of age in the Southampton Women's Survey (SWS) cohort (section 3.2). The key findings are summarised below:

- Significant differential methylation changes in umbilical cord blood DNA associated with BMD at birth and eight years of age.
- There was overlap of the top differentially methylated cytosine-phosphate-guanine dinucleotides (dmCpGs) associated with BMC and BMD at four years of age, but the overlap was minimal between the different age groups.
- Transcription factor enrichment analysis found significant enrichment of the top 100 dmCpGs associated with BMC at four years, BMD at birth, four and six years of age with transcription factor binding motifs including T cell factor 7 (TCF7), enhancer of zeste homolog 2 (EZH2), ten-eleven translocase (TET), and octamer-binding transcription factor 4 (OCT4).

Aim 2, investigated in chapter 4 was to investigate i) Whether the supplementation of mothers with a placebo or 1000 IU/day vitamin D intervention during pregnancy altered umbilical cord tissue DNA methylation in the Maternal Vitamin D Osteoporosis Study (MAVIDOS) randomised controlled trial (RCT) and ii) whether this was similar for births where mothers were insufficient for vitamin D in the early stages of pregnancy also within the MAVIDOS RCT. Finally, iii) Whether associations exist between umbilical cord blood DNA methylation and maternal late-stage pregnancy vitamin D status in the SWS cohort. The key findings are summarised below:

- Differential methylation analyses with respect to both intervention group and intervention group for mothers insufficient for vitamin D in early pregnancy found vitamin D supplementation induced changes in umbilical cord tissue DNA methylation within the MAVIDOS RCT. Significant differential methylation changes associated the intervention analysis were potentially pro-osteogenic, one of which overlapped with the intervention maternal early-stage pregnancy vitamin D (MESP D) insufficient analysis.
- The dmCpGs associated with the intervention group analysis were enriched for pathways integral to skeletal and general development such as Wnt/ β -catenin, nuclear factor kappa B (NF- κ B) signalling but also for peroxisomal proliferator-activated receptor- α (PPAR α)/retinoid X receptor- α (RXR α) activation, a heterodimer primarily involved in the regulation of lipid metabolism and homeostasis, all of which can be influenced by vitamin D.
- Significant differential methylation changes in umbilical cord blood DNA were associated with maternal late-stage pregnancy vitamin D (MLSP D) insufficiency in the SWS cohort. DmCpGs were found to be only weakly associated with the MLSP D status (FDR<0.20) and MLSP D sufficiency (FDR<0.15) analyses.
- The most significant dmCpG associated with the MLSP D insufficient analysis was within Hexokinase1 (*HK1*), a rate-limiting gene in glycolysis. The most dmCpG most strongly associated with MLSP D status was Thioredoxin-interacting protein (TXNIP), where TXNIP deficiency is associated with an increased cellular glucose uptake and metabolism.

Aim 3, investigated in chapter 5 was to identify DNA methylation signatures in MAVIDOS RCT umbilical cord DNA associated with postnatal BMC or BMD at the ages of birth and four years of age. The key findings are summarised below:

- Significant differential methylation changes were found to be associated with BMC at four years of age and BMD at both birth and four years of age.
- The dmCpGs associated with BMC at four years of age and BMD at birth and four years of age showed little overlap with the SWS study. However an analysis of the overlap between BMC or BM D and intervention-related analyses within the MAVIDOS RCT saw a number of differential methylation changes in the *PTPRN2* gene, which encodes both a gene which facilitates glucose homeostasis and a miRNA, miR-153 which is a negative

regulator of insulin secretion and anti-osteogenic in human bone marrow mesenchymal stromal cells (BM-MSCs).

Aim 4, investigated in chapter 6 was to utilise umbilical cord mesenchymal stromal cells (UC-MSCs) obtained from American type culture collections (ATCC) and umbilical cord tissue from the Southampton Pregnancy Intervention for the Next Generation (SPRING) study to determine whether short or long-term supplementation of UC-MSCs with vitamin D alters the differentiation potential and DNA methylation of UC-MSCs *in vitro*. The key findings are summarised below:

- Cytochrome P450 Family 24 Subfamily A Member 1 (CYP24A1) mRNA induction provides a reliable indication of the $1\alpha,25\text{-dihydroxyvitamin D}_3$ ($1,25(\text{OH})_2\text{D}$)-mediated induction of vitamin D responsive pathways in both UC-MSC and osteosarcoma cell lines.
- There were 79 significant differential methylated CpGs in the DNA of UC-MSCs obtained from ATCC associated with short-term $1,25(\text{OH})_2\text{D}$ treatment *in vitro*. The most significant differentially methylated CpGs included myosin light chain kinase 3 (MYLK3), transforming growth factor receptor β 2 (TGF β R2) and twist basic helix-loop-helix transcription factor 1 (TWIST1).
- Pathway enrichment analysis of the top 100 dmCpGs associated with the short-term vitamin D treatment of UC-MSCs showed enrichment for processes related to cellular respiration and the glycolytic pathway. Vitamin D has been seen to upregulate glucose transporter type 4 (GLUT4) expression, translocation to the cell surface and glucose utilisation in a pre-adipocytic cell line. This could have implications for osteogenic differentiation, or simply enhance basal respiration if replicated in UC-MSCs.

Aim 5, investigated in chapter 7 was to calculate both epigenetic age and epigenetic gestational age in both MAVIDOS RCT umbilical cord tissue DNA and SWS umbilical cord blood DNA. Using previously published methods we then aimed to estimate both age acceleration (AA) and gestational age acceleration (GAA) and assess i) the effects of maternal vitamin D supplementation on AA and GAA in the MAVIDOS RCT, ii) the relationship between AA and GAA with postnatal bone measures of BMC and BMD at the ages of birth and four years of age in the MAVIDOS RCT, iii) the effect maternal vitamin D status on AA and GAA in the SWS cohort, iv) the relationship between AA and GAA with postnatal measures of BMC and BMD at the ages of birth, four, six and eight years of age in the SWS cohort. The key findings are summarised below:

- Increased acceleration of the gestational age clock was seen to be associated with a decreased BMD at four years of age in the MAVIDOS RCT.
- Increased acceleration of the gestational age clock was seen to be associated with a decreased BMC at birth in SWS.
- There was no association between AA or GAA and vitamin D supplementation in the MAVIDOS RCT or MLSP D status in the SWS study.

8.2 Discussion of main findings

8.2.1 Biomarkers

Maximising peak bone mass (PBM) in early life can significantly reduce the risk of osteoporotic fracture in adulthood, such that increasing PBM by 10% could reduce the risk of osteoporotic fracture in post-menopausal women by up to 50% (5,1020–1022). A prognostic biomarker identifiable from birth tissues that could identify a deficiency in the accumulation of bone mass before PBM was achieved could therefore be invaluable in determining whether an intervention, such as supplementation or medication could be used to bolster skeletal development and so reduce the chance of developing osteoporosis later in life. Epigenetic biomarkers based on methylation are currently in use for early cancer diagnosis and approved by the US Food and Drug Administration such as Epi proColon, an assay that detects methylated Septin-9 in DNA obtained from peripheral blood samples for the early detection of colorectal cancer (1023). As a biomarker, a dmCpG would require extensive validation, but this is becoming more viable with the 450k and 850k arrays.

8.2.1.1 Individual dmCpGs associated with postnatal BMC or BMD

Work within the SWS study has previously identified perinatal methylation changes in umbilical cord blood DNA within the cyclin-dependant kinase N2A (*CDKN2A*) gene and the promoter region of the *RXRα* gene as markers associated with postnatal bone outcomes (2,3,745). Whilst we are not able to validate these methylation changes as the CpG sites are not present on the Illumina Infinium MethylationEPIC BeadChip (EPIC) array, here, we have found further epigenetic signatures associated with postnatal measures BMC and BMD within the SWS cohort and MAVIDOS RCT which could potentially act as prospective biomarkers of bone development.

Interrogation of umbilical cord blood DNA extracted from the SWS cohort found significant perinatal methylation changes associated with both BMD at birth and eight years of age. Within the MAVIDOS RCT interrogation of umbilical cord tissue DNA found significant associations with BMC at four years, BMD at birth and four years of age. A biomarker associated with BMD at birth may be of limited clinical utility as BMC and BMD can both be measured at birth and reference ranges exist for what may be considered appropriate for gestational age (1024). However, a prognostic measure of BMC or BMD at four or eight years of age could prospectively be compared against a reference range for the age to indicate the trajectory for bone growth. Furthermore, dual energy X-ray absorptiometry (DXA) at eight years has been suggested to be predictive of fracture risk during puberty suggesting that whilst greatest bone growth has been indicated as between 12-15 years for girls and 14-17 for boys, this prepubertal period of bone growth is still crucial in skeletal development (1025,1026).

However, the epigenetic markers identified here may be both age and tissue specific. Within the SWS cohort and MAVIDOS RCT, the dmCpGs associated with each analysis with respect to BMC or BMD typically overlapped very little between age groups. This may be due to this period of early life being one of rapid developmental progression. Between the ages birth and four years of age a child will begin to walk, increasing the mechanical loading of the skeleton; additionally, puberty can begin as early as eight years of age. Both mechanical loading and sex hormones play a major role in facilitating skeletal development and both mechanical loading and the onset of puberty have been associated with changes in methylation (694–696,718,1027–1031). This may explain at least in part the apparently distinct set of dmCpGs for these analyses and furthermore, this suggests the activity of distinct processes or pathways behind bone development within each age group, an area which could potentially be explored further. In contrast, comparisons between BMC and BMD within a particular age group typically saw a reasonable overlap between the top 100 dmCpGs associated with each analysis, BMC is however a metric of BMD per unit area, so this would be expected and suggests the epigenome-wide association study (EWAS) has identified consistent changes in DNA methylation associated with these bone measures. To assess whether dmCpGs were common among birth tissues, a comparison of the overlap within age groups between the SWS cohort and MAVIDOS RCT was conducted, finding an overlap of a single cytosine-phosphate-guanine (CpG). This may be largely due to the fact that DNA methylation was analysed in umbilical cord blood in SWS and umbilical cord tissue in the MAVIDOS RCT and DNA methylation is known to be largely cell-type specific (693). Studies comparing the epigenetic profile of both umbilical cord tissue DNA and umbilical cord blood DNA indeed found their DNA methylation profiles were largely distinct. In addition, umbilical cord tissue showed higher inter-individual variability in umbilical cord tissue, potentially making an equitable comparison more difficult (649,693). The next steps to translate the current utility of these markers from associative to predictive tissue and age specific markers of postnatal bone growth would be to replicate further and validate in larger, multiple cohorts. In addition, we have the potential to extend the longitudinal measurements with respect to this work. Whilst DXA measurements at eight years of age have been seen to be potentially predictive of fracture risk during puberty and so relevant as an indicator postnatal bone growth (1025,1026), DXA measurements are currently being cleaned for the SWS study at twelve years of age, within the pubertal period for both boys and girls. Puberty is a period of accelerated bone growth where approximately half of adult skeletal mass is accrued (1032,1033), so a potential estimation of bone growth during this critical period of growth would be invaluable to determining potential risk for future diseases such as osteoporosis and perhaps the need for an early intervention.

8.2.1.2 *Methylation clocks*

In contrast to the individual differential methylation changes described so far, Chapter 7 investigated a burgeoning area of research in the field of epigenetics which utilises the differential

methylation of multiple CpG sites in the genome as markers of epigenetic ageing. Epigenetic age acceleration has been proposed as early biomarker of disease risk, as well as predictors of life expectancy and mortality. AA of the Horvath clock has been seen to associate with obesity, lower physical and cognitive function, Alzheimer's disease, HIV, menopause and all-cause mortality (990–992,1002–1004). However, there are different interpretations of what GAA or gestational age deceleration (GAD) of the Knight clock may mean. In the original publication of the gestational age clock by *Knight et al.* (2016), the authors showed a high correlation between epigenetic GA and GA and hypothesised that the acceleration or deceleration of the clock may correspond with a greater or lesser developmental maturity at birth (672), suggesting deceleration may encompass the risks of a premature birth. However further work by *Girchenko et al.* (2017) indicated variations in GAA of the offspring at birth was associated with a number of maternal and offspring characteristics known to reflect exposure to prenatal environmental adversity (1008). GAA has been associated with maternal characteristics such as maternal body mass index (BMI), smoking during pregnancy but also foetal characteristics such as high birthweight, Down's syndrome and a lower 1-minute APGAR among others (1008,1013,1034). Within this study we found GAA was inversely associated with BMD at four years of age in the SWS cohort, which according to *Knight et al.* (2016) would suggest that a higher developmental maturity at birth is associated with a lower BMD at four years (672). This initially seems counter-intuitive as a higher maturity at birth has been linked to a greater birthweight, which typically correlates with a greater bone mass in childhood (1,515,516,518,519,1011). However, in an analysis of the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort, *Bright et al.* (2019) found the relationship between GAA and birthweight attenuates and inverts by five years of age, such that by ten years of age greater GAA is associated with lower childhood weight. The authors further suggest that whilst those with a greater GAA may have a higher birthweight, this may activate biological compensatory mechanisms which lead to a slower rate of growth during childhood (996). With respect to our study, this may suggest a decreased BMD at four years of age in the SWS study is associated with a greater acceleration and vice versa, which may be indication of compensatory mechanisms attempting to bring bone growth back into equilibrium. As a new area of research, the number of studies investigating the relationship between GAA and postnatal bone growth with which to compare this finding are limited, making it difficult to contextualise in current research. However, it could be foreseen that if replicated and validated in a large enough cohort, GAA values could be calculated at birth and if they fall into a particular reference range, potentially provide a prospective estimation of BMD at four years of age. This is a developing field, and more research is required for methylation clocks to move towards being used as predictive biomarkers in a clinical setting.

8.2.2 *Biological implications of differential methylation changes within umbilical cord blood and tissue DNA*

Whilst we identified a number of differential methylation changes in both the SWS cohort and MAVIDOS RCT that could act as potential prospective biomarkers of postnatal BMC or BMD, these markers may be the result of a bystander effect, a consequence of change in phenotype or may represent differential methylation changes on a functional pathway which could have potential biological and functional implications for skeletal development. However, discerning the functional implications of differential methylation changes within both umbilical cord tissue and umbilical cord blood can be problematic. These tissues are composed of a number of cell types and this mixture of DNA will have been interrogated by the EPIC array, making it difficult to attribute these methylation changes to a single cell type, especially when factors such as vitamin D can affect the activity of a number of cell types present in both umbilical cord tissue and blood (781,1035–1039). However, as the largest proportion of the structure of the umbilical cord, the connective tissue, or Wharton's Jelly has been found to be a comparatively rich in mesenchymal stromal cells genetically derived from the foetus (412,431,917,1040). Furthermore, when comparing the epigenetic profiles of both umbilical cord tissue and blood to 26 primary tissue or cell types mapped by the Epigenome Roadmap project, a study by *Lin et al.* (2017) found umbilical cord tissue most similar to tissues derived from the MSC-derived mesoderm, the germ layer from which bone develops. Umbilical cord blood however was found to cluster more closely with cells of a hematopoietic stem cell-derived mesoderm. MSCs have been reproducibly cultured from umbilical cord blood, but with a much lower yield than umbilical cord tissue (458,465,1041–1043). This suggests that at least in umbilical cord tissue, the methylation changes may reflect changes within UC-MSCs. Such changes may also potentially therefore reflect early environmental exposures on either the three germ layers or similar epigenetic changes exerted across tissues during *in utero* life. It is also intriguing that any of the methylation changes observed in umbilical cord tissue, umbilical cord blood and UC-MSC cells affected genes in similar pathways.

8.2.2.1 *Glucose homeostasis*

The EWAS investigating the effects of short-term treatment of UC-MSCs with 1,25(OH)₂D *in vitro*, along with the EWAS investigating MLSP D status and MLSP D insufficiency in SWS all indicated possible associations between vitamin D status and the differential methylation of CpGs associated with glucose homeostasis and metabolism. When investigating the effect of short-term 1,25(OH)₂D treatment of ATCC UC-MSC cells for 24 hours in section 6.3.1, EPIC array interrogation of the cellular DNA saw the differential methylation of CpGs enriched for processes related to cellular respiration and the glycolytic pathway. Vitamin D has previously been identified to upregulate the expression of the glucose transporter GLUT4 and enhance glucose utilisation in a number of cell types, including pre-adipocytic cells (961–963). When investigating associations

between umbilical cord blood DNA methylation and MLSP D insufficiency (<50 nmol/l) in the SWS study (section 4.2.5) the most significant dmCpG was *HK1*, a rate-limiting gene in glycolysis which was negatively associated with maternal vitamin D status. In addition, the dmCpG most strongly associated with the MLSP D status, an analysis where the cohort was sufficient for vitamin D (>50 nmol/l) (Table 5) was *TXNIP*, a gene of which deficiency has been associated with an increased cellular glucose uptake and metabolism and positively associated with maternal vitamin D status.

The dmCpGs within *HK1* and *TXNIP* were both within the gene body. This raises an issue for interpretation in relation to the potential effects of these methylation changes on transcriptional activity as gene body or intragenic methylation has been suggested to be, but not always associated with an increase in transcriptional activity. However, assuming intragenic methylation increases transcriptional activity, these analyses suggest that a vitamin D may facilitate differential methylation changes which allow for a greater level of glucose uptake and metabolism. When replete with vitamin D *TXNIP* expression may act as negative feedback to glucose uptake and metabolism, whereas when insufficient with vitamin D, we may see differential methylation changes which may upregulate glycolytic activity. However these differential methylation changes do not imply causality and this potential interrelationship would need therefore to be validated with further functional experimentation. If this is established and these changes are indeed occurring within UC-MSCs, this could be consequential for the osteoblastic differentiation of MSCs which has been seen to favour high glucose (1044,1045).

8.2.2.2 Vitamin D induced changes in genes required for osteogenesis

EPIC array interrogation of DNA taken from MAVIDOS RCT umbilical cord tissue saw significant, maternal vitamin D supplementation induced differential methylation changes within two potentially pro-osteogenic genes, Solute Carrier Family 38 Member 10 (*SLC38A10*) and Tenascin C (*TNC*). The knockout of *SLC38A10* in a murine model, *Bassett et al.* (2012) found mice had a reduced BMC, shorter stature and a weakened, brittle bone matrix as a result of a markedly impaired bone formation. This was suggested to be due to the potential role of *SLC38A10* as a regulator of cell volume expansion during the hypertrophy of chondrocytes during endochondral ossification. Further work by the same group *in vitro* showed osteoblasts extracted from these mice exhibited reduced proliferation, downregulation of five integrin subunits and two chemokines which regulate integrin expression in a number of cell types (759,1046). Integrins are essential for osteoblast proliferation, adhesion to extracellular matrix, differentiation and mineralisation suggesting *SCL38A10* may in-part regulate osteoblast proliferation and differentiation through altering integrin expression. Furthermore, the intervention within this analysis showed enrichment for a wide range of cell adhesion and binding processes and in a separate study *Posa et al.* (2018) suggest that vitamin D can promote the osteogenic differentiation of MSCs through the modulation of integrin expression (1047). This work i)

potentially supports part two of the hypothesis for this thesis that maternal vitamin D supplementation alters DNA methylation in genes within birth tissues which may be associated with improved BMC or BMD in postnatal development, but also ii) collectively presents an avenue of research to further explore the potential mechanisms through which vitamin D may facilitate the osteoblastic differentiation of MSCs.

8.2.2.3 *The differential methylation of PTRN2*

Whilst we identified potentially pro-osteogenic, maternal vitamin D supplementation induced methylation changes in umbilical cord tissue DNA within the MAVIDOS RCT, we also potentially identified an indirect interaction between both maternal vitamin D supplementation and offspring postnatal bone growth. A comparison of the overlap between the top dmCpGs associated with the differential methylation analyses for BMC at four years, BMD at birth and both the intervention-related analyses within the MAVIDOS RCT showed the differential methylation of multiple CpG sites within the *PTPRN2* gene locus. The protein encoded by the protein tyrosine phosphatase receptor type N2 (*PTPRN2*) gene, known as IA-2 β , has been implicated insulin secretion and so plays a role in glucose homeostasis, but curiously the gene also encodes an intronic miRNA which is a negative regulator of insulin secretion and also of osteoblastogenesis in BM-MSCs. The coding region of miR-153 has been seen to lack a classical TSS but has been shown to contain a CpG island, the differential methylation of which has been seen to regulate its expression, suggesting miR-153 expression can be regulated independently of IA-2 β expression in a tissue-specific manner (908–910). Further work will therefore be critical to establish i) where these CpGs lie both relative to *IA-2 β* gene structure and the transcribed region of miR-153, ii) the effect of differential methylation on transcriptional activity. Without this information it would be difficult to infer the biological implications of these changes. This, along with the other methylation changes of potential biological consequence in this thesis provide a basis from which to investigate the functional relevance of these changes *in vitro*.

8.2.3 *Short and long-term supplementation of UC-MSCs with 1,25(OH) $_2$ D*

Within this study we are using umbilical cord cells, blood and tissue as they are comparably easy to obtain when compared to foetal BM-MSCs or bone marrow. Additionally, umbilical cord blood and UC-MSCs are genetically derived from the foetus and a study by *Lin et al.* (2017) has suggested the methylation profile of umbilical cord blood and tissue makes them a good surrogate for tissues derived from the mesoderm, such as bone (458). This makes these cells an attractive tissue by which to investigate the potential influence of the environment *in-utero* on postnatal development. However ultimately, due to the heterogenous cellular composition of umbilical cord tissue and blood, an EPIC array interrogation of DNA extracted from these tissues and a subsequent differential methylation analysis may not reflect methylation changes in UC-MSCs alone. Therefore, when investigating the effect of maternal vitamin D supplementation on

umbilical cord tissue methylation within the MAVIDOS RCT in section 4.2.4, this would make interpreting the potential biological implications of these changes problematic. To circumvent this issue we aimed to isolate and enrich UC-MSCs from umbilical cord tissue taken from the SPRING RCT as described in section 6.2.2. Similar to the MAVIDOS RCT, SPRING RCT mothers were supplemented with 1000 IU/day vitamin D or a placebo, but half of the vitamin D and placebo supplemented groups were given healthy conversation skills support, aimed at improving the quality of the pregnant mothers diet (626). Whilst the study design is not identical, we could therefore have potentially compared the methylation profile of umbilical cord tissue DNA from the MAVIDOS RCT and the methylation profile of UC-MSC DNA extracted from umbilical cord tissue taken from the SPRING RCT to identify similarities. This may have allowed us to identify methylation changes in umbilical cord tissue which we could suggest may potentially be originating within the UC-MSC fraction of the tissue as a result of maternal vitamin D supplementation. As umbilical cord tissue is foetal and a surrogate for tissue derived from the mesoderm (458) this may have allowed us to infer the potential consequence of vitamin D supplementation on skeletal growth. However, as we are investigating skeletal development, the implications of methylation changes may need to be further validated *in vitro* within BM-MSCs and *in vivo*. MSCs have been suggested to have an epigenetic ‘memory’ which may predispose them to differentiate along a particular lineage, depending on their tissue of origin (915). Additionally, BM-MSC differentiation *in vitro* mimics that of intramembranous ossification and not endochondral ossification where an osteochondral progenitor, perichondral and preosteoblastic cells will also be intermediates in the differentiation process, therefore these changes would have to be ultimately modelled *in vivo* (13).

Whilst this portion of the work plan for this thesis wasn’t possible due to unforeseen circumstances meaning a lack of access to the lab, we did observe enrichment of significant differential methylation changes in UC-MSCs suggesting an enhancement of cellular respiration and glycolytic activity as a result of short-term vitamin D supplementation which complemented previous work in the SWS cohort, which found maternal vitamin D status potentially linked to glycolytic activity (section 4.2.5). Furthermore, we saw significant differential methylation in the promoter region of *TWIST1* as a result of vitamin D supplementation. Overexpression of *TWIST1* within BM-MSCs both *in vivo* and *in vitro* has been shown to decrease their osteogenic differentiation potential and enhance their adipogenic differentiation potential (953,954).

8.2.4 Further work

8.2.4.1 Osteoclast, osteocyte methylation and the bone remodelling process

The bone remodelling cycle involves the interplay of various cell types, including osteoblasts, osteoclasts and osteocytes. Whilst this dissertation has focused on MSC and osteoblastic biology, examples have been presented of methylation changes which could also potentially influence

osteoclastic and osteocytic biology and so possibly impact the bone remodelling process later in development. When assessing the top 100 dmCpGs associated with BMC at four years of age in the SWS cohort differential methylation of cg18397653 (FDR=0.2131) was seen within the five prime untranslated region (5'UTR) region of the DMP-1 gene (appendix 9.4.1.1.1). Methylation was increased at this CpG, a mark typically associated with repression of expression. Methylation of the dmCpG also showed a positive association with increasing BMC at four years of age (appendix 9.13). As discussed previously, dentin matrix acidic phosphoprotein 1 (DMP1) is expressed predominantly by osteocytes and deletion of DMP1 has been seen to lead to X-linked hypophosphatemic rickets, perhaps owing to its role in facilitating the mineralisation of bone through the regulation of phosphate homeostasis (103,108). However whilst its primary role is in regulating mineralisation, a study by Sun *et al.* (2015) demonstrated mice generated with an irregular glycosylation of the gene showed developmental alterations which included changes in the speed of bone remodelling and mineralisation, irregular osteocytic morphology and abnormal osteocytic, osteoblast and osteoclast activity (1048). This suggests the expression of this gene may play an as of yet unknown role in the regulation of the bone remodelling process which could potentially be explored further. Within section 4.2.5.3 there was enrichment of nuclear factor of activated T cells 1 (NFATC1) transcription factor binding sites in the top 100 dmCpGs associated with MLSP D sufficient mothers. Depending on the location and direction of the methylation changes within these genes, this may have implications for NFATC1 activity which as the master regulator of osteoclastic differentiation (1049) may either significantly enhance or suppress osteoclastic differentiation, potentially leading to an imbalance in the bone remodelling process. Similarly, when assessing the most significantly differentially methylated CpGs in cord tissue associated with BMC at four years of age in the MAVIDOS RCT (section 5.2.2.1) we saw the differential methylation of CD82. In a mouse model generated by Bergsma *et al.* (2018), CD82 deletion led to the development of bones smaller in diameter and weaker, but no changes were observed in bone density. This was suggested by authors to be both due to a fall in osteoclast number and deficiencies in osteoblast function (860,861). The reduction in methylation observed at the CpG site in the 5'UTR region of the CD82 gene is typically a change associated with an increase in expression, therefore we may see effects reciprocal to deletion, however this does not preclude the existence on a negative feedback mechanism to limit its expression.

The interpretation of these marks with respect to either osteoclastic or osteocytic biology and so the potential effect on the bone remodelling process however is heavily reliant on a number of factors. To interpret the implications of a differential methylation mark on osteocytic biology it would initially need to be established whether we have observed the differential methylation within the MSC fraction of the tissue. Furthermore, it would need to be established whether the methylation of a mark established in an MSC population could persist into the osteocytic stage of differentiation for this cell type lineage. Similarly, if attempting to interpret the effect of

differential methylation on osteoclastic biology, it would initially need to be established whether we have observed the differential methylation within an HSC or osteoclast precursor population fraction of the tissue. Perhaps most significantly however, it would need to be ascertained whether differential methylation of these marks within the studied cell type influences active transcription of the gene in which differential methylation has occurred, or whether the change may be as a result of a bystander effect, unrelated to the transcription of the gene entirely.

Nonetheless, whilst there was not scope within the context of this dissertation to fully address the potential implication of these methylation changes in occurring in osteoclastic precursors or the implications of changes in osteocytic cells, here, we have seen examples of findings which could provide a base from which to further explore this in the future.

8.2.4.2 *Chromatin conformation*

The EPIC array also interrogates a number of CpGs within enhancer regions (647), many of which are currently not annotated to any known gene. All but one differential methylation analysis within this thesis found intergenic CpG sites differentially methylated. These dmCpGs are valuable in their utility as potential biomarkers, however, understanding the biological relevance of these changes will be difficult without knowing how these sites functionally interact with the rest of the genome.

Enhancer regions can increase transcription of their target genes in *cis* but can be 100 bp to Mb away from the regulated gene on the same chromosome. This means a change in chromatin conformation, or looping is often required for activation to occur, which can be identified by chromatin conformation capture technologies (1050,1051). Techniques such as 4C, or one vs. all could be used to validate these intergenic, or potential enhancer region CpG in the future alongside any other methylation array work (1052).

8.2.4.3 *Chromatin accessibility, methylome, transcriptome*

An issue when trying to interpret differential methylation changes is discerning whether these changes are functionally inconsequential or whether they may have a significant effect on the transcriptional activity of a gene with which it is associated. Furthermore, whilst there is a minimal criteria for the classification of multipotent mesenchymal stromal cells defined by the ISCT (443,446), these are far from definitive. MSCs are a heterogeneous population stromal cells the characteristic of which can vary by donor source, tissue source or isolation and culture technique which can lead to the selection of a mixture of cells rather than a homogenous culture. Single-cell sequencing technologies such as single-cell nucleosome, methylation and transcription sequencing (scNMT-seq) may alleviate both these issues (851). When discerning the effect of an extrinsic factor such as vitamin D on a cultured set of cells, we would potentially be able to obtain methylation and corresponding gene expression data for a given CpG locus in one instance, but

also chromatin accessibility data to ascertain whether the region is transcriptionally active. The additional benefit of single-cell sequencing would be the ability to cluster cells into distinct populations based on their transcriptome. Until more definitive criteria, or reliable, reproducible techniques are developed for the isolation, enrichment and culture of a homogenous population of MSCs, techniques such as this may allow for the researcher to gain an appreciation of the composition of their MSC culture. This could help a researcher refine their isolation procedure to reach a more homogenous, consistent culture of cells, identify commonalities in cultures from different extraction sites, but also potentially allow for an equitable comparison of cultures between labs.

8.2.4.4 *Gene, environment, methylation (GEM)*

Phenotype ultimately depends on the interaction between specific combinations of epigenetic components, genome composition and environmental inputs (676). Epigenetic modifications can occur in response to environmental stimuli, however the epigenome can also be influenced by genetic variants. Both SNPs and copy-number variations and other modifications can lead to epigenetic variation. SNPs that associate with quantitative changes in methylation level are known as methylation quantitative trait loci (meQTL). Whilst only a small proportion of all CpGs are known to be associated with meQTLs, they can have a dramatic effect on methylation level, between 10-97%. Therefore it is important to determine which methylation changes in a study have a genetic contribution (1053). Integral parts of the vitamin D metabolism and signalling pathway have polymorphisms which can affect vitamin D activity *in vivo*. Polymorphisms in the VDR have been linked to gestational diabetes mellitus, foetal growth restriction and preterm birth (1054–1056). Polymorphisms in maternal vitamin D binding protein (VDBP) have been linked to reduced neonatal birthweight (1057), metabolism of vitamin D in pregnant women and subsequent alterations in bone forming markers in their neonates, suggesting an effect on offspring bone growth (1058). Genes, environment, methylation (GEM) is an R package that would allow for an exploration of the interaction between gene, environment and methylation (1059). Methylation data, genotype variants and environmental factors, including covariates are all supplied to the program and the analysis allows for a determination of whether significantly differentially methylated probes have a genetic contribution.

8.2.4.5 *Long-term effects of vitamin D in UC-MSCs obtained from the SPRING study*

Within this study both umbilical cord blood and tissue has been interrogated to assess for any associations that may exist in between DNA methylation and maternal vitamin D status or supplementation over the course of pregnancy respectively. Whilst umbilical cord tissue has a relatively high yield of MSCs when compared to bone marrow or cord blood, the heterogeneous mixture of cells in the tissue makes it difficult to attribute differential methylation changes to this cell type alone. Therefore we aimed to isolate and enrich cells from the birth tissues of neonates

born to mothers in the SPRING RCT in which mothers were also supplemented with 1000 IU/day vitamin D. This would allow for a direct comparison with the methylation changes in the MAVIDOS RCT, a platform for further functional experimentation and a more reliable insight into the potential functional consequences for MSC function and differentiation in the tissue and these cells.

When assessing the role of human mesenchymal stromal cells (hMSCs) in the pathogenesis of osteoporosis, *Real et al.* (2017) obtained hMSCs obtained from the femoral head of those undergoing hip replacement surgery due to hip fractures. When these cells were cultured in an osteogenic medium these hMSCs showed an impaired differentiation capacity, reduced alkaline phosphatase activity and a poor accumulation of a mineralised matrix when compared to control (997). This is an indication hMSC phenotype will reflect environment *in vivo*, suggesting that we could potentially see a similar effect in long-term vitamin D supplemented UC-MSCs isolated from the umbilical of the SPRING study participants. However, this would be dependant on both time and money remaining in the project.

8.3 Final comment

In addressing the first part of the hypothesis of this study, whether DNA methylation in birth tissues is associated with BMC or BMD in postnatal development, we have identified DNA methylation changes present within birth tissues associated with both postnatal BMC and BMD in the MAVIDOS RCT and SWS studies. These dmCpGs, with extensive reproduction and validation outside these cohorts could potentially qualify as prognostic biomarkers, indicating the trajectory of bone growth in early life and so those potentially at risk for fracture or conditions such as osteoporosis later in life.

Addressing the second part of the hypothesis, whether maternal vitamin D supplementation alters DNA methylation in genes within birth tissues and whether the changes may be associated with improved BMC or BMD in postnatal development, we have found maternal vitamin D supplementation induced differential methylation changes within umbilical cord tissue in the MAVIDOS RCT. We have also identified a more indirect potential interaction between both vitamin D supplementation and postnatal bone growth where the differential methylation of multiple sites was identified in the *PTPRN2* gene associated with both postnatal bone and intervention group analyses. These differential methylation changes are recognised with the understanding that umbilical cord tissue is acting as a tissue surrogate to those of mesodermal origin such as bone, therefore further functional validation of biological findings will be required. Nonetheless, these changes present potential evidence of prenatal epigenetic links between maternal vitamin D supplementation and postnatal bone growth.

9 Chapter 9 - Appendix

9.1 ISCT MSC standards

1. Adherence to plastic in standard culture conditions
2. In vitro culture phenotype

Positive ($\geq 95\%$)	Negative ($\leq 2\%$)
CD105, CD73, CD90	CD45, CD34, CD14 or CD11b, CD79 α or CD19, HLA-DR
3. In-vitro differentiation: osteoblasts, adipocytes, chondroblasts (as demonstrated by the staining of *in vitro* cell culture)

9.2 Correlation matrices to determine phenotypic characteristics contributing to variance in methylation datasets

9.2.1 SWS cohort

Table 40. Correlation matrix indicating the significance of the correlation between the top 100,000 most variable CpGs in SWS methylation data and SWS cohort phenotypic characteristics

Significant variance in the top 100,000 CpGs was found to be significantly (p -value <0.05) associated with sample plate, Sentrix id, birthweight, sex, gestational age, BMC at birth and all cell types.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Sample_Well	0.753358	0.074474	9.26E-07	0.438494	0.023724	0.662549	0.000177	0.090657	0.195772	0.275928
Sample_Plate	0.012379	0.714863	0.014938	0.3969	0.222269	0.023874	0.000153	0.801763	0.123922	0.326525
Sentrix_ID	0.049049	0.893495	0.062271	0.115626	0.388721	0.024353	0.002178	0.533655	0.450784	0.962299
Sentrix_Position	0.26273	1.72E-08	8.55E-21	0.039556	0.001015	0.323674	4.98E-10	0.000296	0.067245	0.009
Batch	0.400367	0.085103	0.136289	0.057549	0.380941	0.00506	0.009735	0.847509	0.341343	0.646153
Basename	0.491269	0.491269	0.491269	0.491269	0.491269	0.491269	0.491269	0.491269	0.491269	0.491269
Smoking during pregnancy	0.156049	0.635215	0.071832	0.288771	0.599514	0.170785	0.689618	0.500842	0.023172	0.398327
EP BMI	0.700386	0.320573	0.116623	0.569855	0.137053	0.137292	0.249436	0.777107	0.852461	0.329489
Birthweight	0.000393	0.032751	0.277069	0.227167	7.75E-10	3.22E-05	0.588758	0.996043	0.028497	0.480055
Sex	0.000264	0.009951	9.03E-07	2.25E-57	0.001274	1.59E-15	0.108449	0.000129	9.52E-10	0.336943
EP maternal vitamin D	0.990817	0.406765	0.134255	0.544213	0.713175	0.895484	0.549067	0.19895	0.058037	0.210902
LP maternal vitamin D	0.477504	0.781174	0.860061	0.772938	0.84222	0.016995	0.84577	0.640797	0.301954	0.062878
LP VDBP	0.224876	0.474803	0.676334	0.332167	0.751017	0.449409	0.357146	0.918808	0.080147	0.663889
LP calcium	0.075658	0.972609	0.271493	0.427496	0.431871	0.342376	0.920076	0.027729	0.907726	0.130396
LP ALP	0.150408	0.15304	0.776277	0.399402	0.022135	0.149585	0.321327	0.794422	0.145757	0.172144
Gestaional age	0.001347	0.786932	0.058259	2.23E-06	3.83E-31	0.00022	5.30E-05	0.083846	0.441438	0.100575
Cord vitamin D	0.360912	0.356852	0.217365	0.49124	0.629269	0.257965	0.817686	0.647845	0.951922	0.439321
Month of birth	0.17974	0.032633	0.133702	0.88584	0.013416	0.507366	0.893605	0.964871	0.915709	0.652831
Bi season	0.252511	0.026432	0.549468	0.414808	0.958406	0.109099	0.929269	0.265925	0.847444	0.414808
Quad season	0.075531	0.051859	0.075072	0.521224	0.983877	0.101442	0.622684	0.716554	0.763619	0.64095
Birth DXA	0.757649	0.229441	0.908131	0.289137	0.159202	0.776901	0.591337	0.751145	0.974414	0.767113
BMC at birth	0.044972	0.128247	0.773918	0.71986	2.34E-07	0.001796	0.933387	0.236079	0.390185	0.473113
BMD at birth	0.67894	0.205837	0.97901	0.331278	0.003756	0.014926	0.806263	0.217176	0.698385	0.978646
4 year DXA	0.529658	0.697861	0.006	0.076042	0.87316	0.262444	0.764643	0.862651	0.050134	0.536663
BMC at 4 years	0.327429	0.967043	0.396071	0.794907	0.52604	0.136201	0.080789	0.113406	0.131696	0.189916
BMD at 4 years	0.874602	0.231476	0.742	0.072721	0.622166	0.432044	0.085973	0.38218	0.220663	0.312153
6 year DXA	0.926979	0.552071	0.263793	0.735317	0.382061	0.153784	0.032848	0.178295	0.030312	0.398873
BMC at 6 years	0.487694	0.807423	0.53123	0.315443	0.30842	0.633625	0.11309	0.099342	0.073989	0.004217
BMD at 6 years	0.750885	0.332426	0.782821	0.016685	0.615874	0.917048	0.116282	0.201656	0.128647	0.003561
8 year DXA	0.332616	0.746022	0.581599	0.864569	0.498446	0.396068	0.001375	0.41445	0.078767	0.885098
BMC at 8 years	0.135119	0.261592	0.873822	0.065087	0.160332	0.96176	0.00864	0.024479	0.076841	0.000778
BMD at 8 years	0.702003	0.27018	0.609854	0.004443	0.640692	0.902845	0.112354	0.15735	0.401655	0.054252
Bcell	1.74E-46	1.43E-28	0.065608	0.550132	0.001577	0.179159	0.006982	0.009853	0.990056	0.328027
CD14	0.037697	5.42E-11	0.004443	6.12E-05	1.58E-07	0.786984	2.06E-05	0.094509	0.073285	0.063799
CD4T	3.63E-37	8.78E-09	0.717988	0.94398	0.042315	0.034649	0.335261	2.14E-06	0.24499	1.83E-06
CD8T	0.047627	3.41E-40	4.94E-06	0.204208	3.52E-05	0.003265	0.495104	0.008302	0.177927	0.000431
Granulocyte	#####	0.398785	0.027873	0.573741	0.203519	0.774959	0.001074	0.121224	0.111738	0.432937
NK	2.21E-37	1.74E-24	0.015476	0.020206	0.166274	0.219025	2.98E-06	6.07E-19	0.032062	1.62E-09

9.2.2 MAVIDOS RCT

Table 41. Correlation matrix indicating the significance of the correlation between the top 100,000 most variable CpGs in SWS methylation data and MAVIDOS RCT phenotypic characteristics

Significant variance in the top 100,000 CpGs was found to be significantly (p -value <0.05) associated with Sentrix position, sex, gestational age, early pregnancy BMI, and all cell types, excluding endothelial cells.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Sample_Well	0.115218	0.054529	0.165993	0.121024	0.637049	0.661415	0.270005	0.316493	0.037521	0.02497
Sample_Plate	0.99943	0.444601	0.787497	0.013741	0.958002	0.639913	0.971229	0.005383	0.607055	0.933226
Sample_Group	0.49021	0.49021	0.49021	0.49021	0.49021	0.49021	0.49021	0.49021	0.49021	0.49021
Sentrix_ID	0.960991	0.888794	0.585957	0.058608	0.618165	0.607771	0.906274	0.527502	0.903972	0.466783
Sentrix_Position	0.003047	1.07E-06	0.135035	2.11E-07	0.576028	0.056552	0.681597	0.001632	0.002946	0.243268
Batch	0.962916	0.174297	0.520726	0.000582	0.474514	0.19991	0.780148	0.006193	0.330904	0.515441
Treatment group	0.73292	0.598914	0.283131	0.00422	0.246114	0.551851	0.601619	0.291054	0.223528	0.00057
Smoking during pregnancy	0.594035	0.097207	0.849641	0.102524	0.189806	0.53112	0.249179	0.298713	0.453633	0.003272
sex	0.005327	0.001923	0.654625	0.070897	0.014186	1.58E-05	8.53E-12	0.159699	0.004571	4.35E-21
Gestational age	0.002241	0.011417	0.211626	0.415654	0.002817	0.30511	0.955339	0.985167	0.010485	0.522149
Birth DXA	0.486055	0.524937	0.750504	0.663217	0.633927	0.210197	0.789864	0.575016	0.05346	0.01785
Four-year DXA	0.854855	0.331746	0.776589	0.633718	0.26864	0.235323	0.711997	0.372004	0.944475	0.299588
BMC at birth	0.750321	0.540321	0.462311	0.243975	0.806813	0.375758	0.390931	0.121526	0.037809	0.70966
BMD at birth	0.201001	0.257726	0.585382	0.858116	0.115806	0.980142	0.02153	0.739891	0.557543	0.819655
BMC at four years of age	0.678504	0.591894	0.664139	0.522064	0.364166	0.559492	0.008078	0.867011	0.156293	0.716072
BMD at four years of age	0.320227	0.343877	0.338341	0.178072	0.514212	0.173123	0.087246	0.658097	0.096046	0.385324
Early pregnancy maternal BMI	0.037439	0.729661	0.64551	0.211572	0.512515	0.257402	0.860328	0.351756	0.902591	0.031986
Early pregnancy maternal vitamin D	0.90987	0.050047	0.012995	0.221784	0.33779	0.109926	0.948058	0.36817	0.112501	0.063043
Late pregnancy maternal vitamin D binding protein	0.566716	0.675939	0.534913	0.981696	0.622041	0.948006	0.733323	0.801859	0.181153	0.045523
Early pregnancy maternal calcium	0.404409	0.079508	0.799116	0.313635	0.122888	0.460011	0.837593	0.909279	0.946182	0.771038
Late pregnancy maternal calcium	0.275916	0.630405	0.018186	0.07966	0.824315	0.551257	0.222559	0.90204	0.129281	0.540439
Early pregnancy maternal vitamin D binding protein	0.241575	0.767453	0.115543	0.476363	0.384575	0.144629	0.48231	0.620342	0.971391	0.489036
Late pregnancy maternal vitamin D binding protein	0.175767	0.400063	0.555216	0.267151	0.22005	0.971161	0.145519	0.825702	0.713283	0.484312
Quad season	0.948512	0.954044	0.071558	0.208065	0.693245	0.965064	0.232952	0.721331	0.278627	0.010461
Bi season	0.752177	0.901121	0.427142	0.096052	0.791999	0.60576	0.153125	0.655308	0.130268	0.801855
Bcell	1.21E-31	0.03217	0.954935	6.53E-13	1.26E-11	0.025783	0.112102	0.945871	0.530404	0.330841
CD4T	5.73E-08	0.466975	0.614653	0.022263	0.880346	0.227808	0.52288	0.223577	0.175477	0.447341
CD8T	6.48E-16	0.749963	0.67193	1.90E-05	0.002178	0.000953	0.182146	0.17024	0.957952	0.220517
Endothelial	0.134588	1.87E-18	1.78E-14	0.000261	0.001004	0.133781	0.395535	0.124275	0.001224	0.079605
Epithelial	7.40E-55	0.000272	0.730253	0.275158	2.72E-09	0.676822	0.027039	0.004234	0.001891	0.028558
Gran	#####	0.311099	0.258088	0.075306	0.331169	0.560819	0.454316	0.144307	0.060165	0.854404
Mono	5.61E-56	0.12631	0.024259	0.283426	0.363974	0.188877	0.040984	0.205491	0.029275	0.577974
NK	1.06E-45	0.005405	0.682431	6.64E-06	3.59E-05	0.109684	0.096557	0.67766	0.300254	0.406919
Stromal	#####	0.665992	0.035472	0.004635	0.002712	0.636186	0.197705	0.315221	0.547885	0.377615

9.3 Definitions of CpG island shore, shelf and open-sea regions

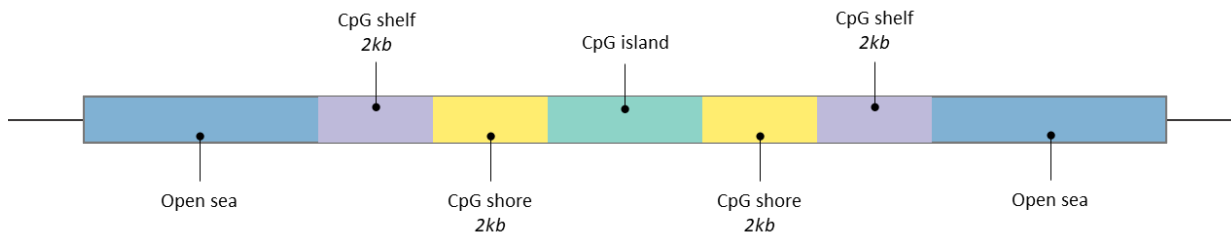


Figure 76. The relative genomic arrangement of a CpG island and associated shores, shelves and open sea regions

CpG shores are regions of 2kb immediately flanking both a CpG island start and end. CpG shelves are 2kb regions flanking CpG shores. An open sea CpG is any CpG not associated with a CpG island, shore or shelf.

9.4 Differential methylation analyses top 100 dmCpGs

9.4.1 BMC-related methylation analyses in the SWS cohort

9.4.1.1 BMC at four years of age

9.4.1.1.1 Top 100 dmCpGs associated with BMC at four years of age

Table 42. The top 100 dmCpGs associated with BMC at four years of age in the SWS cohort

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	-0.1104	0.06567	IGF-1R	51	-0.08555	0.5812	TCAF1
2	-0.09849	0.07	POU2F1	52	-0.1182	0.5812	cg16150900
3	-0.1469	0.09369	cg06957851	53	-0.1273	0.5812	ZNF253
4	-0.239	0.1627	cg13649309	54	0.02586	0.5812	REST
5	-0.1477	0.1627	PLXNA4	55	-0.04086	0.5812	SEC11A
6	-0.1544	0.1981	cg02634733	56	0.1341	0.5812	COL8A1
7	-0.1007	0.1981	cg04164351	57	0.1914	0.5812	NADK
8	0.04344	0.1981	ZNF669	58	-0.1364	0.5812	OCA2
9	0.1362	0.1981	SSH3	59	0.09424	0.5888	CALHM1
10	-0.1005	0.2068	TBCD	60	-0.06371	0.5888	cg10196258
11	-0.05283	0.2131	AGPAT3	61	-0.02951	0.5924	NAA50
12	0.0937	0.2131	DMP1	62	-0.09198	0.5924	ZNF608
13	-0.04496	0.2131	UBE4A	63	-0.05624	0.5965	DYRK1A
14	0.02756	0.2233	LINC00939	64	-0.2472	0.6008	DDX43
15	-0.04575	0.2233	cg07601720	65	-0.4599	0.6037	ABR
16	-0.08831	0.2233	NCOR2	66	-0.1229	0.6194	MIR183
17	-0.05973	0.2233	TNFRSF10A	67	-0.06418	0.6194	TRPS1
18	0.1044	0.2528	cg00880583	68	0.02742	0.6194	RG9MTD1
19	-0.05183	0.3272	ALDH18A1	69	-0.09934	0.6194	cg17749710
20	0.04992	0.3272	EPM2AIP1	70	0.1226	0.6406	IGF-2R
21	0.04627	0.3272	C2orf49	71	0.03611	0.6406	cg17256073
22	0.1006	0.3272	cg21369890	72	-0.06199	0.6529	TRAPPC10
23	0.02981	0.3447	RPA1	73	-0.02371	0.6645	PLCG2
24	-0.1676	0.3727	cg04064631	74	-0.1052	0.6717	SPOP
25	-0.1409	0.3727	CCDC62	75	0.06571	0.6717	MIR548F5
26	-0.1125	0.3727	MYT1L	76	0.0392	0.6717	EIF4G2
27	-0.171	0.3769	PRKCZ	77	0.04021	0.6717	PAPPA
28	-0.1603	0.4118	EPHA5	78	-0.04576	0.6717	SLC12A7
29	0.1499	0.4118	LOC101928231	79	0.03226	0.6717	SRBD1
30	-0.1455	0.4525	PTPRO	80	-0.06765	0.6717	SP100
31	0.04932	0.4525	ZIC2	81	-0.05733	0.6717	TCF7L1
32	0.04425	0.4825	PRSS27	82	0.2544	0.6717	cg21419404
33	-0.08477	0.5236	TRIO	83	-0.04555	0.6717	CENPE
34	-0.08768	0.5236	STUB1	84	-0.1498	0.6809	cg01518259
35	0.1567	0.5236	KCNH7	85	0.06632	0.6809	cg01902998
36	-0.08039	0.5236	USH1C	86	0.13	0.6809	cg02548571
37	-0.1658	0.5414	PAN3	87	-0.1061	0.6809	TRDMT1
38	-0.09278	0.5812	PI4KA	88	-1.04	0.6809	STK4
39	-0.1258	0.5812	cg01022012	89	-0.06759	0.6809	HDAC4
40	-0.0703	0.5812	cg02519452	90	0.03239	0.6809	TMEM65
41	0.04551	0.5812	NUBP1	91	-0.06758	0.6809	ZNF423
42	-0.5554	0.5812	ARFIP1	92	-0.1229	0.6809	cg08437936
43	-0.1262	0.5812	cg05177060	93	-0.1104	0.6809	CASC18
44	-0.1047	0.5812	cg06139720	94	-0.02436	0.6809	LOC641367
45	-0.1451	0.5812	cg08823383	95	0.02979	0.6809	UST
46	-0.08056	0.5812	SLC39A7	96	0.04194	0.6809	ZC3H6
47	-0.04132	0.5812	FAM65A	97	-0.0559	0.6809	C1orf213
48	-0.1552	0.5812	NHP2L1	98	0.18	0.6809	cg16612503
49	-0.1725	0.5812	RAPH1	99	-0.1496	0.6809	HECW1
50	-0.0832	0.5812	IARS2	100	-0.05976	0.6809	ZCCHC7

9.4.1.1.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age

Table 43. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with BMC at four years of age in the SWS cohort

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
cisbp__M1579	TCF7	4.74	Inferred	19	ABR, COL8A1, DYRK1A, EIF4G2, HDAC4, IGF1R, JARID2, KCNH7, PAN3, PAPP, POU2F1, REST, TCF7L1, TRAPPC10, TRIO, TRPS1, UST, ZIC2, ZMAT4
dbcorrd__CHD1__ENCSTR000AQK__1__m6	CHD1	4.52	Direct	14	ABR, C1orf213, CENPE, COL8A1, FBXO18, IGF1R, JARID2, PAN3, PAPP, POU2F1, TRIO, TRPS1, UST, ZNF608
hdp__MSRA	MSRA	4.47	Direct	13	COL8A1, DYRK1A, HDAC4, IGF1R, MAEA, PAN3, PAPP, POU2F1, PRKCZ, REST, TCF7L1, TRIO, UST
cisbp__M1252	HSF1	4.35	Inferred	22	C1orf213, COL8A1, DYRK1A, EIF4G2, FBXO18, HDAC4, IGF1R, JARID2, KCNH7, NCOR2, PAN3, PAPP, POU2F1, PRKCZ, PTPRO, RAPH1, REST, TRAPPC10, TRIO, UST, ZIC2, ZMAT4
cisbp__M0988	IRX6	4.29	Inferred	23	ABR, C1orf213, COL8A1, DYRK1A, EIF4G2, FBXO18, HDAC4, IGF1R, IGF2R, JARID2, KCNH7, NADK, PAN3, PAPP, POU2F1, PRKCZ, REST, TCF7L1, TRAPPC10, TRIO, UST, ZIC2, ZMAT4
cisbp__M1549	SP100	4.13	Inferred	37	ABR, AGPAT3, ALDH18A1, ARFIP1, C1orf213, C2orf49, CENPE, COL8A1, DYRK1A, EIF4G2, FBXO18, HDAC4, IGF1R, IGF2R, JARID2, KCNH7, MAEA, NAA50, NADK, NCOR2, PAN3, PAPP, POU2F1, PRKCZ, PTPRO, RAPH1, REST, SLC39A7, SRBD1, TCF7L1, TRAPPC10, TRIO, TRPS1, UST, ZC3H6, ZIC2, ZNF253
cisbp__M1598	TCF7L1	4.08	Inferred	20	ABR, COL8A1, DYRK1A, EIF4G2, HDAC4, IGF1R, JARID2, KCNH7, PAN3, PAPP, POU2F1, REST, SRBD1, TCF7L1, TRAPPC10, TRIO, TRPS1, UST, ZIC2, ZMAT4
cisbp__M0408	ZKSCAN1	4.06	Inferred	21	ABR, COL8A1, DYRK1A, FBXO18, HDAC4, IGF1R, IGF2R, JARID2, KCNH7, MIR548F5, PAN3, PAPP, POU2F1, PRKCZ, RAPH1, REST, TCF7L1, TRAPPC10, TRIO, UST, ZIC2
cisbp__M0290	MAFK	4.04	Inferred	11	DYRK1A, HDAC4, IGF1R, JARID2, KCNH7, PAN3, PAPP, REST, TRIO, UST, ZMAT4

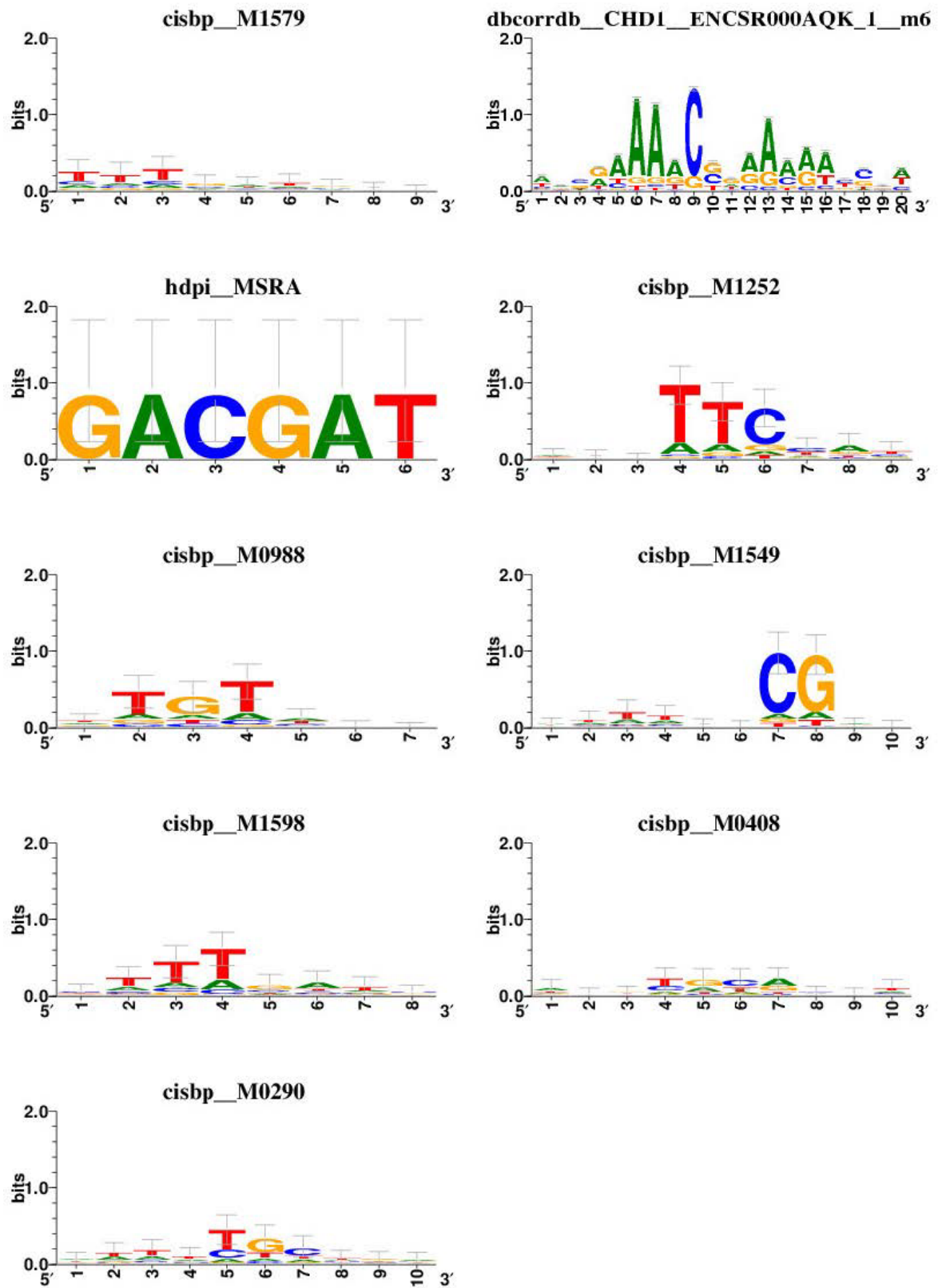


Figure 77. The sequence graphs for transcription factor motifs significantly enriched (NES >4) in the top 100 dmCpGs associated with BMC at four years of age in the SWS cohort

9.4.1.1.3 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age

Table 44. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with BMC at four years of age in the SWS cohort

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
9987	2.26E-02	cellular process	SMARCE1 EPHA5 HDAC4 DYNC1H1 POU2F1 TRIO SRD5A1 KCNH7 MYT1L RPA1 DMP1 STK4 SSH3 CENPE DPYSL2 UST ARFIP1 ALDH18A1 HARS SPG20
4713	4.26E-02	protein tyrosine kinase activity	DYRK1A IGF2R IGF1R

9.4.2 BMD-related methylation analyses in the SWS cohort

9.4.2.1 BMD at birth

9.4.2.1.1 The top 100 dmCpGs associated with BMD at birth

Table 45. The top 100 dmCpGs associated with BMD at birth in the SWS cohort

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	-0.5028	0.01412	TMPRSS11F	51	0.1122	0.384	EGR2
2	0.3223	0.1336	cg02270765	52	-0.3651	0.384	ALLC
3	0.1133	0.1394	HIRA	53	0.07719	0.384	KIF1B
4	-0.08469	0.1394	HEBP2	54	-0.1926	0.384	LSAMP
5	0.2122	0.1394	NAV2	55	-0.09198	0.384	FAM3C
6	0.1158	0.2645	ZNF512B	56	-0.05476	0.4144	ARPC5L
7	0.09476	0.2645	cg00412749	57	-0.1206	0.4144	CACYBP
8	0.1295	0.2645	OLIG3	58	-0.3141	0.4144	CADPS
9	0.2314	0.2645	ESRRA	59	0.1544	0.4144	cg02074878
10	0.225	0.2645	cg06906230	60	0.1374	0.4144	LMF1
11	-0.1046	0.2645	SPTB	61	-0.1301	0.4144	CDV3
12	0.4882	0.2645	cg09651845	62	-0.3057	0.4144	RPL31
13	-0.114	0.2645	SIN3A	63	-0.2959	0.4144	ZHX2
14	-0.1084	0.2645	WDR60	64	0.5616	0.4144	CORO1C
15	-0.08059	0.2645	CALM3	65	0.1359	0.4144	PAX6
16	0.1408	0.2645	FGF10	66	-0.1835	0.4144	CEP128
17	-0.1623	0.2645	LAMTOR4	67	-0.1399	0.4144	cg05276315
18	0.3064	0.2645	PEAR1	68	-0.3002	0.4144	PTPN3
19	-0.2787	0.2645	cg17203448	69	-0.7021	0.4144	GMDS
20	0.159	0.2645	cg18095129	70	0.1884	0.4144	NEU4
21	-0.1136	0.2645	NFYC	71	0.1673	0.4144	cg07337717
22	0.1311	0.2645	C13orf35	72	0.2516	0.4144	cg07482226
23	0.2869	0.2645	cg25582541	73	0.305	0.4144	HCG27
24	0.4005	0.2645	GDE1	74	0.1442	0.4144	INTU
25	0.1141	0.2645	MYH11	75	0.4229	0.4144	CDH4
26	-0.4187	0.2698	cg06328165	76	0.2755	0.4144	cg09597806
27	0.1653	0.2698	cg19355079	77	0.3283	0.4144	LONRF1
28	0.2528	0.2698	PCDH21	78	0.1097	0.4144	JAKMIP1
29	0.2215	0.2726	cg12840070	79	0.1495	0.4144	ALDH18A1
30	0.1272	0.2726	RPTOR	80	-0.4045	0.4144	cg12549493
31	-0.1386	0.2726	TNFAIP3	81	-0.08444	0.4144	TANC2
32	-0.8751	0.2924	cg05785424	82	0.913	0.4144	VIPR2
33	-0.1116	0.3206	SMAD2	83	0.24	0.4144	MRAS
34	0.1749	0.3206	FMN2	84	-0.2417	0.4144	cg14740295
35	-0.2956	0.3206	ESRRA	85	0.08275	0.4144	KIAA0947
36	-0.134	0.3232	BIRC5	86	-0.1246	0.4144	FLJ41350
37	0.1858	0.3252	USP49	87	-0.06793	0.4144	STAT3
38	-0.1868	0.3412	FAM173B	88	-0.3338	0.4144	cg16041412
39	0.4786	0.3419	cg17651613	89	-0.1307	0.4144	POLD3
40	0.1167	0.3419	ATP5G3	90	-0.236	0.4144	SOX6
41	0.1239	0.3421	cg13645791	91	0.1387	0.4144	cg16603064
42	0.07578	0.3421	TRMT61B	92	-0.2693	0.4144	cg17427836
43	-0.3718	0.3421	cg22246785	93	-0.3476	0.4144	KIAA0040
44	-0.4925	0.3422	INSR	94	0.2382	0.4144	TSHZ2
45	0.1532	0.3422	LAMC3	95	-0.06259	0.4144	ABHD5
46	0.1967	0.3533	SLC35F3	96	0.2487	0.4144	SYCE3
47	0.1488	0.3533	GBAS	97	-0.1417	0.4144	ZNF426
48	-0.07769	0.3533	cg23414392	98	-0.06183	0.4144	C6orf120
49	0.1301	0.3635	cg18224693	99	-0.07976	0.4144	CCDC63
50	-0.05091	0.3801	TRIM37	100	0.2494	0.4144	TMEM179

9.4.2.1.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at birth

Table 46. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with BMD at birth in the SWS cohort

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
dbcorrd__EZH2__ENCsR000ARK_1__m1	EZH2	4.22	Direct	43	ABHD5, ACACA, ADAMTS2, ALDH18A1, ARPC5L, ATP5G3, CACYBP, CADPS, CALM3, CDV3, CPEB4, DUSP6, EGR2, ESRRA, FAM173B, FGF10, FLJ41350, FMN2, HEBP2, HIRA, ITGA3, JAKMIP1, KIAA0947, LMF1, MYH11, NAV2, NFYC, OLIG3, PDE11A, PTPN3, RPTOR, SIN3A, SLC35F3, SMAD2, SNX19, STAT3, TMTC4, TRIM37, TSPAN17, USP49, WWOX, ZHX2, ZNF512B

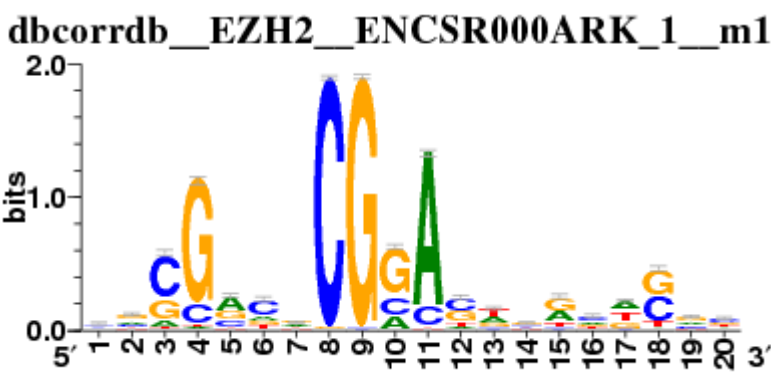


Figure 78. The sequence graph for the transcription factor motif significantly enriched (NES >4) in the top 100 dmCpGs associated with BMD at birth in the SWS cohort

9.4.2.1.3 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMD at birth

Table 47. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with BMD at birth in the SWS cohort

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
7229	1.91E-02	integrin-mediated signaling pathway	LAMA5 ITGB4 ITGA2
48871	2.11E-02	multicellular organismal homeostasis	ITGA2 STAT3 ACACA
7160	2.11E-02	cell-matrix adhesion	LAMA5 ITGB4 ITGA2
31589	2.49E-02	cell-substrate adhesion	LAMA5 ITGB4 ITGA2
1654	3.89E-02	eye development	NFYC STAT3 PTF1A
14070	3.89E-02	response to organic cyclic substance	ITGA2 STAT3 ACACA
30528	3.89E-02	transcription regulator activity	PHF2 OLIG3 NFYC STAT3 TSHZ2 MYB PTF1A
16874	3.89E-02	ligase activity	TSPAN17 FBXO3 TRIM37 ACACA
16563	3.89E-02	transcription activator activity	NFYC STAT3 MYB PTF1A
6928	4.84E-02	cellular component movement	LAMA5 CDH4 ITGA2 STAT3

9.4.2.2 BMD at four years of age

9.4.2.2.1 The top 100 dmCpGs associated with BMD at four years of age

Table 48. The top 100 dmCpGs associated with BMD at four years of age in the SWS cohort

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	0.1949	0.205	cg02548571	51	0.1875	0.4638	TPST1
2	0.173	0.205	IGF2R	52	-0.04673	0.4742	UBE2I
3	0.05542	0.205	ZNF669	53	0.2118	0.4742	CASP8
4	-0.159	0.205	C17orf75	54	0.184	0.4742	LOC101928231
5	-0.1367	0.205	IGF1R	55	0.05534	0.4793	PAPPA
6	-0.2283	0.205	NHP2L1	56	0.2137	0.4793	KCNH7
7	-0.3059	0.205	cg13649309	57	0.03887	0.4793	PCYOX1
8	-0.1186	0.205	POU2F1	58	0.05792	0.4906	NUBP1
9	0.1611	0.205	CRADD	59	-0.08603	0.4906	KCTD4
10	-0.2981	0.205	cg23376847	60	-0.1944	0.4906	LOC101927694
11	0.04107	0.205	RPA1	61	-0.154	0.5208	cg06957851
12	-0.1282	0.205	TBCD	62	-0.2325	0.5208	TDRD12
13	-0.1041	0.2294	cg02519452	63	-0.173	0.5208	LY6K
14	-0.7975	0.2294	ARFIP1	64	0.1162	0.5208	LIPE-AS1
15	-0.06716	0.2294	AGPAT3	65	-0.1728	0.5208	cg25793785
16	-0.05501	0.2294	UBE4A	66	-0.1841	0.5383	cg10682833
17	-0.181	0.2723	MIR183	67	-0.1278	0.5633	ACTN4
18	0.1736	0.279	SSH3	68	-0.2455	0.5633	ST8SIA2
19	-0.126	0.2797	cg04164351	69	0.04857	0.5633	EBLN3
20	0.04144	0.2797	HNRNPH3	70	-0.196	0.5633	FBXW11
21	0.05119	0.2797	EPM2A	71	-0.1101	0.5633	DYX1C1
22	-0.09035	0.2926	cg04398893	72	0.0592	0.5633	C7orf55
23	-0.1535	0.2926	TRDMT1	73	0.03443	0.5633	ZC3H8
24	-0.08149	0.2926	TRIO	74	-0.07832	0.5633	FBN2
25	0.1408	0.2926	VAMP3	75	-0.1489	0.5633	BOP1
26	-0.2193	0.2926	EPHA5	76	-0.1622	0.5685	cg01022012
27	-0.1345	0.2926	cg15754499	77	-0.2216	0.5735	cg00892419
28	0.03705	0.2926	REST	78	0.09008	0.5735	cg01902998
29	-0.1255	0.2926	cg22910769	79	-0.1027	0.5735	FLJ43879
30	-0.1213	0.3097	MEGF11	80	-0.08669	0.5735	cg03263115
31	-0.0716	0.3097	TNFRSF10A	81	-0.1122	0.5735	cg04692555
32	-0.06943	0.3164	CD80	82	0.1915	0.5735	CEND1
33	0.1961	0.3164	cg27546797	83	-0.07392	0.5735	ACTR1B
34	-0.1393	0.3482	cg06139720	84	0.04456	0.5735	CTD-2194D22.4
35	-0.07707	0.365	NLRP2	85	-0.1177	0.5735	DIXDC1
36	-0.06427	0.365	ALDH18A1	86	-0.1408	0.5735	FIBCD1
37	-0.05516	0.365	cg07601720	87	-0.0823	0.5735	DAGLB
38	-0.1998	0.365	cg09469540	88	-0.1615	0.5735	cg21146170
39	-0.09123	0.365	HTT	89	0.04888	0.5735	CCDC88C
40	-0.1764	0.3776	ZMIZ1	90	-0.04235	0.5735	FDPS
41	-0.0857	0.4147	TRAPPC10	91	-0.1715	0.5928	ARL9
42	-0.152	0.4383	CASC18	92	0.1484	0.5928	cg10362800
43	-0.2973	0.4462	FAM3D	93	-0.1034	0.5928	FNIP2
44	0.06226	0.4462	EPM2AIP1	94	-0.1268	0.5928	DSC2
45	-0.1462	0.4462	SERTAD2	95	-0.1772	0.5991	PTPRO
46	-0.03798	0.4462	NAA50	96	0.1024	0.5991	SIRT6
47	-0.1154	0.4462	cg10892979	97	-0.3466	0.5991	LOC728095
48	0.0519	0.4462	SNRNP35	98	-0.07501	0.6016	AGAP1
49	-0.1788	0.4462	AKIRIN1	99	-0.2143	0.6016	DNAJB9
50	0.04801	0.4462	VAPA	100	-0.2397	0.6142	cg01249071

9.4.2.2.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at four years of age

Table 49. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with BMD at four years of age in the SWS cohort

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
transfac_pro__M07909	OSR1	4.67	Inferred	13	CD80, DAXX, DNAH17, FAM66B, FDPS, FNIP2, KCNH7, MEGF11, POU2F1, RG9MTD1, SIRT6, SPTBN1, TRIO
dbcorrd__POLR2A__ENCSTR000DY	POLR2A	4.65	Direct	30	ACTN4, AGPAT3, ARFIP1, BOP1, BTRC, CCDC88C, CEND1, DIXDC1, FBXW11, FIBCD1, FNIP2, HNRNPH3, HTT, IGF2R, LETM2, MAEA, MEGF11, NHP2L1, NUBP1, POU2F1, PTPRO, REST, SIRT6, SPTBN1, TRAPPC10, TRIO, UBE4A, VAPA, ZC3H8, ZMIZ1
hdpi__POLI	POLI	4.38	Direct	12	ACTN4, DIXDC1, FNIP2, KCNH7, MEGF11, POU2F1, REST, SPTBN1, ST8SIA2, TRIO, VAPA, ZMIZ1
taipale_tf_pairs__ETV5_CLOCK_NCACGTGNNNNNSCGGAWRN_CAP_repr	CLOCK	4.29	Direct	13	CASP8, DAXX, DIXDC1, DNAJB9, FBXW11, IGF2R, KCNH7, NLRP2, NPHP4, PAPPA, POU2F1, TAAR6, TRAPPC10
cisbp__M0610	TET1	4.19	Direct	38	ACTN4, AGAP1, AGPAT3, C17orf75, CCDC88C, CENPE, CRADD, DIXDC1, DNAJB9, FBXW11, FDPS, FNIP2, HNRNPH3, IGF1R, IGF2R, KCNH7, MAEA, MEGF11, MICAL3, NAA50, NHP2L1, NPHP3, NUBP1, PABPC1L, POU2F1, REST, SERTAD2, SH3BP4, SNRNP35, SPTBN1, SSH3, TBCD, TRIO, UBE2I, UBE4A, VAMP3, VAPA, ZMIZ1
tfdimers__MD00483	ZBTB14	4.19	Direct	32	ACTN4, ACTR1B, AGPAT3, BTRC, C17orf75, CCDC88C, DAXX, DIXDC1, DNAJB9, FBN2, FBXW11, FDPS, FIBCD1, FNIP2, HNRNPH3, IGF1R, IGF2R, KCNH7, MEGF11, NHP2L1, NUBP1, POU2F1, REST, SERTAD2, SIRT6, SPTBN1, ST8SIA2, TRAPPC10, UBE2I, VAPA, ZC3H8, ZMIZ1
cisbp__M0198	CCDC169-SOHLH2	4.1	Inferred	11	BTRC, C17orf75, CCDC88C, DIXDC1, DNAJB9, FNIP2, IGF2R, KCNH7, POU2F1, SPTBN1, TRIO

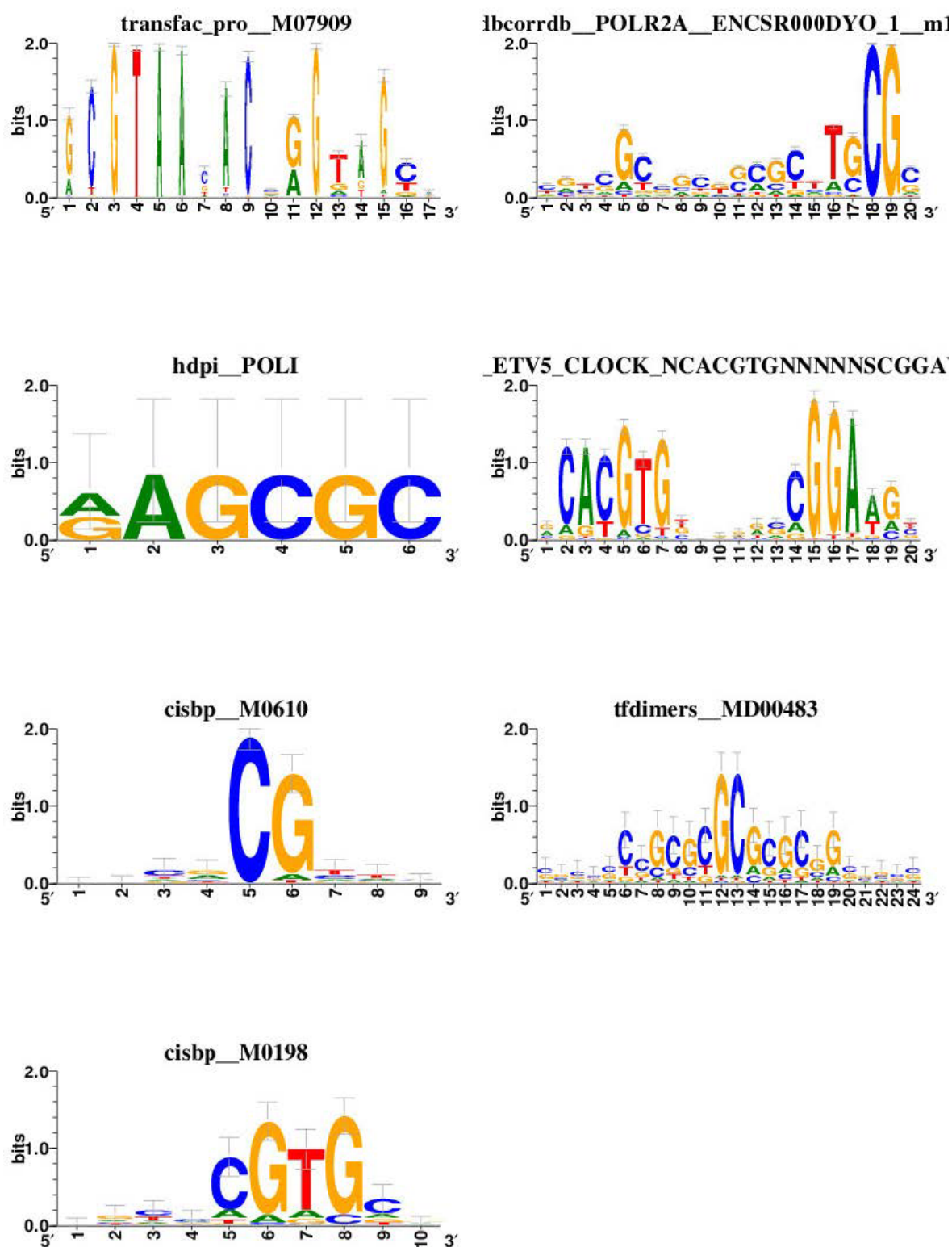


Figure 79. The sequence graphs for transcription factor motifs significantly enriched ($NES > 4$) in the top 100 dmCpGs associated with BMD at four years of age in the SWS cohort

9.4.2.2.3 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMD at four years of age

Table 50. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with BMD at four years of age in the SWS cohort

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
6917	6.11E-04	induction of apoptosis	CASP8 CRADD FAS HTT
12502	6.11E-04	induction of programmed cell death	CASP8 CRADD FAS HTT
8624	1.05E-03	induction of apoptosis by extracellular signals	CASP8 CRADD FAS
43065	1.05E-03	positive regulation of apoptosis	CASP8 CRADD FAS HTT
43068	1.05E-03	positive regulation of programmed cell death	CASP8 CRADD FAS HTT
10942	1.05E-03	positive regulation of cell death	CASP8 CRADD FAS HTT
51704	7.35E-03	multi-organism process	FDPS CASP8 PAPPA HTT
42981	7.59E-03	regulation of apoptosis	CASP8 CRADD FAS HTT
43067	7.59E-03	regulation of programmed cell death	CASP8 CRADD FAS HTT
10941	7.59E-03	regulation of cell death	CASP8 CRADD FAS HTT
4175	7.59E-03	endopeptidase activity	CASP8 CRADD PAPPA
16787	9.89E-03	hydrolase activity	CASP8 CRADD PAPPA FAS CHD1L
70011	1.25E-02	peptidase activity, acting on L-amino acid peptides	CASP8 CRADD PAPPA
6915	1.32E-02	apoptosis	CASP8 FAS HTT
8233	1.32E-02	peptidase activity	CASP8 CRADD PAPPA
12501	1.34E-02	programmed cell death	CASP8 FAS HTT
19899	1.49E-02	enzyme binding	CRADD FAS HTT
42802	1.60E-02	identical protein binding	CASP8 FAS HTT
8219	1.64E-02	cell death	CASP8 FAS HTT
16265	1.64E-02	death	CASP8 FAS HTT
3824	2.09E-02	catalytic activity	FDPS CASP8 CRADD PAPPA FAS CHD1L
44238	2.23E-02	primary metabolic process	FDPS CASP8 CRADD FAS HTT CHD1L
48522	2.49E-02	positive regulation of cellular process	CASP8 CRADD FAS HTT
48518	3.08E-02	positive regulation of biological process	CASP8 CRADD FAS HTT
8152	3.32E-02	metabolic process	FDPS CASP8 CRADD FAS HTT CHD1L
5739	4.13E-02	mitochondrion	FDPS CASP8 FAS
16043	4.16E-02	cellular component organization	CASP8 FAS HTT CHD1L
5829	4.29E-02	cytosol	CASP8 FAS HTT
44281	4.70E-02	small molecule metabolic process	FDPS FAS HTT

9.4.2.3 BMD at six years of age

9.4.2.3.1 The top 100 dmCpGs associated with BMD at six years of age

Table 51. The top 100 dmCpGs associated with BMD at six years of age in the SWS cohort

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	-0.1001	0.1118	IGDCC3	51	0.1556	0.5598	BCAS1
2	-0.08761	0.1235	cg14662769	52	-0.0832	0.5598	GYPC
3	-0.085	0.207	cg00062285	53	-0.1073	0.5598	MLPH
4	-0.1037	0.2382	cg04692555	54	-0.08872	0.5635	cg00025526
5	-0.2125	0.4482	IFT140	55	0.1314	0.5635	SPNS3
6	-0.05369	0.4482	PRPF8	56	-0.1584	0.5635	cg21659431
7	0.09188	0.4482	cg05497124	57	0.04038	0.5746	MOGS
8	-0.1036	0.4482	cg06751408	58	-0.05509	0.5746	cg00570548
9	-0.118	0.4482	cg06957851	59	-0.1406	0.5746	cg04747693
10	-0.1689	0.4482	PUM1	60	-0.04772	0.5746	cg05840338
11	0.08689	0.4482	SPTBN1	61	-0.05909	0.5746	TRIO
12	0.03568	0.4482	RELB	62	-0.1898	0.5746	cg13649309
13	-0.1468	0.4482	cg10682833	63	-0.08486	0.5746	CDK14
14	-0.05285	0.4482	ST6GAL1	64	-0.03925	0.5746	DCTN5
15	-0.1398	0.4482	TANGO2	65	0.1131	0.5746	PARP3
16	-0.1183	0.4482	ZDHHC21	66	0.2056	0.5746	cg21595342
17	-0.1053	0.4482	HLA-DOA	67	-0.121	0.5746	cg27387307
18	-0.1104	0.4482	cg14546718	68	-0.09613	0.5957	cg21039331
19	-0.114	0.4482	TRAPPC6A	69	-0.1345	0.5957	OCA2
20	-0.0487	0.4482	AGPAT3	70	0.03545	0.6002	MIF-AS1
21	-0.1316	0.4482	FANCC	71	-0.1467	0.6215	ADRBK2
22	-0.07839	0.4482	TAOK3	72	-0.1587	0.6641	HEBP1
23	-0.2229	0.4482	SYCE1	73	-0.09739	0.6641	SHISA5
24	0.07231	0.4482	SPARCL1	74	-0.07805	0.6774	cg07705594
25	-0.07688	0.4482	STK19	75	-0.1024	0.6857	NOSTRIN
26	0.1141	0.4482	cg20841733	76	-0.08407	0.6857	cg22910769
27	-0.4309	0.4482	ABR	77	-0.1271	0.6903	cg01893450
28	-0.1662	0.4482	NOS1	78	-0.07954	0.6903	BLNK
29	0.2781	0.4482	cg21419404	79	0.02702	0.6903	BHLHE40
30	0.1283	0.4482	LOC100133669	80	0.101	0.6903	cg06342842
31	-0.4929	0.4482	CRY2	81	0.1154	0.6903	cg08426898
32	-0.03087	0.4482	RFX1	82	-0.1951	0.6903	MTHFS
33	-0.04091	0.4482	AGRN	83	0.05417	0.6903	C12orf70
34	-0.1352	0.4482	cg25536696	84	-0.1146	0.6903	cg10734972
35	0.03493	0.4482	VAPA	85	0.02534	0.6903	CYC1
36	-0.09968	0.4482	cg26251604	86	0.1174	0.6903	cg18676615
37	-0.1183	0.4482	cg27363280	87	-0.1095	0.6903	CSNK1G3
38	0.1132	0.4569	cg01869606	88	-0.07932	0.6931	BAT1
39	-0.03582	0.4569	CERS2	89	-0.1425	0.6931	SLC19A3
40	0.2078	0.4588	cg04106857	90	-0.04421	0.6931	ZNF337
41	-0.09639	0.4638	NKAIN2	91	-0.07224	0.6995	cg12908614
42	-0.1215	0.5117	TLE1	92	-0.07951	0.6995	MICAL3
43	0.153	0.5137	LOC101928231	93	-0.05547	0.7049	ZNF329
44	-1.118	0.5154	STK4	94	0.09658	0.708	cg00880583
45	-0.03925	0.5598	C5orf56	95	-0.105	0.7127	SLC4A3
46	-0.1079	0.5598	GYS2	96	-0.06072	0.7127	STARD13
47	-0.1378	0.5598	CCDC42B	97	-0.1689	0.7127	cg15042870
48	-0.1698	0.5598	cg08730239	98	0.1272	0.7127	RHOBTB3
49	-0.08056	0.5598	MAP3K9	99	0.153	0.7127	STX12
50	-0.1698	0.5598	HIP1	100	-0.1147	0.7127	C7orf55-LUC7L2

9.4.2.3.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at six years of age

Table 52. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with BMD at six years of age in the SWS cohort

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
taipale_cyt_meth __POU5F1_NWW TATGCTAATKARN _FL_meth	POU5F1	4.23	Direct	10	BLNK, CDK14, KCTD9, NOS1, NOSTRIN, SLC19A3, SPTBN1, ST6GAL1, STX12, TCF12
transfac_pro__M 07851	POU3F1	4.17	Inferred	9	BLNK, CDK14, KCTD9, NOS1, SLC19A3, SPTBN1, ST6GAL1, STX12, TCF12
transfac_pro__M 07817	POU2F1	4.09	Inferred	12	BLNK, CDK14, KCTD9, LPIN2, NOS1, NOSTRIN, SLC19A3, SPTBN1, ST6GAL1, STX12, TCF12, UBLN1
taipale_cyt_meth __POU3F2_NTAT GCWAATKAG_eD BD_meth	POU3F2	4.03	Direct	10	BLNK, CDK14, KCTD9, NOS1, SLC19A3, SPTBN1, ST6GAL1, STX12, TCF12, VPS37C

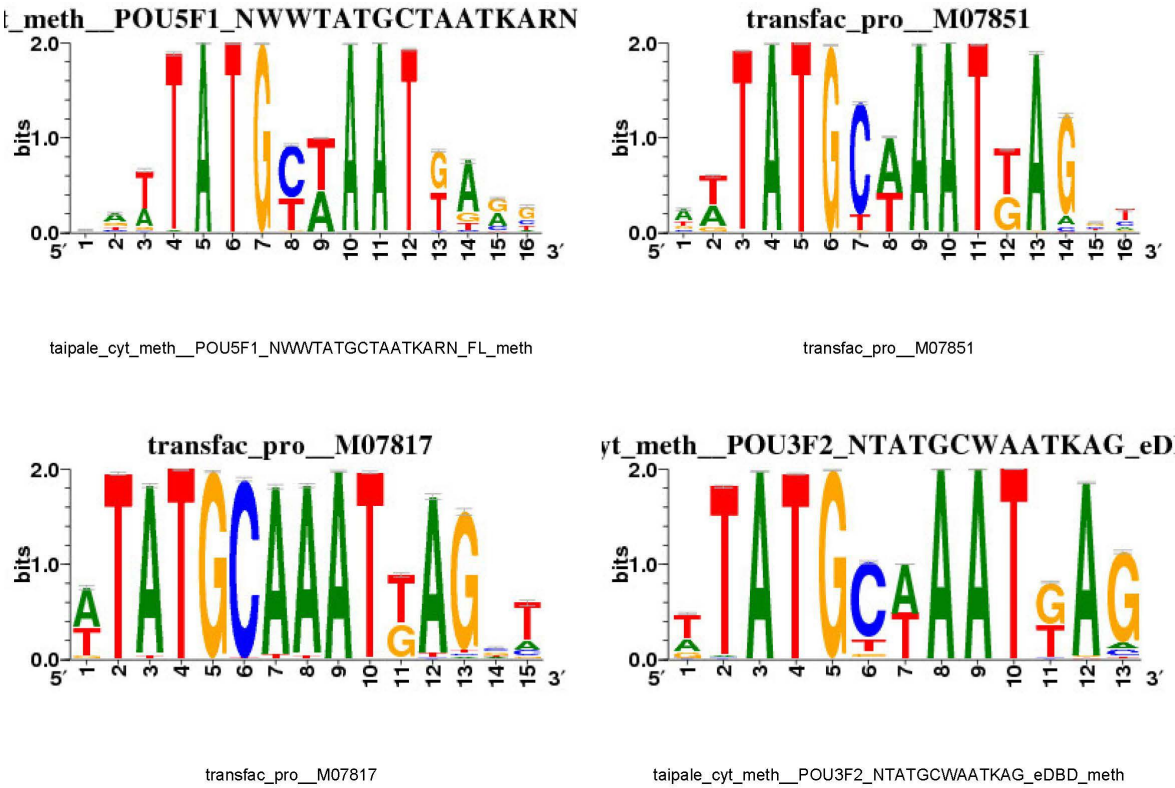


Figure 80. The sequence graphs for transcription factor motifs significantly enriched (NES >4) in the top 100 dmCpGs associated with BMD at six years of age in the SWS cohort

9.4.2.4 BMD at eight years of age

9.4.2.4.1 The top 100 dmCpGs associated with BMD at eight years of age

Table 53. The top 100 dmCpGs associated with BMD at eight years of age in the SWS cohort

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	0.02604	0.04005	MPP3	51	0.1073	0.2596	cg19686759
2	0.03091	0.04005	cg07666211	52	-0.0944	0.2596	KIR3DL3
3	0.04128	0.04005	CKB	53	0.01775	0.2596	RFX3
4	0.09211	0.08219	cg15416279	54	-0.2409	0.2596	UGT8
5	-0.1694	0.09622	SLC15A1	55	0.02174	0.2596	EREG
6	0.1308	0.1713	CAMK2D	56	-0.1682	0.2596	SYCE1
7	-0.1179	0.1713	ABR	57	0.01936	0.2596	CCDC66
8	0.02365	0.1713	COCH	58	0.03122	0.2752	BAT3
9	0.02224	0.1798	KANSL2	59	-0.09174	0.2752	MANF
10	0.03672	0.1798	LRR8D	60	0.1051	0.2752	cg02878043
11	0.1059	0.1798	GJB2	61	0.02737	0.2752	KDR
12	0.1656	0.1798	cg20245641	62	0.03973	0.2752	RTBDN
13	0.02963	0.1798	ASB1	63	-0.1159	0.2752	cg12018888
14	0.0218	0.1798	EFEMP2	64	-0.1421	0.2752	NHP2L1
15	0.02715	0.2333	RELB	65	0.02823	0.2752	PHC1
16	0.02956	0.2367	VAPA	66	-0.05718	0.2752	ZCCHC7
17	0.2211	0.2596	SIGIRR	67	-0.4403	0.2752	cg23719516
18	0.04196	0.2596	SH2D3C	68	0.02732	0.2815	RBM34
19	-0.4547	0.2596	cg03555881	69	-0.1352	0.2815	ADRBK2
20	-0.4777	0.2596	ARFIP1	70	0.03757	0.2815	ZNF580
21	0.03985	0.2596	GALR2	71	0.02783	0.2815	NPBWR1
22	0.02812	0.2596	FST	72	0.05969	0.2851	CEND1
23	-0.1706	0.2596	MEIS2	73	0.02048	0.286	LINC01132
24	0.08203	0.2596	cg06342842	74	0.1405	0.286	cg16553125
25	-0.09589	0.2596	FAM125B	75	0.03081	0.286	BCAR1
26	-0.1251	0.2596	MIR518B	76	-0.07566	0.286	FNIP2
27	-0.1026	0.2596	CSF1	77	0.02585	0.286	CACUL1
28	0.03365	0.2596	FADS6	78	0.08535	0.286	cg01100542
29	-0.1429	0.2596	NUP153	79	0.04958	0.286	TMEM248
30	0.03347	0.2596	cg08302913	80	0.1353	0.286	LINC00544
31	-0.1212	0.2596	HINFP	81	-0.1425	0.286	cg09781931
32	0.02797	0.2596	HOXB7	82	0.01878	0.286	ACAD9
33	-0.1133	0.2596	ABCA1	83	-0.04875	0.286	ATG2B
34	0.03733	0.2596	CALCA	84	-0.04468	0.286	ST6GAL1
35	-0.1198	0.2596	TANGO2	85	-0.02856	0.286	cg11814059
36	0.07953	0.2596	VAMP3	86	0.06004	0.286	CYSLTR2
37	0.02908	0.2596	EPM2A	87	-0.1761	0.286	cg13649309
38	-0.05812	0.2596	DNAJC12	88	0.02391	0.286	EHBP1
39	0.1279	0.2596	TRERF1	89	0.02464	0.286	TOR1A
40	0.1805	0.2596	SH3D19	90	-0.1278	0.286	cg19083474
41	-0.08652	0.2596	HLA-DOA	91	0.02939	0.286	SCARA3
42	0.1592	0.2596	cg14534520	92	0.02286	0.286	ADRM1
43	0.03027	0.2596	EBLN3	93	0.09737	0.286	cg23958988
44	0.06453	0.2596	LAMA2	94	0.03936	0.286	PNPLA6
45	0.04142	0.2596	SYT14	95	0.03385	0.286	ADAMTS12
46	0.1063	0.2596	SESTD1	96	0.03009	0.2876	MIR124-1
47	0.02014	0.2596	CISH	97	0.05022	0.2876	XRCC4
48	0.07313	0.2596	ST8SIA2	98	0.07358	0.2878	CTLA4
49	0.06223	0.2596	SMAD9	99	0.04844	0.2878	EN1
50	0.02901	0.2596	PIP5KL1	100	0.01876	0.2878	SETD4

9.4.2.4.2 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMD at eight years of age

Table 54. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with BMD at eight years of age in the SWS cohort

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
5829	4.00E-02	cytosol	ABR CAMK2D XRCC4 CKB SH3D19

Chapter 9

9.4.3 Intervention related analyses in the MAVIDOS RCT

9.4.3.1 Intervention

9.4.3.1.1 The top 100 dmCpGs associated with intervention

Table 55. The top 100 dmCpGs associated with intervention group in the MAVIDOS RCT

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	0.02326	0.006943	cg21023307	51	0.006456	0.2408	CEBPZ
2	0.0183	0.02741	SLC38A10	52	-0.00548	0.2442	GPRIN2
3	0.02806	0.02741	TNC	53	0.004572	0.2448	PCBP1
4	-0.01194	0.06227	ABCA7	54	0.004715	0.2476	ZNF488
5	-0.02296	0.06227	KLK15	55	-0.01688	0.2477	TIAM1
6	0.02293	0.06227	cg06629252	56	0.01862	0.2477	cg16439608
7	0.006756	0.09155	AMN1	57	-0.00655	0.2772	MLLT4
8	-0.0039	0.1138	ARL4C	58	-0.01198	0.2772	DLL4
9	-0.01402	0.1138	NR2C1	59	-0.03493	0.2772	MUC4
10	-0.01107	0.1138	cg00554871	60	-0.00673	0.2772	cg08759359
11	-0.01304	0.1138	cg14172302	61	-0.01464	0.2772	TDRD12
12	0.0248	0.1138	cg08985530	62	-0.00659	0.2772	RAP1GAP2
13	0.01893	0.1138	EMX2	63	-0.0038	0.2772	PLAGL1
14	0.01308	0.1138	cg26780108	64	-0.01348	0.2772	PDE4D
15	0.02536	0.1138	ANXA6	65	-0.00239	0.2772	DDX23
16	0.02526	0.1138	IL33	66	-0.00691	0.2772	PC
17	-0.01384	0.1161	PTPRN2	67	-0.00383	0.2772	WNK4
18	-0.00881	0.1161	MTR	68	-0.00885	0.2772	DSG1-AS1
19	0.009676	0.1161	ACY3	69	0.02085	0.2772	C1QL1
20	0.004903	0.1161	ZMIZ1	70	0.003605	0.2772	PAWR
21	0.01103	0.1161	IFFO1	71	0.006125	0.2772	cg02418473
22	0.01507	0.1281	TSNARE1	72	0.008489	0.2772	cg03277842
23	0.02724	0.1281	EXTL3	73	0.01306	0.2772	OSBP2
24	-0.01377	0.1408	CSF1R	74	0.009681	0.2772	cg04343459
25	-0.00928	0.1466	NCOA3	75	0.003024	0.2772	cg04474222
26	0.003086	0.1532	FGFR2	76	0.01519	0.2772	cg05917797
27	0.02065	0.1599	ZADH2	77	0.01587	0.2772	cg05921140
28	0.01799	0.1655	RASGRP3	78	0.0103	0.2772	ARAP1
29	0.01665	0.1798	cg08987855	79	0.01985	0.2772	D2HGDH
30	0.01992	0.1835	cg22696984	80	0.008243	0.2772	RMND1
31	0.01352	0.1916	PSD	81	0.02643	0.2772	cg08782510
32	0.01653	0.1916	cg12909727	82	0.009938	0.2772	RAP1A
33	0.004139	0.1916	RWDD3	83	0.01563	0.2772	cg10781632
34	0.03409	0.1916	CYFIP1	84	0.004153	0.2772	HYI
35	0.0218	0.2037	cg19692149	85	0.00451	0.2772	cg12458913
36	0.006119	0.2051	GTDC1	86	0.01269	0.2772	E2F8
37	0.01059	0.2051	cg25530992	87	0.02299	0.2772	HOXC10
38	0.01934	0.2051	cg27457087	88	0.01124	0.2772	TCF4
39	-0.00885	0.2214	GRN	89	0.01609	0.2772	SLC17A6
40	-0.00782	0.2214	CCR3	90	0.009418	0.2772	EGFR
41	0.01384	0.2214	ZNF607	91	0.01219	0.2772	cg17885144
42	0.001805	0.2214	LHFPL5	92	0.006042	0.2772	NFATC1
43	0.007092	0.2214	cg17043444	93	0.01168	0.2772	FAM45A
44	0.01167	0.2214	cg22222526	94	0.01033	0.2772	cg21381504
45	0.01365	0.2214	LOC100507487	95	0.01908	0.2772	cg23903701
46	0.01461	0.2361	cg10528700	96	0.02731	0.2772	cg24733398
47	-0.0121	0.2392	cg10741568	97	0.0168	0.2772	SUSD4
48	-0.01192	0.2392	MGC27382	98	0.003905	0.2772	POLL
49	-0.0123	0.2408	PRKAG3	99	0.0217	0.2772	LOC100506860
50	0.007681	0.2408	MCTP2	100	-0.00895	0.2795	DOCK8

9.4.3.1.2 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with intervention

Table 56. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with the analysis of intervention in the MAVIDOS RCT

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
3779	1.27E-02	actin binding	CYFIP1 TPM4 TNC TLN2 TLN1 EGFR
45216	1.27E-02	cell-cell junction organization	TLN2 TLN1 PLEKHA7
9966	1.27E-02	regulation of signal transduction	RAP1GAP2 CCL11 GOT1 TCF4 LMCD1 EGFR ADA VAV2
35466	1.27E-02	regulation of signaling pathway	RAP1GAP2 CCL11 GOT1 PAWR TCF4 LMCD1 EGFR ADA VAV2
23051	1.27E-02	regulation of signaling process	RAP1GAP2 CCL11 GOT1 TCF4 LMCD1 EGFR ADA VAV2
1726	1.27E-02	ruffle	CYFIP1 PC TLN2 TLN1
51015	1.31E-02	actin filament binding	CYFIP1 TNC EGFR
5912	1.46E-02	adherens junction	TLN2 TLN1 MLLT4 PLEKHA7
70161	1.62E-02	anchoring junction	TLN2 TLN1 MLLT4 PLEKHA7
9967	1.62E-02	positive regulation of signal transduction	GOT1 TCF4 LMCD1 EGFR ADA
23056	1.62E-02	positive regulation of signaling process	GOT1 TCF4 LMCD1 EGFR ADA
10646	1.76E-02	regulation of cell communication	RAP1GAP2 CCL11 GOT1 PAWR TCF4 LMCD1 EGFR ADA VAV2
22610	1.81E-02	biological adhesion	CCL11 TNC TLN2 MLLT4 EGFR PLEKHA7 ADA
7155	1.81E-02	cell adhesion	CCL11 TNC TLN2 MLLT4 EGFR PLEKHA7 ADA
34330	1.81E-02	cell junction organization	TLN2 TLN1 PLEKHA7
31252	1.81E-02	cell leading edge	CYFIP1 PC TLN2 TLN1
8092	1.81E-02	cytoskeletal protein binding	CYFIP1 TPM4 TNC TLN2 TLN1 EGFR
5200	1.81E-02	structural constituent of cytoskeleton	PC TLN2 TLN1
30054	1.87E-02	cell junction	CYFIP1 TLN2 TLN1 MLLT4 PLEKHA7 ADA
22612	1.98E-02	gland morphogenesis	CCL11 TNC EGFR
5911	1.99E-02	cell-cell junction	TLN2 TLN1 MLLT4 PLEKHA7
42995	2.03E-02	cell projection	ARL4C CYFIP1 PC GOT1 TLN2 TLN1 ADA CYFIP1 CCL11 GALNT18 DOCK8 TNC MTR MLLT4 EGFR INPP5A PCBP1 FAM65A MAPK6 LMCD1 E2F8 IL33 GOT1 TPM4 PAWR TEX14 PPP2R5C PLEKHA7 VAV2 ARL4C PC ZADH2 CPE TCF4 TLN2 TLN1 STAM2 MOCS1 ADA
5488	2.04E-02	binding	TNC LMCD1 ADA
90257	2.04E-02	regulation of muscle system process	CCL11 PC TNC EGFR
2009	2.24E-02	morphogenesis of an epithelium	GOT1 TCF4 LMCD1 EGFR ADA
35468	2.48E-02	positive regulation of signaling pathway	CCL11 PC EGFR ADA
30334	2.48E-02	regulation of cell migration	TNC TCF4 EGFR
32386	2.73E-02	regulation of intracellular transport	PC PAWR TCF4 PPP2R5C EGFR ADA VAV2
42981	2.92E-02	regulation of apoptosis	

10647	2.97E-02	positive regulation of cell communication	GOT1 TCF4 LMCD1 EGFR ADA
10941	2.97E-02	regulation of cell death	PC PAWR TCF4 PPP2R5C EGFR ADA VAV2
51270	2.97E-02	regulation of cellular component movement	CCL11 PC EGFR ADA
43067	2.97E-02	regulation of programmed cell death	PC PAWR TCF4 PPP2R5C EGFR ADA VAV2
40012	2.98E-02	regulation of locomotion	CCL11 PC EGFR ADA
9653	3.06E-02	anatomical structure morphogenesis	CYFIP1 CCL11 PC TNC TCF4 EGFR ADA VAV2
6928	3.06E-02	cellular component movement	PC TPM4 TLN1 EGFR VAV2
30335	3.06E-02	positive regulation of cell migration	CCL11 PC EGFR
5102	3.06E-02	receptor binding	IL33 CCL11 TNC TCF4 TLN2 TLN1 VAV2
65008	3.06E-02	regulation of biological quality	CYFIP1 CCL11 GOT1 TNC CPE TCF4 TLN2 TLN1 ADA
48729	3.06E-02	tissue morphogenesis	CCL11 PC TNC EGFR
19842	3.09E-02	vitamin binding	PC GOT1 MTR
51272	3.43E-02	positive regulation of cellular component movement	CCL11 PC EGFR
40017	3.43E-02	positive regulation of locomotion	CCL11 PC EGFR
32879	3.48E-02	regulation of localization	CCL11 PC TNC TCF4 EGFR ADA
31406	3.80E-02	carboxylic acid binding	PC GOT1 MTR
6725	3.80E-02	cellular aromatic compound metabolic process	MTR MOC51 ADA
44271	3.80E-02	cellular nitrogen compound biosynthetic process	GOT1 MTR MOC51 ADA
5829	3.80E-02	cytosol	GOT1 MTR TCF4 TLN1 MLLT4 STAM2 ADA VAV2
60429	3.80E-02	epithelium development	CCL11 PC TNC EGFR
5615	3.80E-02	extracellular space	IL33 CCL11 TNC LMCD1 EGFR ADA
6936	3.80E-02	muscle contraction	TPM4 TNC TLN1
48522	3.80E-02	positive regulation of cellular process	CCL11 PC GOT1 PAWR TCF4 PPP2R5C LMCD1 EGFR ADA VAV2
44057	3.80E-02	regulation of system process	TNC LMCD1 EGFR ADA
3012	3.95E-02	muscle system process	TPM4 TNC TLN1
44463	4.23E-02	cell projection part	PC GOT1 TLN1 ADA
43066	4.60E-02	negative regulation of apoptosis	PC TCF4 EGFR ADA
42127	4.60E-02	regulation of cell proliferation	CCL11 PAWR TCF4 PPP2R5C EGFR ADA
6979	4.60E-02	response to oxidative stress	TPM4 EGFR ADA
43069	4.63E-02	negative regulation of programmed cell death	PC TCF4 EGFR ADA
48732	4.67E-02	gland development	CCL11 TNC EGFR
19904	4.87E-02	protein domain specific binding	INPP5A PAWR TCF4 TLN1
60548	4.88E-02	negative regulation of cell death	PC TCF4 EGFR ADA

9.4.3.1.3 Ingenuity pathway canonical pathway analysis of the top 100 dmCpGs associated with intervention

Table 57. The full IPA canonical pathway enrichment analysis results conducted with the top 100 dmCpGs associated with the analysis of intervention in the MAVIDOS RCT

Ingenuity Canonical Pathways	p-value	Enriched genes
Epithelial Adherens Junction Signaling	0.000407	AFDN EGFR RAP1A TCF4 VAV2
Rac Signaling	0.001549	CYFIP1 PIP5K1C RAP1A TIAM1
MSP-RON Signaling In Cancer Cells Pathway	0.002239	EGFR KLK15 RAP1A TCF4
Actin Cytoskeleton Signaling	0.002399	CYFIP1 PIP5K1C RAP1A TIAM1 VAV2
Glucocorticoid Receptor Signaling	0.002884	EGFR KRT36 NCOA3 NFATC1 PC PRKAG3 RAP1A
Th1 and Th2 Activation Pathway	0.00537	CCR3 DLL4 IL33 NFATC1
Senescence Pathway	0.005495	E2F8 MAPK6 NFATC1 PPP2R5C RAP1A
Acute Myeloid Leukemia Signaling	0.005888	CSF1R RAP1A TCF4
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	0.006026	NFATC1 RAP1A VAV2
NF-kB Signaling	0.00631	EGFR FGFR2 IL33 RAP1A
Regulation of the Epithelial-Mesenchymal Transition Pathway	0.007943	EGFR FGFR2 RAP1A TCF4
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	0.009333	CEBPZ IL33 NFATC1 RAP1A TCF4
T Cell Receptor Signaling	0.009333	NFATC1 RAP1A VAV2
Telomerase Signaling	0.009772	EGFR PPP2R5C RAP1A
CDK5 Signaling	0.01	MAPK6 PPP2R5C RAP1A
Glioma Signaling	0.010471	E2F8 EGFR RAP1A
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	0.012303	CSF1R IL33 NFATC1 TCF4
Cholecystokinin/Gastrin-mediated Signaling	0.012882	EGFR IL33 RAP1A
Sperm Motility	0.01349	CSF1R EGFR FGFR2 PDE4D
IL-15 Production	0.01349	CSF1R EGFR FGFR2
p70S6K Signaling	0.015849	EGFR PPP2R5C RAP1A
STAT3 Pathway	0.018197	EGFR FGFR2 RAP1A
PTEN Signaling	0.018197	EGFR FGFR2 RAP1A
PI3K Signaling in B Lymphocytes	0.019055	NFATC1 RAP1A VAV2
Ovarian Cancer Signaling	0.019498	EGFR RAP1A TCF4
PKC δ Signaling in T Lymphocytes	0.025704	NFATC1 RAP1A VAV2
Systemic Lupus Erythematosus In B Cell Signaling Pathway	0.026303	IL33 NFATC1 RAP1A RASGRP3
Glioblastoma Multiforme Signaling	0.0302	E2F8 EGFR RAP1A
Tight Junction Signaling	0.031623	AFDN PPP2R5C TIAM1
Wnt/ β -catenin Signaling	0.033884	PPP2R5C SOX21 TCF4

T Cell Exhaustion Signaling Pathway	0.035481	NFATC1 PPP2R5C RAP1A
Tumor Microenvironment Pathway	0.035481	RAP1A TIAM1 TNC
Acute Phase Response Signaling	0.038019	IL33 RAP1A TCF4
B Cell Receptor Signaling	0.040738	NFATC1 RAP1A VAV2
Regulation Of The Epithelial Mesenchymal Transition By Growth Factors Pathway	0.041687	EGFR FGFR2 RAP1A
PPAR α /RXR α Activation	0.043652	NCOA3 PRKAG3 RAP1A
Leukocyte Extravasation Signaling	0.044668	AFDN RAP1A VAV2
Estrogen Receptor Signaling	0.045709	EGFR NCOA3 PRKAG3 RAP1A
Natural Killer Cell Signaling	0.046774	NFATC1 RAP1A VAV2
Adrenomedullin signaling pathway	0.046774	IL33 MAPK6 RAP1A
Superpathway of Inositol Phosphate Compounds	0.047863	INPP5A PAWR PIP5K1C
ERK/MAPK Signaling	0.050119	NFATC1 PPP2R5C RAP1A
Calcium Signaling	0.052481	NFATC1 RAP1A TPM4
Cardiac Hypertrophy Signaling (Enhanced)	0.052481	FGFR2 IL33 NFATC1 PDE4D RAP1A
mTOR Signaling	0.054954	PPP2R5C PRKAG3 RAP1A
AMPK Signaling	0.057544	CHRM2 PPP2R5C PRKAG3
cAMP-mediated signaling	0.067608	CHRM2 PDE4D RAP1A
Systemic Lupus Erythematosus Signaling	0.067608	IL33 NFATC1 RAP1A
Insulin Secretion Signaling Pathway	0.079433	CPE PC RAP1A
Protein Kinase A Signaling	0.081283	NFATC1 PDE4D RAP1A TCF4
Colorectal Cancer Metastasis Signaling	0.085114	EGFR RAP1A TCF4
Breast Cancer Regulation by Stathmin1	0.095499	CCR3 CHRM2 E2F8 PPP2R5C RAP1A
CREB Signaling in Neurons	0.097724	CCR3 CHRM2 EGFR FGFR2 RAP1A
G-Protein Coupled Receptor Signaling	0.102094	CHRM2 PDE4D RAP1A
Neuroinflammation Signaling Pathway	0.125026	CSF1R MAPK6 NFATC1
Synaptogenesis Signaling Pathway	0.136144	AFDN RAP1A TIAM1
Hepatic Fibrosis Signaling Pathway	0.199986	IL33 RAP1A TCF4
Molecular Mechanisms of Cancer	0.224905	E2F8 RAP1A TCF4

9.4.3.2 Intervention maternal early stage vitamin D insufficient

9.4.3.2.1 The top 100 dmCpGs associated with intervention group for mothers vitamin D insufficient in early stage pregnancy

Table 58. The top 100 dmCpGs associated with intervention group MESP D insufficient in the MAVIDOS RCT

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	0.03039	0.001612	cg21023307	51	0.02727	0.2002	FBLL1
2	-0.02339	0.007983	POLRMT	52	0.009318	0.2002	FNDC1
3	0.03833	0.01136	LOC102723376	53	0.01384	0.2002	SNPH
4	0.02114	0.01568	TSNARE1	54	0.02165	0.2002	cg13886580
5	-0.00335	0.01633	C14orf102	55	-0.00521	0.2002	TPRKB
6	0.02533	0.02085	DCC	56	0.006738	0.2002	ATF7IP
7	0.00588	0.03618	POLL	57	0.01223	0.2002	LPIN1
8	0.007381	0.05292	SUPT5H	58	-0.03184	0.2002	cg17974166
9	0.009277	0.05292	cg20990091	59	0.01706	0.2002	LTBR
10	-0.00788	0.05408	APH1B	60	-0.01348	0.2002	C17orf56
11	0.01475	0.08352	INPP4A	61	0.00633	0.2002	COX19
12	0.01328	0.09706	cg14736819	62	0.0321	0.2002	cg23320368
13	-0.05125	0.1021	MUC4	63	-0.00549	0.2002	CADPS
14	0.006257	0.1021	cg12458913	64	0.01998	0.2002	CLIP1
15	0.01273	0.1021	CAMK2B	65	-0.01542	0.2027	TRPS1
16	0.01756	0.1021	cg26780108	66	-0.01593	0.2028	cg09363736
17	-0.01876	0.1323	PTPRN2	67	0.01123	0.2044	cg01701689
18	0.009863	0.1323	cg17043444	68	0.01212	0.2044	BRAF
19	0.008169	0.1323	SLC45A2	69	0.0479	0.2044	SMIM19
20	0.01012	0.1323	SLC1A7	70	0.006498	0.2044	RAC1
21	-0.0142	0.1377	RAET1L	71	0.01507	0.2079	cg07240086
22	-0.01219	0.1377	TSHZ3	72	0.007933	0.2141	cg24092135
23	0.01416	0.1402	CENPT	73	0.01877	0.2149	ANK1
24	-0.00571	0.1402	RAET1L	74	0.01048	0.2149	SUN1
25	0.0141	0.1403	PHF3	75	0.01024	0.2149	SMYD3
26	0.005415	0.1403	PACS2	76	0.02037	0.2149	IPO8
27	0.02554	0.1406	KBTBD6	77	-0.00448	0.2149	CPEB3
28	-0.00427	0.1406	MIR3197	78	0.02395	0.2149	SDHAF1
29	0.02156	0.1634	SLC25A29	79	0.003954	0.2159	NID1
30	0.01516	0.1765	SPG7	80	0.01867	0.2159	ABLIM3
31	0.0251	0.1765	RASGRP3	81	0.01347	0.2159	ZNF598
32	-0.02686	0.1765	cg06446456	82	-0.01027	0.2159	MX1
33	0.01544	0.1765	RALGDS	83	-0.00358	0.2159	C17orf85
34	-0.00289	0.1765	KIF11	84	0.006881	0.2159	cg11560589
35	-0.01126	0.1797	cg05351147	85	0.04005	0.2162	cg16691186
36	0.007366	0.1797	cg09120960	86	0.01246	0.2221	PCSK6
37	-0.01451	0.1797	PDS5B	87	0.008697	0.224	SNRPE
38	0.01684	0.1797	cg17689721	88	-0.00484	0.224	CRTAP
39	0.009515	0.1803	MCTP2	89	0.01574	0.224	cg03234839
40	0.01983	0.1896	LTBP1	90	0.01051	0.224	MGRN1
41	-0.00522	0.1931	TSGA14	91	0.01176	0.224	C15orf38
42	-0.00765	0.1989	UBXN11	92	-0.00489	0.224	RAP1A
43	-0.01499	0.1989	cg14172302	93	-0.00475	0.224	cg08339771
44	0.006399	0.2002	VWA3B	94	0.006832	0.224	GAK
45	0.02106	0.2002	LINC00925	95	0.0125	0.224	RAP1A
46	-0.03806	0.2002	SLC6A18	96	0.01515	0.224	TEX38
47	0.01951	0.2002	TAF4	97	-0.00602	0.224	cg12317470
48	-0.0352	0.2002	OGFR	98	0.0186	0.224	cg14025581
49	0.0258	0.2002	TRIM9	99	0.005109	0.224	RWDD3
50	0.0201	0.2002	SLC38A10	100	0.02965	0.224	TMEM100

9.4.3.2.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with intervention group for mothers vitamin D insufficient in early stage pregnancy

Table 59. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with the analysis of MESP D insufficient mothers in the MAVIDOS RCT

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
dbcorrd__RBBP5__EN CSR000AQC_1__m5	RBBP5	5.01	Direct	16	AATK, ADAMTS2, BRAF, CAMK2B, CLIP1, FBLL1, IRS1, OGFR, PACS2, PTPRN2, RAC1, RALGDS, SLC25A29, TAF4, TRPS1, TSHZ3
homer__ACAGCTGCTG _Tcf12	TCF12	4.84	Direct	14	ABLIM3, ALDH1L2, ANK1, DCC, IRS1, LTBP1, PDS5B, RASGRP3, SCRNI, SLC1A7, SNPH, SUN1, SUPT5H, TMEM100
factorbook__TCF12	TCF12	4.62	Direct	15	ABLIM3, ANK1, DCC, IRS1, LTBP1, PDS5B, PLEKHG6, RALGDS, RASGRP3, SCRNI, SLC1A7, SNPH, SUN1, SUPT5H, TMEM100
cisbp__M6353	MYF6	4.43	Direct	16	ABLIM3, ANK1, DCC, IRS1, LTBP1, MGRN1, PCSK6, PDS5B, PLEKHG6, RALGDS, RASGRP3, SCRNI, SLC1A7, SNPH, SUN1, TMEM100
cisbp__M4479	TCF12	4.38	Direct	11	ABLIM3, ANK1, DCC, LTBP1, PDS5B, RASGRP3, SCRNI, SLC1A7, SUN1, SUPT5H, TMEM100
taipale__NHLH1_full_C GCAGCTGCG	NHLH1	4.32	Direct	17	AATK, ANK1, DCC, IRS1, LTBP1, MIR3197, PDS5B, PLEKHG6, RALGDS, RASGRP3, SCRNI, SLC38A10, SLC45A2, SNPH, SUN1, SUPT5H, TMEM100
taipale_cyt_meth__HES 5_GGCACGTGY_eDBD _meth	HES5	4.27	Direct	9	ABLIM3, ANK1, LTBP1, MUC4, PRDM13, RALGDS, SLC1A7, SLC45A2, TRIM9
homer__ACAGCTGTTN _Ptf1a	PTF1A	4.24	Inferred	21	ABLIM3, ALDH1L2, ANK1, CADPS, DCC, IRS1, LTBP1, LTBR, MUC4, PDS5B, PLEKHG6, RAP1A, RASGRP3, SCRNI, SLC1A7, SLC38A10, SLC45A2, SNPH, SUN1, SUPT5H, TMEM100
cisbp__M5671	NHLH1	4.18	Direct	17	AATK, ANK1, DCC, IRS1, LTBP1, PDS5B, PLEKHG6, RALGDS, RASGRP3, SCRNI, SLC1A7, SLC38A10, SLC45A2, SNPH, SUN1, SUPT5H, TMEM100
hocomoco__BHA15_H UMAN.H11MO.0.B	BHLHA15	4.11	Direct	12	ABLIM3, ANK1, DCC, LTBP1, PDS5B, RASGRP3, SCRNI, SETD8, SLC1A7, SUN1, SUPT5H, TMEM100
taipale_cyt_meth__ASC L2_NRCAGCTGYN_eDB D	ASCL2	4.06	Direct	14	ANK1, DCC, IRS1, LTBP1, PDS5B, PLEKHG6, RASGRP3, SCRNI, SLC38A10, SLC45A2, SNPH, SUN1, SUPT5H, TMEM100
taipale__Ascl2_DBD_R RCAGCTGY_repr	ASCL2	4.06	Inferred	8	ANK1, DCC, LTBP1, PDS5B, SCRNI, SETD8, SUPT5H, TMEM100
transfac_pro__M00641	HSF1	4.02	Direct	9	CADPS, DCC, LTBP1, MIR3197, RAC1, SLC25A29, TMEM100, TRIM9, UBXN11
taipale_cyt_meth__ASC L2_NRCAGCTGYN_FL_ meth	ASCL2	4.01	Direct	24	ABLIM3, ANK1, DCC, GPR111, IRS1, LTBP1, LTBR, MCTP2, PDS5B, PLEKHG6, RAP1A, RASGRP3, SCRNI, SETD8, SLC1A7, SLC38A10, SLC38A2, SLC45A2, SNPH, SUN1, SUPT5H, TMEM100, TRIM9, TSGA14

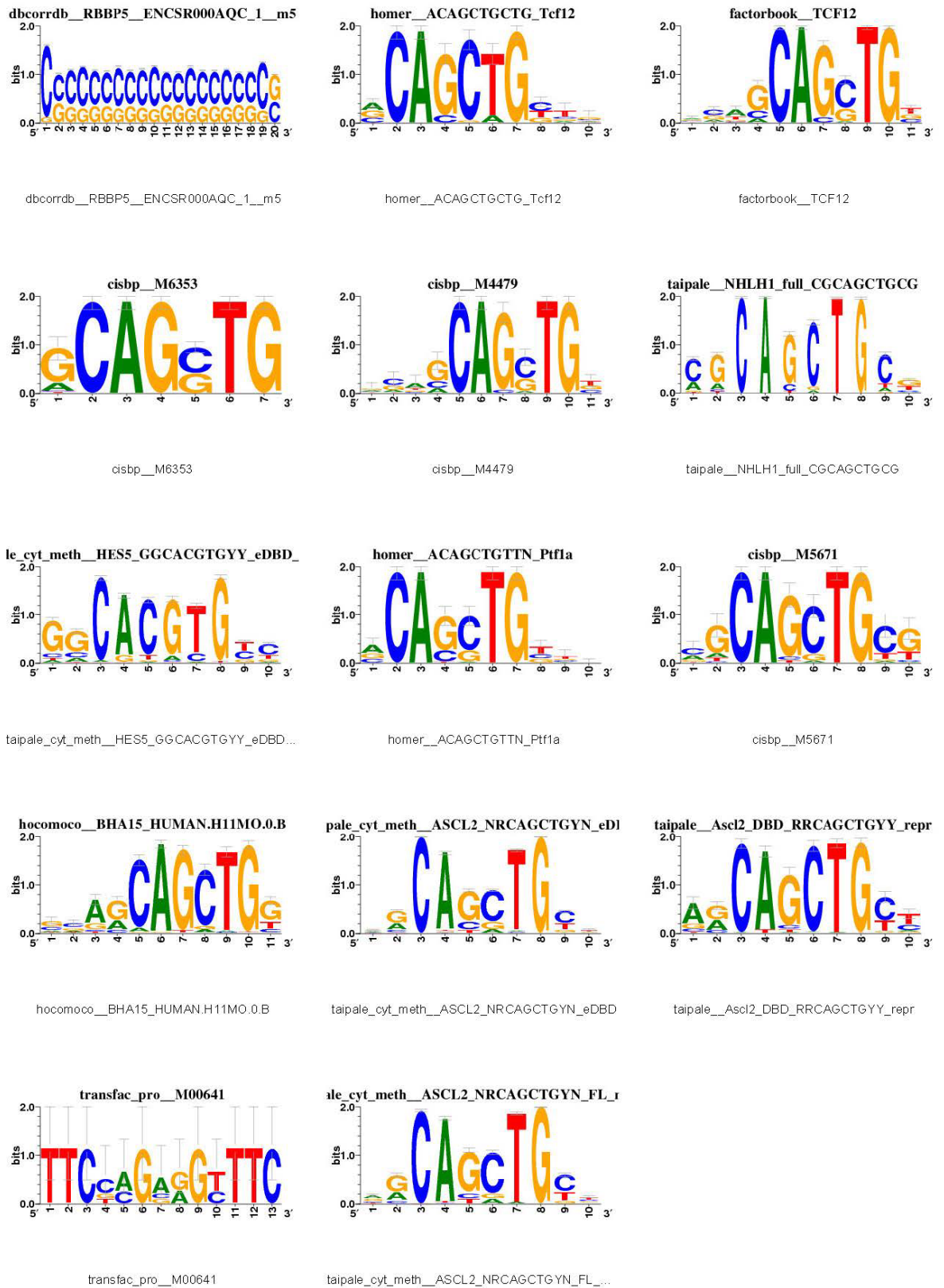


Figure 81. The sequence graphs for transcription factor motifs significantly enriched (NES >4) in the top 100 dmCpGs associated with the analysis of MESP D insufficient mothers in the MAVIDOS RCT

9.4.3.2.3 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with intervention group for mothers vitamin D insufficient in early stage pregnancy

Table 60. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with the analysis of intervention MESP D insufficient in the MAVIDOS RCT

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
776	1.93E-03	kinetochore	CENPT CLIP1 NDE1 CENPA
775	4.67E-03	chromosome, centromeric region	CENPT CLIP1 NDE1 CENPA
7163	4.67E-03	establishment or maintenance of cell polarity	NDE1 CENPA ANK1
777	5.89E-03	condensed chromosome kinetochore	CENPT NDE1 CENPA
779	5.89E-03	condensed chromosome, centromeric region	CENPT NDE1 CENPA
44427	5.89E-03	chromosomal part	CENPT CLIP1 NDE1 PDS5B CENPA
5694	1.19E-02	chromosome	CENPT CLIP1 NDE1 PDS5B CENPA
7049	1.61E-02	cell cycle	GAK CLIP1 NDE1 PDS5B SUPT5H CENPA
793	2.32E-02	condensed chromosome	CENPT NDE1 CENPA
5085	3.11E-02	guanyl-nucleotide exchange factor activity	RGL1 RALGDS RASGRP3
278	3.77E-02	mitotic cell cycle	CLIP1 NDE1 PDS5B CENPA
16043	4.73E-02	cellular component organization	CLIP1 IRS1 NDE1 PDS5B TGFB1 SUPT5H CELSR2 CENPA ANK1
5515	4.77E-02	protein binding	PHF3 RWDD3 PTPRN2 MGRN1 IRS1 NDE1 PDS5B SUPT5H CELSR2 CENPA LTBP1 ANK1 RASGRP3 GAK CLIP1 TGFB1 RGL1
280	4.77E-02	nuclear division	CLIP1 NDE1 PDS5B
7067	4.77E-02	mitosis	CLIP1 NDE1 PDS5B
87	4.77E-02	M phase of mitotic cell cycle	CLIP1 NDE1 PDS5B
48285	4.77E-02	organelle fission	CLIP1 NDE1 PDS5B
51649	4.77E-02	establishment of localization in cell	SCRN1 MGRN1 NDE1 CENPA ANK1

9.4.4 Maternal late-stage pregnancy vitamin D status-related analyses in the SWS cohort

9.4.4.1 Maternal late-stage pregnancy vitamin D status

9.4.4.1.1 The top 100 dmCpGs associated with maternal late-stage pregnancy vitamin D status

Table 61. The top 100 dmCpGs associated with MLSP vitamin D status in the SWS cohort

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	0.000524	0.1821	TXNIP	51	0.000123	0.3995	C19orf71
2	-3.8E-05	0.2217	HMOX2	52	8.81E-05	0.3995	ZNF775
3	-4.7E-05	0.2218	LRRC34	53	0.000202	0.3995	cg13092315
4	0.000498	0.2218	TXNIP	54	0.000134	0.3995	SEMA4B
5	0.000182	0.2218	HMCN2	55	-4.9E-05	0.3995	MIR155HG
6	0.000257	0.2218	SLC6A18	56	0.000121	0.3995	TRPM1
7	0.000217	0.2218	cg15959921	57	0.000288	0.3995	FAM208A
8	0.000139	0.2319	cg04525002	58	0.000229	0.3995	ROBO1
9	0.000167	0.2682	CCDC49	59	0.000179	0.3995	CDKN2B-AS1
10	0.000208	0.2682	PRSS21	60	0.000188	0.3995	cg16583536
11	8.99E-05	0.2682	cg19323811	61	7.91E-05	0.3995	SLC39A11
12	0.000238	0.2761	TSGA10IP	62	5.88E-05	0.3995	ZCCHC7
13	-4.6E-05	0.2761	WHAMM	63	8.25E-05	0.3995	TNKS
14	7.28E-05	0.2761	cg10574377	64	0.000108	0.3995	cg21204230
15	0.000172	0.3283	cg00858150	65	0.000199	0.3995	BPI
16	0.000149	0.3283	TCERG1L	66	0.000162	0.3995	cg22872992
17	0.000161	0.3283	cg04733196	67	0.000173	0.3995	CEND1
18	0.000257	0.3283	cg05507654	68	8.66E-05	0.3995	ANXA3
19	0.000144	0.3283	CD300E	69	7.84E-05	0.3995	VPS13D
20	0.000159	0.3283	MYLK	70	9.87E-05	0.4018	ELOVL1
21	-4.9E-05	0.3283	TMBIM4	71	0.000182	0.4018	PDF
22	0.000144	0.3283	GALNT9	72	0.000191	0.4018	NOS1
23	0.000152	0.3283	PXDNL	73	0.000178	0.4018	cg03341576
24	-0.00029	0.3537	RCCD1	74	0.000102	0.4018	cg03620964
25	0.000156	0.3593	FGF21	75	0.000161	0.4018	cg03662347
26	0.000272	0.3749	TMEM176B	76	0.000166	0.4018	cg04112181
27	-6.1E-05	0.3749	APLP1	77	-9.9E-05	0.4018	SLC43A2
28	-5.2E-05	0.3995	ABCC3	78	6.79E-05	0.4018	MRPS18C
29	-7.3E-05	0.3995	PTPN4	79	7.3E-05	0.4018	MADD
30	4.84E-05	0.3995	CIITA	80	0.000249	0.4018	PPP2R2C
31	0.000185	0.3995	CPLX1	81	0.000238	0.4018	cg05329578
32	-4.9E-05	0.3995	ZBTB10	82	8.53E-05	0.4018	ATP13A1
33	8.87E-05	0.3995	WDR33	83	-3.2E-05	0.4018	LRTOMT
34	0.000127	0.3995	cg03470331	84	-0.0001	0.4018	cg06064588
35	-3.9E-05	0.3995	SNX3	85	0.000147	0.4018	cg06488388
36	0.000162	0.3995	DHX37	86	0.000119	0.4018	cg06696063
37	0.000152	0.3995	cg04730551	87	-3.7E-05	0.4018	CDK4
38	0.000191	0.3995	cg04735773	88	-0.00057	0.4018	ABI3BP
39	8.22E-05	0.3995	SOLH	89	0.000148	0.4018	MYL10
40	-5.6E-05	0.3995	TRRAP	90	6.26E-05	0.4018	BAP1
41	9.54E-05	0.3995	TNS2	91	0.000204	0.4018	WNK2
42	9.96E-05	0.3995	cg06411987	92	-3.2E-05	0.4018	ROGDI
43	0.000133	0.3995	ZNF563	93	9.04E-05	0.4018	cg11438730
44	4.22E-05	0.3995	LRPAP1	94	-3.2E-05	0.4018	RPRD1B
45	4.57E-05	0.3995	cg07296279	95	-0.00021	0.4018	cg12467478
46	6.07E-05	0.3995	KIF26A	96	-6E-05	0.4018	ZUFSP
47	-0.00018	0.3995	cg09191149	97	0.000125	0.4018	cg12910107
48	0.000203	0.3995	NEURL3	98	0.000178	0.4018	CATSPERD
49	0.000155	0.3995	NOTUM	99	0.000137	0.4018	cg13520575
50	-3.9E-05	0.3995	NFATC2	100	9.77E-05	0.4018	LRRC43

9.4.4.1.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with maternal late-stage pregnancy vitamin D status

Table 62. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with MLSP D status in the SWS cohort

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
taipale_cyt_meth__OVOL1_NRWA CCGTTATNYN_FL_meth_repr	OVOL1	5.02	Direct	12	ABI3BP, ATP13A1, CD300E, GNE, KIF26A, LRRC43, NFATC2, OVOL1, ROBO1, TRPM1, YWHAE, ZBTB10
taipale_cyt_meth__TBX18_NRAGG TGTGAAN_eDBD_meth	TBX18	4.3	Direct	10	ABI3BP, C7orf51, CD300E, CDK4, ITGA1, MADD, NAV1, NKAIN2, OVOL1, ROBO1
taipale__TBX20_full_NAGGTGTGAA N	TBX20	4.29	Direct	10	ABI3BP, C7orf51, CD300E, HMOX2, ITGA1, NAV1, NFATC2, NKAIN2, OVOL1, RAP1GAP2
transfac_pro__M07940	OVOL1	4.2	Inferred	13	ABI3BP, ATP13A1, FRMD4A, KIF26A, MYLK, NFATC2, OVOL1, ROBO1, SEMA4B, SLC6A18, WNK2, YWHAE, ZBTB10
cisbp__M5886	TBX20	4.08	Direct	10	ABI3BP, C7orf51, CD300E, HMOX2, ITGA1, NAV1, NFATC2, NKAIN2, OVOL1, RAP1GAP2
cisbp__M0438	ZNF8	4.01	Inferred	11	AFF1, C7orf51, CELF4, FAM49B, FRMD4A, LMBR1, MADD, OVOL1, PRRX2, WDR33, ZBTB25

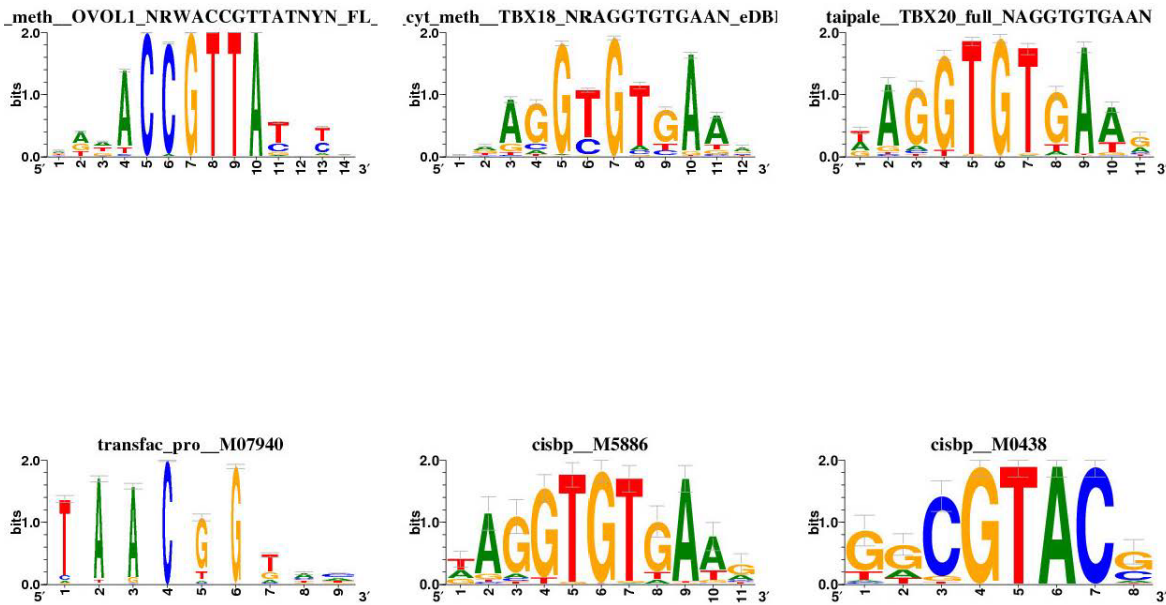


Figure 82. The sequence graphs for transcription factor motifs significantly enriched (NES >4) in the top 100 dmCpGs associated with MLSP D status in the SWS cohort

9.4.4.1.3 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with maternal late-stage pregnancy vitamin D status

Table 63. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with MLSP D status in the SWS cohort

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
5635	4.54E-02	nuclear envelope	RANBP2 TNKS TMEM176B
43005	4.54E-02	neuron projection	ANXA3 NOS1 ROBO1

9.4.4.2 Maternal late-stage pregnancy vitamin D insufficiency

9.4.4.2.1 The top 100 dmCpGs associated with maternal late-stage pregnancy vitamin D insufficiency

Table 64. The top 100 dmCpGs associated with MLSP vitamin D insufficiency in the SWS cohort

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	-0.00095	0.005895	HK1	51	0.000594	0.1542	ZNF423
2	-0.00095	0.008254	cg08431985	52	-0.00088	0.1542	cg25881032
3	0.001431	0.01375	cg18940084	53	-0.00031	0.1568	TRADD
4	0.000572	0.02479	SPECC1	54	-0.00025	0.1617	BTBD1
5	-0.00215	0.02851	SLC22A3	55	0.001069	0.1617	PLXNA4
6	0.000863	0.02851	cg02379973	56	0.001194	0.1651	TMEM66
7	-0.00097	0.02851	HCCA2	57	-0.00051	0.1651	NFIX
8	-0.00066	0.02851	PDIK1L	58	-0.00049	0.1723	NCRNA00173
9	-0.00028	0.02851	NMU	59	-0.00079	0.1723	TCF23
10	0.001079	0.02851	GALNT13	60	0.001012	0.1723	BPIFB1
11	-0.0003	0.02851	SALL1	61	0.000845	0.1725	PTPN1
12	-0.00032	0.03386	C17orf49	62	-0.00068	0.1725	ZNF549
13	0.000308	0.05331	APTX	63	0.000394	0.1747	DCTN4
14	-0.00028	0.06148	KIAA1217	64	-0.00025	0.1747	GFPT1
15	0.001594	0.06785	C8A	65	-0.00152	0.1747	ATP5A1
16	0.000609	0.07426	UBE2L6	66	0.001047	0.1791	PKNOX2
17	-0.00029	0.07426	cg11179308	67	0.000701	0.1791	DSPP
18	-0.001	0.07426	cg19353294	68	-0.00022	0.1791	USP42
19	-0.00172	0.104	EIF4E3	69	-0.00172	0.1791	WHAMM
20	-0.00027	0.104	cg08957925	70	0.000342	0.1791	GATAD2A
21	-0.00054	0.104	ZNF461	71	-0.00019	0.1791	RASA1
22	-0.00036	0.1156	CDK13	72	-0.00022	0.1861	HNRNPU
23	0.001136	0.1156	cg09516677	73	0.000895	0.1861	cg01439945
24	0.001405	0.1156	cg11543883	74	0.000593	0.1861	cg07136029
25	0.00081	0.1156	PPP2R2D	75	-0.00091	0.1861	TRMT2A
26	-0.00194	0.1156	NOTCH1	76	0.000428	0.1884	LOC401463
27	0.000639	0.1156	cg16101118	77	-0.00069	0.1884	GCC1
28	-0.0005	0.1156	CUX2	78	0.000382	0.1884	cg02812408
29	0.001043	0.1177	C8orf34	79	0.000694	0.1884	NIN
30	-0.00062	0.1177	RHOJ	80	-0.00025	0.1884	TACR1
31	0.001514	0.1197	cg02059509	81	-0.00072	0.1884	DERA
32	0.002652	0.1197	FRAS1	82	0.000648	0.1884	C9orf144
33	0.00117	0.1197	KIAA1462	83	-0.00028	0.1884	DAZAP2
34	-0.00282	0.1276	KBTBD11	84	0.000613	0.1884	PAPD4
35	-0.00122	0.132	TNIP1	85	0.000938	0.1884	SBK3
36	-0.00054	0.1379	AXIN1	86	0.002265	0.1884	cg26134300
37	0.001266	0.1386	ROS1	87	0.00066	0.1952	cg02098816
38	-0.0007	0.1499	LETM1	88	0.00105	0.1952	cg05228120
39	-0.00103	0.1507	EDC4	89	-0.00185	0.1952	RTEL1
40	-0.00067	0.1507	ATP2B4	90	0.000748	0.1952	cg07274516
41	-0.00044	0.1507	SLC16A10	91	-0.00077	0.1952	FAM129C
42	0.004485	0.1507	ABCC13	92	0.000949	0.1952	cg09155904
43	-0.00073	0.1517	C3orf27	93	0.000479	0.1952	SFTA2
44	0.001084	0.1527	cg01292139	94	0.001324	0.1952	DDAH1
45	0.000841	0.1527	cg01543452	95	0.00054	0.1952	STAG1
46	-0.00097	0.1527	cg07202441	96	0.000415	0.1952	cg11476326
47	-0.00123	0.1527	GNAS	97	-0.00035	0.1952	HAS2
48	0.001809	0.1527	TMF1	98	0.000749	0.1952	cg13259502
49	0.000478	0.1542	MYT1L	99	0.000692	0.1952	cg13455509
50	0.000662	0.1542	KIF13A	100	-0.00038	0.1952	GTPBP10

9.4.4.2.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with maternal late-stage pregnancy vitamin D insufficiency

Table 65. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with MLSP D insufficiency in the SWS cohort

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
jaspar__MA0063.1	NKX2-5	4.26	Inferred	18	ATP2B4, ATP5A1, CUX2, DSPP, FRAS1, GATAD2A, GNAS, HAS2, HNRNPU, LOC401463, NMU, PANK4, SALL1, SFTA2, SLC11A2, SLC16A10, TLE4, TRMT2A

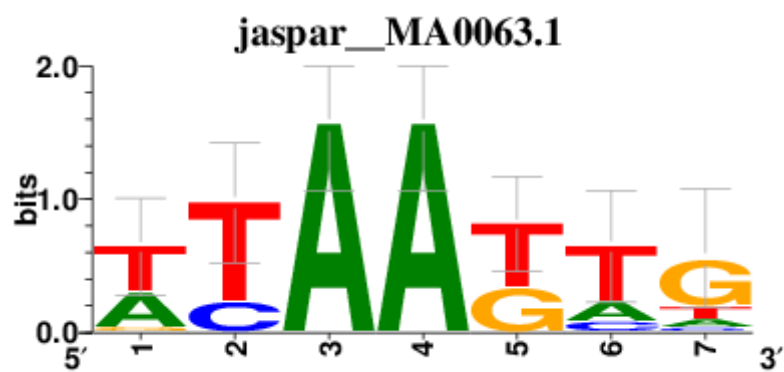


Figure 83. The sequence graph for transcription factor motif significantly enriched (NES >4) in the top 100 dmCpGs associated with MLSP D insufficiency in the SWS cohort

9.4.4.2.3 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with maternal late-stage pregnancy vitamin D insufficiency

Table 66. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with MLSP D insufficiency in the SWS cohort

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
32553	4.92E-02	ribonucleotide binding	NIN ATP5A1 KIF13A HNRNPU ULK2 CDK13 ROS1 GLUL
32555	4.92E-02	purine ribonucleotide binding	NIN ATP5A1 KIF13A HNRNPU ULK2 CDK13 ROS1 GLUL
5524	4.92E-02	ATP binding	ATP5A1 KIF13A HNRNPU ULK2 CDK13 ROS1 GLUL
17076	4.92E-02	purine nucleotide binding	NIN ATP5A1 KIF13A HNRNPU ULK2 CDK13 ROS1 GLUL
32559	4.92E-02	adenyl ribonucleotide binding	ATP5A1 KIF13A HNRNPU ULK2 CDK13 ROS1 GLUL
32879	4.92E-02	regulation of localization	SPAG9 NMU UNC5C TACR1 ROS1
30554	4.92E-02	adenyl nucleotide binding	ATP5A1 KIF13A HNRNPU ULK2 CDK13 ROS1 GLUL
30334	4.92E-02	regulation of cell migration	SPAG9 UNC5C TACR1
34641	4.92E-02	cellular nitrogen compound metabolic process	NFIX DDAH1 ATP5A1 PIR HNRNPU GLUL DERA PNRC2
1883	4.92E-02	purine nucleoside binding	ATP5A1 KIF13A HNRNPU ULK2 CDK13 ROS1 GLUL
5515	4.92E-02	protein binding	SPAG9 DAZAP2 ATP5A1 HNRNPU UNC5C TACR1 DERA PNRC2 LETM1 NIN NMU KIF13A PIR ULK2 HOXB1 ROS1 GLUL
1882	4.92E-02	nucleoside binding	ATP5A1 KIF13A HNRNPU ULK2 CDK13 ROS1 GLUL

9.4.4.3 Maternal late-stage pregnancy vitamin D sufficiency

9.4.4.3.1 The top 100 dmCpGs associated with maternal late-stage pregnancy vitamin D sufficiency

Table 67. The top 100 dmCpGs associated with MLSP vitamin D sufficiency in the SWS cohort

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	0.000224	0.1318	ARRDC2	51	-5.8E-05	0.293	MRPS34
2	-0.00013	0.1318	HCG27	52	0.00029	0.293	MYT1L
3	-0.00011	0.1318	DSEL	53	0.0004	0.293	TNPO1
4	0.000391	0.1318	NEBL	54	0.000294	0.293	cg22346500
5	0.000371	0.1318	cg11182160	55	0.000321	0.293	CNTNAP5
6	0.000229	0.1318	cg12740011	56	0.000345	0.293	cg24546683
7	0.000269	0.1318	cg13708613	57	0.000289	0.293	cg25803918
8	0.000466	0.1318	BPI	58	-0.00015	0.293	ACYP2
9	0.000247	0.2158	LGI2	59	-5.5E-05	0.3115	LRRC34
10	-0.0002	0.2482	cg19090034	60	0.000102	0.3115	ARID4B
11	-7.1E-05	0.289	ABCC3	61	-0.00021	0.3115	cg25008857
12	0.000263	0.289	GALNT9	62	0.000636	0.3115	TXNIP
13	-5.3E-05	0.289	cg03068458	63	-0.00013	0.316	cg27116543
14	-0.00013	0.289	MICAL3	64	0.000339	0.3248	cg16743754
15	0.000325	0.289	cg04735773	65	0.000243	0.3248	CHD4
16	0.000365	0.289	PFKFB3	66	0.000269	0.3271	C16orf5
17	7.41E-05	0.289	cg07296279	67	0.000476	0.3271	FAM208A
18	-5.7E-05	0.289	WHAMM	68	0.000186	0.3271	ITGA7
19	0.000325	0.289	cg07621030	69	0.000241	0.3271	CCND2
20	0.000275	0.289	cg09259843	70	-0.00015	0.3347	PHLDB1
21	0.000271	0.289	PGAM2	71	-9.9E-05	0.3347	DR1
22	-0.00014	0.289	SWT1	72	-0.00014	0.3353	LRRC37B2
23	0.000327	0.289	C2orf85	73	0.000119	0.3432	cg17068819
24	-4.8E-05	0.289	TMEM143	74	0.000255	0.3499	cg01625396
25	-9.5E-05	0.289	DPM2	75	-4.8E-05	0.3499	cg03901351
26	0.000278	0.289	cg15052470	76	-0.00012	0.3499	TMEM14C
27	-4.4E-05	0.289	PPA1	77	8.24E-05	0.3499	SNX19
28	-0.00012	0.289	cg16525035	78	-0.00012	0.3499	IL4
29	-3.8E-05	0.289	NME3	79	-5.4E-05	0.3499	TUBB
30	0.000206	0.289	cg16899991	80	0.00061	0.3541	cg08234653
31	0.000607	0.289	SERPINA6	81	0.000199	0.3543	ZBTB7C
32	-5.4E-05	0.289	SFXN4	82	-0.00015	0.3751	cg00603850
33	0.000249	0.289	HAO2	83	0.000211	0.3751	ADORA2A
34	0.000137	0.289	FGGY	84	-0.00028	0.3751	SNX29
35	-6.2E-05	0.289	KIAA1377	85	0.00027	0.3751	KIAA0284
36	-9.1E-05	0.289	cg19819774	86	0.000133	0.3751	GOSR2
37	0.000155	0.289	RALGPS1	87	0.000404	0.3751	cg10697663
38	0.000551	0.289	CYP4F12	88	-6.5E-05	0.3751	GTPBP8
39	-6.2E-05	0.289	ARPC5	89	-0.00038	0.3751	cg13943773
40	-9.6E-05	0.2893	MGAT4A	90	0.000274	0.3751	cg17361541
41	-7.6E-05	0.293	LOC646851	91	0.000165	0.3751	cg19952829
42	0.000191	0.293	PLCH2	92	0.000268	0.3751	cg21673927
43	7.09E-05	0.293	CIITA	93	0.000322	0.382	PCDHA1
44	-0.00018	0.293	MIOS	94	-0.00023	0.3844	cg03040863
45	-0.0001	0.293	PHC2	95	0.000226	0.3844	cg12814059
46	-4.6E-05	0.293	C20orf194	96	0.000478	0.3844	PRR4
47	0.000256	0.293	cg04733196	97	-0.00015	0.3884	KLHL17
48	-4.8E-05	0.293	LANCL2	98	0.000112	0.3884	MRPS18C
49	0.00044	0.293	NFATC2	99	-0.00022	0.3884	cg04785299
50	-6.3E-05	0.293	SMAD5	100	0.000292	0.3884	RNF223

9.4.4.3.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with maternal late-stage pregnancy vitamin D sufficiency

Table 68. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with MLSP D sufficiency in the SWS cohort

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
taipale_cyt_meth__ZSCAN23_NYCATGTGCTAA TTACAMN_eDBD_meth__repr	ZSCAN23	6.85	Direct	12	AFF1, BPI, C4orf45, GOSR2, HAO2, ITGA7, NEBL, PHLDB1, PLCH2, PPA1, SERPINA6, TMEM143
taipale_cyt_meth__ZSCAN23_NYCATGTGCTAA TTACAMN_eDBD	ZSCAN23	5.78	Direct	15	AFF1, BPI, C2orf85, C4orf45, GOSR2, HAO2, ITGA7, MICAL3, NEBL, PCDHA1, PHLDB1, PLCH2, SERPINA6, TMEM143, ZBTB7C
dbcordb__FOXMI__E NCSR000BRU_1__m2	FOXMI	4.69	Direct	12	ANGPT4, ARSB, BPI, CCND2, FAM53B, GOSR2, LOC619207, MGAT4A, PCDHA1, PHLDB1, RTN4, SERPINA6
dbcordb__NFIC__ENC R000BRN_1__m2	NFIC	4.65	Direct	10	ARSB, BPI, FAM53B, GOSR2, LOC619207, MGAT4A, PCDHA1, PHLDB1, RTN4, SERPINA6
tfdimers__MD00351	IRF8	4.43	Direct	10	AFF1, ARID4B, CCND2, CIITA, CRB2, FGGY, ITGA7, KIAA1377, SEPT9, SLC35D2
tfdimers__MD00336	IRF8	4.29	Direct	16	ACYP2, AFF1, ARID4B, CCND2, CIITA, CRB2, LANCL2, MYT1L, NFATC2, PRR4, RTN4, SERPINA6, SLC35D2, TUBB, TXNIP, ZBTB17
transfac_pro__M05699	ZNF816	4.27	Direct	21	ALOXE3, ANGPT4, ARRDC2, ARSB, CCND2, CHAT, DTHD1, GTPBP8, IL4, LANCL2, LOC619207, MYT1L, PCDHA1, PGAM2, PHC2, PHLDB1, RTN4, SEPT9, SERPINA6, TNPO1, ZBTB17
tfdimers__MD00547	FOXMI	4.25	Direct	12	ANGPT4, CCND2, CHAT, DEFB119, KIAA1377, MYT1L, PCDHA1, PGAM2, PLCH2, TUBB, WHAMM, ZBTB7C
taipale_cyt_meth__NKX3-2_NTCGTTAN_eDBD_meth	NKX3-2	4.21	Direct	13	C20orf194, C6orf165, CCND2, CNTNAP5, GOSR2, GTPBP8, KIAA0284, LGI2, NFATC2, PHC2, PPA1, RTN4, SEPT7
dbcordb__STAT5A__E NCSR000BQZ_1__m2	STAT5A	4.16	Direct	11	ANGPT4, ARSB, BPI, CCND2, FAM53B, GOSR2, LOC619207, MGAT4A, PCDHA1, RTN4, SERPINA6
hocomoco__ZNF85_HUMAN.H11MO.1.C	ZNF85	4.13	Direct	10	ACYP2, CCND2, DTHD1, LANCL2, MICAL3, NFATC2, PHLDB1, SEPT9, SPRED1, TUBB
cisbp__M5449	FOXG1	4.09	Direct	10	ARSB, BPI, C6orf165, CNTNAP5, DTHD1, KIAA1377, NEBL, NFATC2, PRR4, TXNIP
dbcordb__NFATC1__E NCSR000BQL_1__m3	NFATC1	4.09	Direct	16	ANGPT4, ARSB, BPI, C4orf45, CCND2, FAM53B, GOSR2, LOC619207, MGAT4A, PCDHA1, PGAM2, PHC2, PHLDB1, RTN4, SERPINA6, TXNIP
taipale__POU6F2_DBD__WTAATKAGCTMATTA W	POU6F2	4.07	Direct	12	ARSB, ARSG, C4orf45, CCDC57, CCND2, DEFB119, HAO2, LANCL2, MIOS, MRPS18C, NEBL, ZBTB7C
hocomoco__RUNX1_HUMAN.H11MO.0.A	RUNX1	4.07	Direct	9	ARSB, BPI, FAM53B, IL4, LOC619207, MGAT4A, PCDHA1, RTN4, SERPINA6

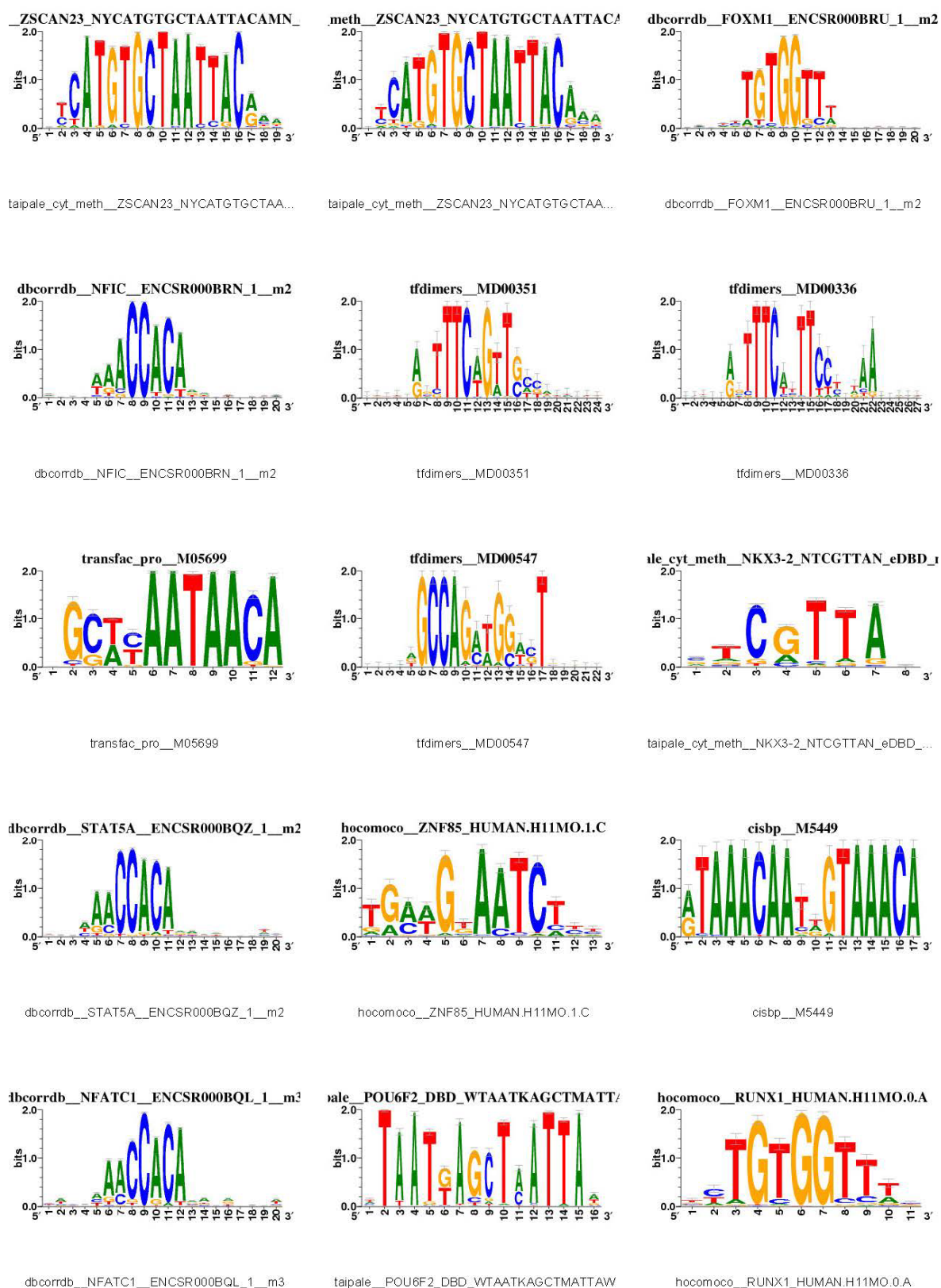


Figure 84. The sequence graphs for transcription factor motifs significantly enriched (NES >4) in the top 100 dmCpGs associated with MLSP D sufficiency in the SWS cohort

9.4.5 BMC-related methylation analyses in the MAVIDOS RCT

9.4.5.1 BMC at four years of age

9.4.5.1.1 Top 100 dmCpGs associated with BMC at four years of age

Table 69. The top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	0.000151	0.00136	C14orf23	51	0.000278	0.1213	PINX1
2	-0.00055	0.003306	cg02973930	52	5.22E-05	0.1213	cg01961134
3	-0.00026	0.0222	CSMD3	53	-0.00019	0.1213	PTPRM
4	8.13E-05	0.03258	TMCC3	54	0.00012	0.1213	ZWINT
5	0.000136	0.03822	POU6F1	55	9.58E-05	0.1223	cg18528591
6	-0.00043	0.03822	CD82	56	0.000268	0.1227	FAM107B
7	0.000155	0.05723	FER1L5	57	9.78E-05	0.1246	cg04580394
8	0.000221	0.05723	DAB2IP	58	-0.00014	0.1246	PABPC1
9	-0.00031	0.05723	cg16702610	59	0.00039	0.1362	FLYWCH1
10	-0.00022	0.05723	cg25106913	60	-0.00019	0.1397	LOC102477328
11	-0.00044	0.06764	STIM1	61	-0.0002	0.1465	UTRN
12	6.77E-05	0.1067	HSD11B1	62	0.000114	0.1482	MPP7
13	-8.7E-05	0.1067	LRFN2	63	-0.00019	0.1482	SDR16C5
14	0.000199	0.1104	USP12	64	-0.00034	0.1482	cg18776206
15	-0.0003	0.1104	RAB37	65	-0.00027	0.1482	cg26790217
16	-0.00019	0.1104	PPP2R5C	66	-0.00038	0.1486	STAT6
17	0.000228	0.1104	FUT5	67	0.000201	0.1486	ARHGAP22
18	8.45E-05	0.1104	FRMD3	68	0.00017	0.1486	MGAT4C
19	-0.00044	0.1104	GMDS	69	-0.00016	0.1526	cg00189927
20	5.12E-05	0.1104	GABRB3	70	6.09E-05	0.1528	TMEM106A
21	-0.00029	0.1104	cg10931627	71	-0.00025	0.1528	MKLN1
22	9.82E-05	0.1104	cg11687934	72	-0.00037	0.1689	cg26111226
23	0.000109	0.1104	DYSF	73	9.86E-05	0.1726	PRDM2
24	-0.00011	0.1104	PLCB1	74	0.000281	0.1735	FEV
25	0.000334	0.1104	NFIX	75	-0.00011	0.1903	ADAM17
26	-0.00054	0.1104	ST6GAL1	76	0.000101	0.1923	cg25286579
27	-0.00047	0.1104	SLC22A23	77	-0.0002	0.2011	MIR25
28	0.000217	0.1104	HECW1	78	-0.00033	0.2011	KIAA1919
29	6.54E-05	0.1104	KIAA1949	79	0.000304	0.2011	CEP68
30	0.000122	0.1104	LINC01122	80	-0.00014	0.2011	cg11022516
31	-0.00012	0.1104	cg25193525	81	0.000124	0.2011	cg11152857
32	-0.00023	0.1137	CLIC5	82	-0.00021	0.2011	cg18657917
33	0.000247	0.115	cg02167856	83	-0.00041	0.2011	TMEM117
34	0.000372	0.1161	CNTN4	84	0.000134	0.2011	RIC8A
35	-0.00037	0.1161	LRRC28	85	0.000363	0.2011	SRGAP1
36	-0.00061	0.1175	cg12273319	86	-0.00016	0.2011	cg26257545
37	0.000121	0.1175	cg21711075	87	0.000234	0.2017	CLEC14A
38	-0.00043	0.1194	KRTAP19-5	88	-0.00019	0.2017	cg14878302
39	8.53E-05	0.1195	MEOX2	89	0.000221	0.2017	NYNRIN
40	-0.00038	0.1195	CD96	90	-0.00032	0.2017	cg09629679
41	-0.00029	0.1195	CPA4	91	-0.00046	0.206	cg09176152
42	0.000123	0.1195	PARK2	92	-0.00025	0.2067	ZNF503
43	-0.00044	0.1195	cg19088503	93	0.00014	0.2069	ST6GALNAC1
44	0.000109	0.1195	EYA2	94	-0.00016	0.2078	SAMD4A
45	-0.00013	0.1195	PTPRN2	95	0.0002	0.2078	FBXL7
46	5.9E-05	0.1195	MAFF	96	0.000143	0.2078	cg09609598
47	0.000155	0.1195	cg22404419	97	9.4E-05	0.2078	cg12920070
48	-0.00019	0.1195	FHL5	98	-0.00031	0.2078	RARS
49	-0.00017	0.1195	DNAJC13	99	0.000274	0.2078	ZFH4
50	-0.0003	0.1195	cg27132553	100	0.00011	0.2078	cg16845483

9.4.5.1.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age

Table 70. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
taipale_cyt_meth_ _FOSL1_RATGAYAC G_FL	FOSL1	5.12	Direct	13	CCR7, CLIC5, GABRB3, GMDS, KRTAP19-5, MAFF, MEOX2, MKLN1, PLCB1, POU6F1, PPP2R5C, WNT11, ZFHX4
cisbp__M2191	TBP	4.38	Inferred	15	C14orf23, CNTN4, DLG2, FAM107B, GABRB3, HECW1, MEOX2, MGAT4C, NAV2, POU6F1, PRDM2, TMCC3, TMEM117, UTRN, WNT11
yetfasco__YER148 W_798	TBP	4.35	Inferred	16	C14orf23, CNTN4, DLG2, FAM107B, FHL5, GABRB3, HECW1, MEOX2, MGAT4C, NAV2, POU6F1, PRDM2, TMCC3, TMEM117, UTRN, WNT11
taipale_cyt_meth_ _ZBTB20_NYAAYGT ATRKN_eDBD_met h	ZBTB20	4.19	Direct	11	CCR7, CNTN4, DYSF, MGAT4C, NAV2, PABPC1, POU6F1, PRDM2, RXRa, TMEM117, WNT11
transfac_pro__M0 1524	TBP	4.18	Inferred	15	C14orf23, CNTN4, DLG2, FAM107B, FHL5, GABRB3, HECW1, MEOX2, NAV2, POU6F1, PRDM2, TMCC3, TMEM117, UTRN, WNT11
jaspar__MA0386.1	TBP	4.16	Inferred	14	C14orf23, CNTN4, DLG2, FAM107B, FHL5, GABRB3, HECW1, MEOX2, NAV2, POU6F1, TMCC3, TMEM117, UTRN, WNT11
taipale_cyt_meth_ _POU3F2_NTATGC GCATAN_eDBD_m eth	POU3F2	4.03	Direct	13	DLG2, DNAJC13, HSD11B1, KRTAP19-5, LRFN2, NAV2, POU6F1, SEPT9, SLC22A23, ST6GAL1, UTRN, WNT11, ZFHX4

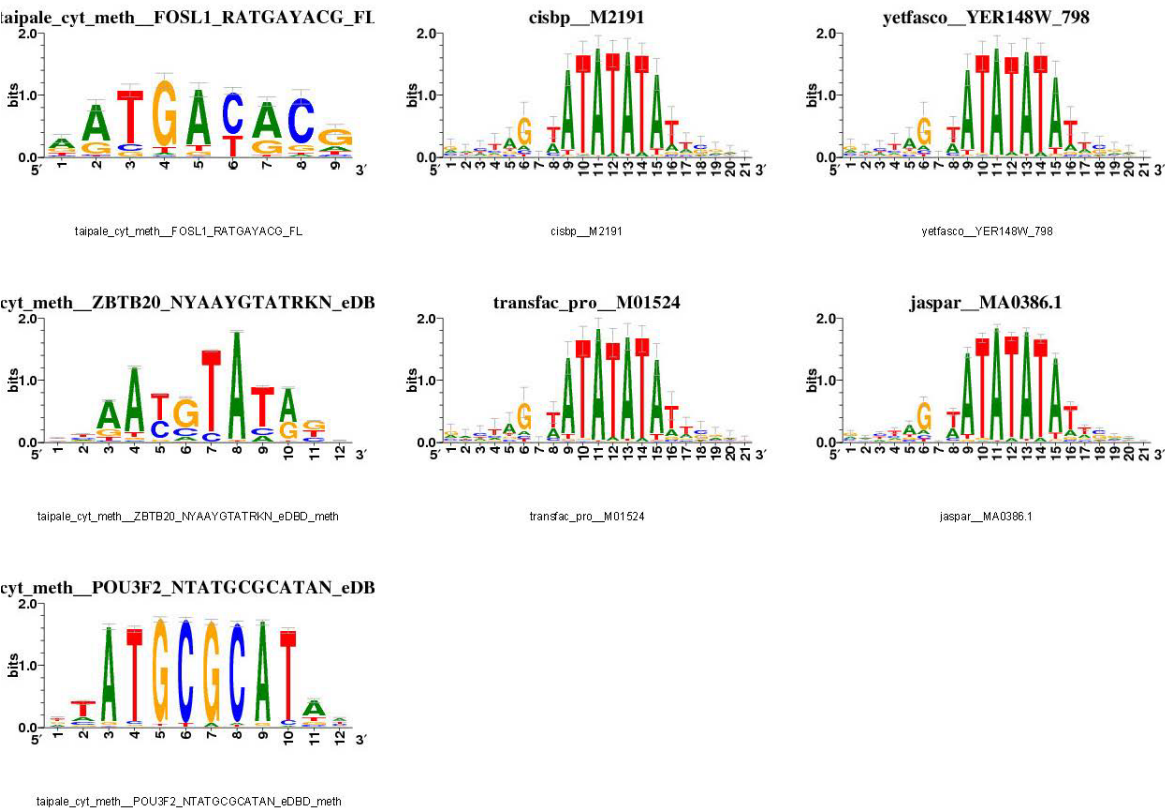


Figure 85. The sequence graphs for transcription factor motifs significantly enriched (NES >4) in the top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT

9.4.5.1.3 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMC at four years of age

Table 71. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with BMC at four years of age in the MAVIDOS RCT

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
19226	2.17E-02	transmission of nerve impulse	GABRB3 CNTNAP2 HTR1E
7165	2.17E-02	signal transduction	GABRB3 CNTNAP2 HTR1E DAB2IP ARHGAP15
23046	2.20E-02	signaling process	GABRB3 CNTNAP2 HTR1E DAB2IP ARHGAP15
23060	2.20E-02	signal transmission	GABRB3 CNTNAP2 HTR1E DAB2IP ARHGAP15
23046	3.68E-02	signaling process	DLG2 PLCB1 SRGAP1 PARK2
23060	3.68E-02	signal transmission	DLG2 PLCB1 SRGAP1 PARK2
9056	3.73E-02	catabolic process	PLCB1 PARK2 FBXL7
7154	4.47E-02	cell communication	GABRB3 CNTNAP2 HTR1E

9.4.6 BMD-related methylation analyses in the MAVIDOS RCT

9.4.6.1 BMD at birth

9.4.6.1.1 Top 100 dmCpGs associated with BMD at birth

Table 72. The top 100 dmCpGs associated with BMD at birth in the MAVIDOS RCT

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	-0.2998	0.007972	CTBP2	51	-0.2002	0.3078	CDIP1
2	-0.1455	0.007972	NEUROD4	52	-0.1983	0.3078	PDIA5
3	-0.4367	0.007972	cg22209601	53	-0.1371	0.3078	TMEM26
4	-0.6329	0.01848	TOP3A	54	-0.5653	0.3078	RADIL
5	-0.2106	0.01848	VAV2	55	0.2485	0.3078	PPP2R2A
6	-0.982	0.01913	cg07524558	56	-0.2413	0.3078	C1orf97
7	0.5307	0.1348	cg15020206	57	-0.118	0.3078	DPYSL5
8	-0.2902	0.1383	SLFN12	58	-0.3661	0.3078	C6orf47
9	-0.175	0.1383	CST5	59	-0.1318	0.3078	CCHCR1
10	-0.3855	0.1383	C15orf27	60	-0.1277	0.3078	WWOX
11	-0.2022	0.1383	cg13026587	61	-0.2538	0.3078	ADAMTS13
12	-0.1457	0.1498	ZNF282	62	-0.6758	0.3078	cg04366628
13	0.07522	0.1498	KCNK10	63	-0.09368	0.3078	MNT
14	-0.5031	0.1498	PARD6G	64	-0.2245	0.3078	TRAF3
15	-0.469	0.173	cg15348679	65	0.5247	0.3078	MAD2L2
16	0.2829	0.1914	cg00691939	66	-0.2536	0.3078	PTPRN2
17	0.6147	0.1914	ADAMTSL5	67	0.3195	0.3078	cg07766916
18	-0.5104	0.1914	SEMA3F	68	-0.2021	0.3078	FGFR1
19	-0.2721	0.1914	NOSTRIN	69	-0.3393	0.3078	GPR123
20	-0.5227	0.1914	cg13292374	70	-0.2742	0.3078	IGF1
21	-0.4644	0.1914	SHB	71	-0.4939	0.3078	BTC
22	0.6151	0.1934	cg27547377	72	0.09055	0.3078	PPP1CC
23	-0.2423	0.1964	NR4A1	73	-0.3333	0.3078	cg13538089
24	0.2727	0.2064	CERS6	74	-0.4917	0.3078	cg14201911
25	-0.2435	0.2064	LSP1	75	-0.3966	0.3078	cg14275429
26	-0.5047	0.2205	WWP2	76	-0.6643	0.3078	RASA3
27	-0.35	0.2205	cg22821013	77	-0.1438	0.3078	SPTA1
28	-0.2086	0.2205	HSF1	78	0.2915	0.3078	TBC1D22A
29	-0.1025	0.2205	BCL2	79	-0.2037	0.3078	LOC90586
30	-0.2503	0.2575	FIBCD1	80	0.3102	0.3078	MYO10
31	-0.1672	0.2575	WDR5	81	-0.07149	0.3078	C9orf91
32	-0.4016	0.2575	cg08123106	82	-0.2359	0.3078	cg21059834
33	0.313	0.2575	TNFRSF17	83	-0.2	0.3078	TENC1
34	-0.1814	0.2575	GLI3	84	-0.1488	0.3078	cg23395024
35	-0.2235	0.2575	FAM188B	85	-0.5829	0.3078	KCNB2
36	-0.2476	0.2575	CSMD3	86	-0.5066	0.3125	TGM7
37	0.1817	0.2575	TSSC1	87	-0.3515	0.3125	cg17254414
38	-0.3274	0.2575	AOC2	88	-0.107	0.3129	SCFD2
39	0.4447	0.2747	SERGEF	89	0.1077	0.3129	FOXK1
40	-0.5774	0.2842	LOC101927865	90	-1.149	0.3129	cg16690299
41	-0.1834	0.2842	PFKL	91	0.4485	0.3189	ANKRD29
42	0.1097	0.2871	cg08490791	92	0.1379	0.3189	C10orf76
43	-0.256	0.3011	LYNX1	93	-0.1017	0.3222	CHIC2
44	-0.2593	0.3011	cg02447638	94	-0.08901	0.3312	FGF22
45	0.2377	0.3011	INO80C	95	0.269	0.3312	PTPRD
46	-0.261	0.3011	TMEM141	96	-0.345	0.3326	SOLH
47	-0.1314	0.3011	COL11A2	97	-0.3246	0.3326	GRM8
48	-0.4596	0.3011	SH3BP2	98	-0.7071	0.3333	cg05177932
49	0.5231	0.3011	MTUS1	99	0.8943	0.3348	KCNAB2
50	-0.3659	0.3078	USH1C	100	0.2782	0.3358	cg07907563

9.4.6.1.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at birth

Table 73. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with BMD at birth in the MAVIDOS RCT

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
hocomoco__SP1_H UMAN.H11MO.0.A	SP1	4.42	Direct	10	COL11A2, DLGAP4, LYNX1, MAD2L2, MNT, MYO10, PPP1CC, PPP2R2A, RADIL, TMEM141
transfac_pro__M0 6676	ZNF665	4.37	Direct	10	ANKRD29, BCL2, C6orf47, CSMD3, GRM8, IGF1, MLL2, MTUS1, TENC1, WWP2
transfac_pro__M0 6296	ZNF568	4.14	Direct	30	ANKRD29, AUTS2, BCL2, CADPS2, CCHCR1, CHIC2, CSMD3, CTBP2, DLGAP4, DPYSL5, FGFR1, GRM8, IGF1, KCNK10, MAD2L2, MTUS1, PARD6G, PDIA5, PTPRD, RAB6B, RGS12, SCAMP5, SH3BP2, SPTA1, TRAF3, USH1C, VAV2, WWOX, WWP2, ZNF282

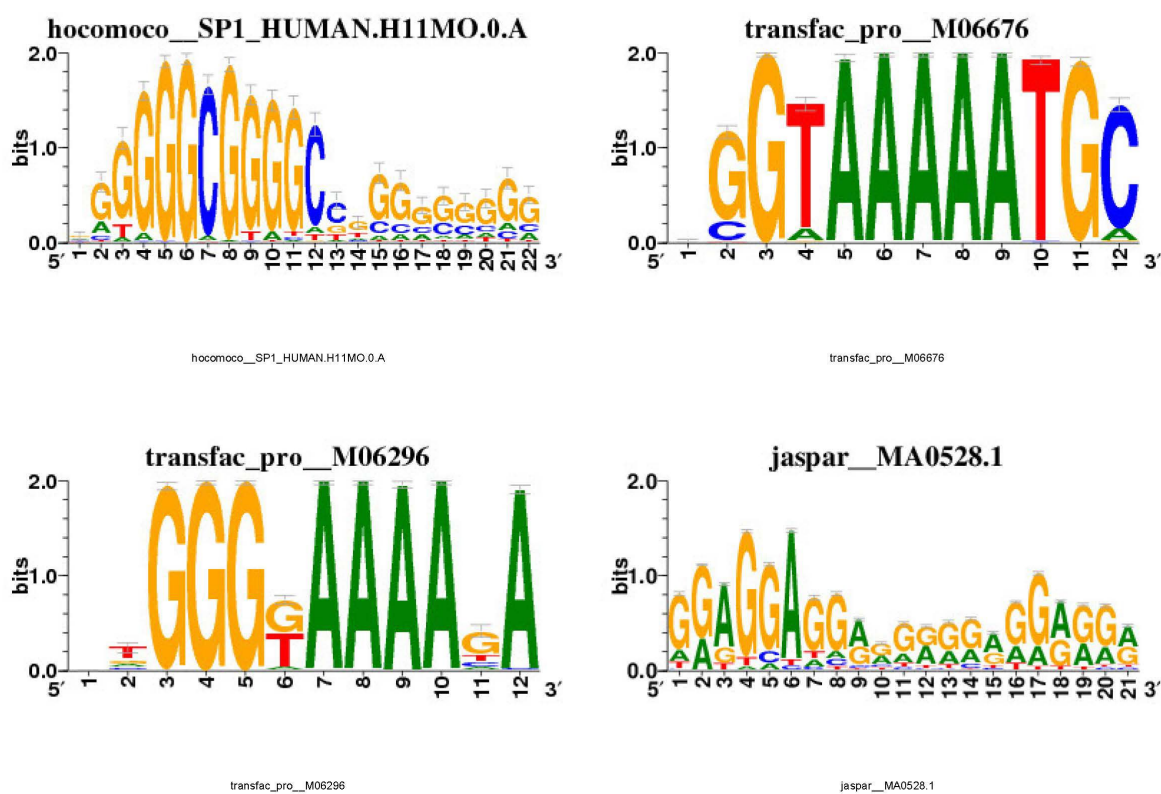


Figure 86. The sequence graphs for transcription factor motifs significantly enriched (NES >4) in the top 100 dmCpGs associated with BMD at birth in the MAVIDOS RCT

9.4.6.2 BMD at four years of age

9.4.6.2.1 Top 100 dmCpGs associated with BMD at four years of age

Table 74. The top 100 dmCpGs associated with BMD at four years of age in the MAVIDOS RCT

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	-0.3297	0.001049	CSMD3	51	-0.4703	0.08252	cg26111226
2	-0.259	0.002496	cg26257545	52	0.2514	0.08252	C19orf57
3	-0.2572	0.01695	PTPRM	53	-0.07297	0.08252	MARCKSL1
4	-0.6914	0.01714	FERMT2	54	-0.3339	0.08345	MKLN1
5	-0.1597	0.0194	cg25193525	55	0.3059	0.08852	FBXL7
6	0.07637	0.02072	GABRB3	56	-0.2392	0.08883	UTRN
7	-0.4172	0.02072	cg16702610	57	0.3113	0.08883	ZDHHHC18
8	-0.4083	0.02874	RAB37	58	0.1087	0.09309	PLA2G12A
9	-0.3406	0.02874	TIGAR	59	-0.315	0.09309	SEMA3B
10	-0.2805	0.03086	HIVEP1	60	-0.4779	0.09575	KCNMA1
11	-0.2129	0.03086	SLC16A12	61	-0.5398	0.1013	SLC22A23
12	0.3437	0.03802	cg02925006	62	-0.2849	0.1042	CLIC5
13	-0.6209	0.04486	cg02973930	63	-0.08249	0.1042	RSF1
14	-0.5434	0.04486	cg06112335	64	0.3857	0.1042	KCNH6
15	-0.6598	0.04486	STT3B	65	-0.2276	0.1052	cg15601996
16	0.1699	0.04486	C14orf23	66	-0.07091	0.1052	ZNF697
17	0.1652	0.04486	LINC01122	67	0.2508	0.1055	LOC100996634
18	-0.1066	0.04612	TPPP	68	-0.3421	0.1115	TLL2
19	-0.2404	0.04786	SAMD4A	69	-0.1845	0.1115	MCCC1
20	0.3465	0.04786	KIF1A	70	0.1885	0.1115	cg04143252
21	-0.4136	0.04786	RARS	71	0.1031	0.1115	FRMD3
22	-0.3952	0.04786	FBXO38	72	-0.1391	0.1115	NDST4
23	-0.1813	0.05133	TTC17	73	-0.1601	0.1115	HTR1E
24	-0.2063	0.05753	FBXL13	74	0.2104	0.1115	VIPR2
25	-0.2184	0.06035	DNAJC3	75	0.1263	0.1115	cg12920070
26	-0.5451	0.06775	cg19088503	76	-0.1974	0.1115	CNTNAP4
27	-0.2159	0.07081	cg00189927	77	-0.1674	0.1115	MED12L
28	-0.4818	0.07081	GMD5	78	-0.5609	0.1115	HACD2
29	0.09129	0.07081	HSD11B1	79	-0.2515	0.1115	FAM82A1
30	-0.4441	0.07081	cg21223344	80	-0.3208	0.1115	MRAP
31	0.2972	0.07193	cg06818582	81	-0.1959	0.1115	GREB1
32	0.104	0.07994	TMCC3	82	-0.2176	0.1115	GPR1
33	-0.1265	0.08073	FGGY	83	-0.1861	0.1116	EDRF1
34	-0.06845	0.0808	FAM60A	84	-0.2769	0.1116	LNX2
35	-0.5674	0.0808	cg21167269	85	-0.2464	0.1116	WDFY3
36	-0.228	0.0808	HOOK3	86	-0.1928	0.1116	RIMS1
37	0.4018	0.08219	PINX1	87	-0.3306	0.1116	NLK
38	0.07855	0.08219	CCR7	88	-0.3981	0.1116	CYP19A1
39	-0.5033	0.08219	STIM1	89	-0.1543	0.1116	cg21740531
40	0.1422	0.08219	WDR55	90	-0.1696	0.1116	cg22252839
41	-0.1105	0.08219	CLASP1	91	0.2671	0.1116	cg26431823
42	-0.2061	0.08219	cg15407900	92	-0.329	0.1116	cg26790217
43	-0.43	0.08219	cg20592075	93	-0.1732	0.1125	CADM1
44	-0.2025	0.08219	IKBKB	94	0.3026	0.1149	FUT5
45	-0.2512	0.08219	RNF13	95	-0.5386	0.1149	KRTAP19-5
46	0.1142	0.08219	SPRED2	96	-0.4332	0.1149	cg13005202
47	-0.3863	0.08219	cg27132553	97	-0.3374	0.1149	C5orf24
48	0.1247	0.08252	SERPINB6	98	-0.07031	0.1149	GLRB
49	-0.381	0.08252	cg13473880	99	-0.5851	0.1149	ARHGAP22
50	0.3253	0.08252	AQP8	100	-0.2633	0.1149	BRAF

9.4.6.2.2 Transcription factor enrichment analysis of the top 100 dmCpGs associated with BMD at four years of age

Table 75. The transcription factors, transcription factor database motif references, NES score, annotation and genes enriched for the top 100 dmCpGs associated with BMD at four years of age in the MAVIDOS RCT

Motif	Transcription factor	NES	Annotation	Genes enriched	Enriched genes
transfac_pro__M02908	SOX30	4.37	Inferred	19	ACACA, CADM1, CREB5, CSMD3, EPB41, FAM60A, FAM82A1, FERMT2, FGGY, GABRB3, HIVEP1, KCNMA1, LRRC33, MARCKSL1, NDST4, NLK, RIMS1, SPRED2, UTRN
flyfactorsurvey__sd_FlyReg_FBgn0003345	TEAD1	4.16	Inferred	14	ACACA, CADM1, CLIC5, CNTNAP4, EPB41, FBXL13, FERMT2, HIVEP1, KCNMA1, MAP2K1, MARCKSL1, MKLN1, NLK, RIMS1
cisbp__M2050	TEAD1	4.02	Inferred	15	ACACA, CADM1, CLIC5, CNTNAP4, EPB41, FBXL13, FERMT2, HIVEP1, KCNMA1, MAP2K1, MARCKSL1, MKLN1, NLK, RAB37, RIMS1

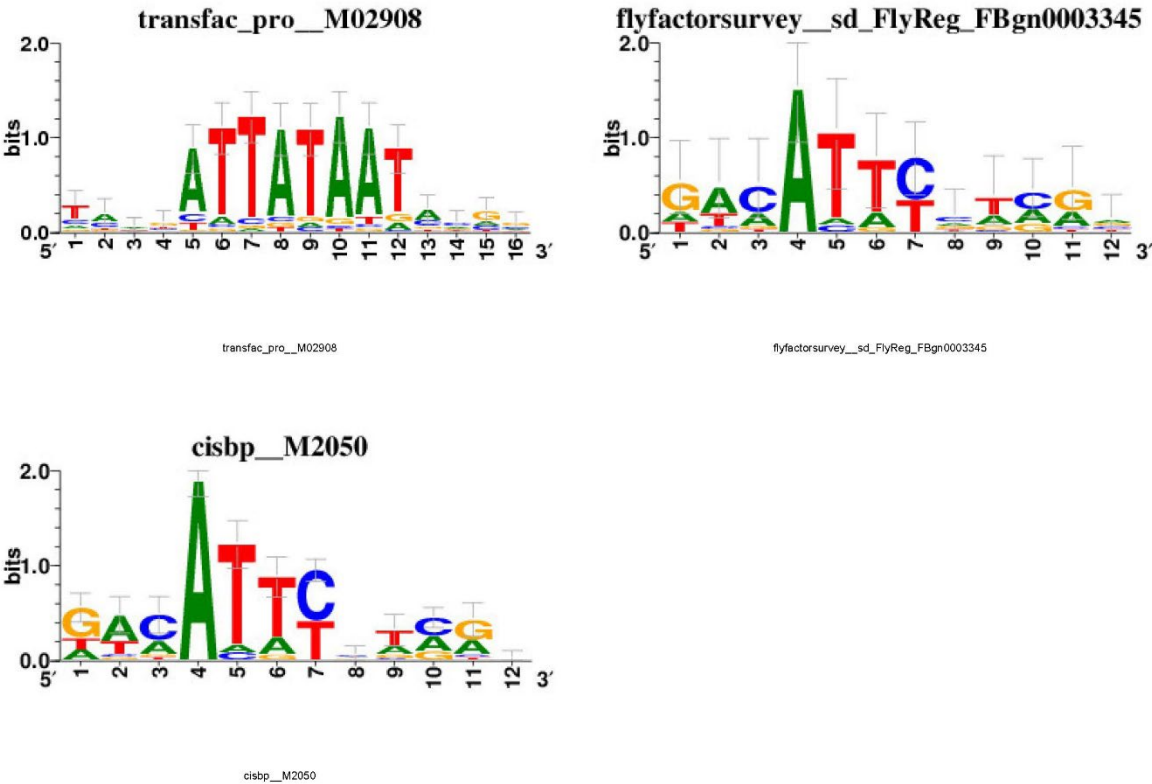


Figure 87. The sequence graphs for transcription factor motifs significantly enriched (NES >4) in the top 100 dmCpGs associated with BMD at four years of age in the MAVIDOS RCT

9.4.6.2.3 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated with BMD at four years of age

Table 76. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with BMD at four years of age in the MAVIDOS RCT

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
4674	5.56E-11	protein serine/threonine kinase activity	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
4672	3.42E-10	protein kinase activity	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
6468	7.35E-10	protein amino acid phosphorylation	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
16773	7.36E-10	phosphotransferase activity, alcohol group as acceptor	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
16301	1.16E-09	kinase activity	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
16310	1.55E-09	phosphorylation	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
35556	2.12E-09	intracellular signal transduction	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
16772	2.81E-09	transferase activity, transferring phosphorus-containing groups	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
6793	4.23E-09	phosphorus metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
6796	4.23E-09	phosphate metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
23034	1.90E-08	intracellular signaling pathway	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
7265	2.76E-08	Ras protein signal transduction	MAP2K1 MAP2K2 CHUK MAPK1 MAPK3
43687	3.29E-08	post-translational protein modification	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
7243	8.43E-08	intracellular protein kinase cascade	IKBKB MAP2K1 MAP2K2 CHUK MAPK1 NLK
23014	8.43E-08	signal transmission via phosphorylation event	IKBKB MAP2K1 MAP2K2 CHUK MAPK1 NLK
5524	9.01E-08	ATP binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
32559	9.60E-08	adenyl ribonucleotide binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
6464	1.06E-07	protein modification process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
30554	1.31E-07	adenyl nucleotide binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
43412	1.42E-07	macromolecule modification	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
1883	1.42E-07	purine nucleoside binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
1882	1.43E-07	nucleoside binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
16740	1.46E-07	transferase activity	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
32553	3.51E-07	ribonucleotide binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
32555	3.51E-07	purine ribonucleotide binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
7165	3.89E-07	signal transduction	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3

17076	4.62E-07	purine nucleotide binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
4707	5.14E-07	MAP kinase activity	MAPK1 NLK MAPK3
23033	8.53E-07	signaling pathway	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
44267	9.58E-07	cellular protein metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
23046	9.58E-07	signaling process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
23060	9.58E-07	signal transmission	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
7264	1.25E-06	small GTPase mediated signal transduction	MAP2K1 MAP2K2 CHUK MAPK1 MAPK3
166	1.28E-06	nucleotide binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
19538	4.05E-06	protein metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
5057	7.33E-06	receptor signaling protein activity	MAP2K1 MAPK1 NLK MAPK3
23052	1.60E-05	signaling	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
71216	1.85E-05	cellular response to biotic stimulus	EIF2AK3 MAPK1 MAPK3
4702	3.03E-05	receptor signaling protein serine/threonine kinase activity	MAPK1 NLK MAPK3
44260	3.51E-05	cellular macromolecule metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
9636	3.88E-05	response to toxin	IKBKB CHUK MAPK1
5829	5.88E-05	cytosol	IKBKB MAP2K1 MAP2K2 CHUK MAPK1 MAPK3
43170	9.50E-05	macromolecule metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
42221	1.03E-04	response to chemical stimulus	IKBKB MAP2K1 CHUK EIF2AK3 MAPK1 MAPK3
10033	1.66E-04	response to organic substance	MAP2K1 CHUK EIF2AK3 MAPK1 MAPK3
32496	1.75E-04	response to lipopolysaccharide	CHUK MAPK1 MAPK3
2237	2.18E-04	response to molecule of bacterial origin	CHUK MAPK1 MAPK3
48513	2.60E-04	organ development	MAP2K1 CHUK EIF2AK3 MAPK1 NLK MAPK3
9607	2.68E-04	response to biotic stimulus	CHUK EIF2AK3 MAPK1 MAPK3
44237	3.72E-04	cellular metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
267	3.74E-04	cell fraction	IKBKB MAP2K1 MAP2K2 CHUK MAPK1
3824	4.28E-04	catalytic activity	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
10646	5.06E-04	regulation of cell communication	IKBKB MAP2K1 CHUK EIF2AK3 NLK
165	5.51E-04	MAPKKK cascade	MAP2K1 MAP2K2 MAPK1
44238	5.51E-04	primary metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
9653	6.21E-04	anatomical structure morphogenesis	MAP2K1 CHUK MAPK1 NLK MAPK3
51098	6.48E-04	regulation of binding	IKBKB EIF2AK3 MAPK3
50896	7.98E-04	response to stimulus	IKBKB MAP2K1 CHUK EIF2AK3 MAPK1 NLK MAPK3

9617	9.23E-04	response to bacterium	CHUK MAPK1 MAPK3
48731	1.08E-03	system development	MAP2K1 CHUK EIF2AK3 MAPK1 NLK MAPK3
8152	1.22E-03	metabolic process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
1568	1.32E-03	blood vessel development	MAP2K1 MAPK1 NLK
1944	1.43E-03	vasculature development	MAP2K1 MAPK1 NLK
9888	1.43E-03	tissue development	MAP2K1 CHUK EIF2AK3 MAPK3
9966	1.59E-03	regulation of signal transduction	IKBKB MAP2K1 CHUK EIF2AK3
23051	1.61E-03	regulation of signaling process	IKBKB MAP2K1 CHUK EIF2AK3
50794	1.62E-03	regulation of cellular process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
48856	1.68E-03	anatomical structure development	MAP2K1 CHUK EIF2AK3 MAPK1 NLK MAPK3
5625	2.11E-03	soluble fraction	MAP2K1 MAP2K2 MAPK1
50789	2.31E-03	regulation of biological process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
32501	2.31E-03	multicellular organismal process	IKBKB MAP2K1 CHUK EIF2AK3 MAPK1 NLK MAPK3
10556	2.48E-03	regulation of macromolecule biosynthetic process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK MAPK3
10468	2.72E-03	regulation of gene expression	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK MAPK3
51707	2.74E-03	response to other organism	CHUK MAPK1 MAPK3
6950	2.75E-03	response to stress	MAP2K1 CHUK EIF2AK3 MAPK1 MAPK3
7275	2.76E-03	multicellular organismal development	MAP2K1 CHUK EIF2AK3 MAPK1 NLK MAPK3
2376	2.85E-03	immune system process	IKBKB CHUK MAPK1 NLK
31326	2.92E-03	regulation of cellular biosynthetic process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK MAPK3
48523	2.98E-03	negative regulation of cellular process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK
9889	2.98E-03	regulation of biosynthetic process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK MAPK3
65007	3.07E-03	biological regulation	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
35466	3.32E-03	regulation of signaling pathway	IKBKB MAP2K1 CHUK NLK
32502	3.98E-03	developmental process	MAP2K1 CHUK EIF2AK3 MAPK1 NLK MAPK3
65009	4.00E-03	regulation of molecular function	IKBKB MAP2K1 EIF2AK3 MAPK3
48519	4.26E-03	negative regulation of biological process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK
60255	4.72E-03	regulation of macromolecule metabolic process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK MAPK3
44444	5.15E-03	cytoplasmic part	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 MAPK3
9719	5.37E-03	response to endogenous stimulus	MAP2K1 CHUK MAPK1
5737	5.81E-03	cytoplasm	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
80090	6.05E-03	regulation of primary metabolic process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK MAPK3

7166	6.90E-03	cell surface receptor linked signaling pathway	EIF2AK3 MAPK1 NLK MAPK3
31323	7.23E-03	regulation of cellular metabolic process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK MAPK3
15630	7.44E-03	microtubule cytoskeleton	MAP2K1 MAPK1 MAPK3
5515	8.07E-03	protein binding	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
33554	8.65E-03	cellular response to stress	EIF2AK3 MAPK1 MAPK3
19222	8.73E-03	regulation of metabolic process	IKBKB MAP2K1 EIF2AK3 MAPK1 NLK MAPK3
19899	9.02E-03	enzyme binding	MAP2K1 MAPK1 NLK
9887	9.08E-03	organ morphogenesis	CHUK MAPK1 MAPK3
44093	9.10E-03	positive regulation of molecular function	IKBKB MAP2K1 EIF2AK3
45449	1.03E-02	regulation of transcription	IKBKB MAP2K1 MAPK1 NLK MAPK3
3008	1.03E-02	system process	IKBKB CHUK MAPK1 MAPK3
42802	1.04E-02	identical protein binding	IKBKB CHUK EIF2AK3
51704	1.35E-02	multi-organism process	CHUK MAPK1 MAPK3
50793	1.37E-02	regulation of developmental process	MAP2K1 EIF2AK3 MAPK1
7049	1.38E-02	cell cycle	MAP2K1 MAPK1 MAPK3
5626	1.49E-02	insoluble fraction	IKBKB CHUK MAPK1
19219	1.52E-02	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	IKBKB MAP2K1 MAPK1 NLK MAPK3
42981	1.56E-02	regulation of apoptosis	IKBKB EIF2AK3 MAPK1
51171	1.56E-02	regulation of nitrogen compound metabolic process	IKBKB MAP2K1 MAPK1 NLK MAPK3
43067	1.58E-02	regulation of programmed cell death	IKBKB EIF2AK3 MAPK1
51649	1.60E-02	establishment of localization in cell	MAP2K1 EIF2AK3 MAPK1
10941	1.60E-02	regulation of cell death	IKBKB EIF2AK3 MAPK1
9987	1.73E-02	cellular process	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
5938	1.96E-02	cell cortex	CLIC5 CLASP1 FERMT2
51641	2.11E-02	cellular localization	MAP2K1 EIF2AK3 MAPK1
51716	2.15E-02	cellular response to stimulus	EIF2AK3 MAPK1 MAPK3
48522	2.15E-02	positive regulation of cellular process	MAP2K1 CHUK EIF2AK3 MAPK1
60089	2.52E-02	molecular transducer activity	MAP2K1 MAPK1 NLK MAPK3
4871	2.52E-02	signal transducer activity	MAP2K1 MAPK1 NLK MAPK3
48518	2.72E-02	positive regulation of biological process	MAP2K1 CHUK EIF2AK3 MAPK1
44430	2.78E-02	cytoskeletal part	CLIC5 PINX1 KIF1A CLASP1 FERMT2

15630	2.78E-02	microtubule cytoskeleton	CLIC5 PINX1 KIF1A CLASP1
32991	3.31E-02	macromolecular complex	CLIC5 TERT PINX1 KCNMA1 RSF1 KIF1A MED12L CLASP1
43228	3.51E-02	non-membrane-bounded organelle	MAP2K1 MAPK1 NLK MAPK3
43232	3.51E-02	intracellular non-membrane-bounded organelle	MAP2K1 MAPK1 NLK MAPK3
31981	3.69E-02	nuclear lumen	MAPK1 NLK MAPK3
16043	3.88E-02	cellular component organization	CLIC5 TERT PINX1 KCNMA1 RSF1 CLASP1 FERMT2
43234	3.88E-02	protein complex	CLIC5 PINX1 KCNMA1 RSF1 KIF1A MED12L CLASP1
43234	4.09E-02	protein complex	IKBKB MAP2K1 CHUK MAPK1
5856	4.14E-02	cytoskeleton	MAP2K1 MAPK1 MAPK3
44424	4.15E-02	intracellular part	IKBKB MAP2K1 MAP2K2 CHUK EIF2AK3 MAPK1 NLK MAPK3
6996	4.16E-02	organelle organization	TERT PINX1 RSF1 CLASP1 FERMT2
44427	4.16E-02	chromosomal part	TERT PINX1 CLASP1
5856	4.20E-02	cytoskeleton	CLIC5 PINX1 KIF1A CLASP1 FERMT2
5694	4.88E-02	chromosome	TERT PINX1 CLASP1

9.4.7 Short-term 1,25(OH)₂D treatment of ATCC UC-MSCs analyses

9.4.7.1 Short-term 1,25(OH)₂D treated ATCC UC-MSCs

9.4.7.1.1 Top 100 dmCpGs associated the short-term 1,25(OH)₂D treatment of ATCC UC-MSCs

Table 77. The top 100 dmCpGs associated with short-term 1,25(OH)₂D treatment in ATCC UC-MSCs

Number	Log fold-change	FDR	Gene name	Number	Log fold-change	FDR	Gene name
1	-0.17	2.56E-05	cg11347955	51	0.075661	0.028491	cg05105889
2	-0.11791	0.00013	cg17523024	52	-0.08169	0.028509	AAAS
3	0.108059	0.000163	MYLK3	53	-0.0801	0.028509	LASS6
4	0.101307	0.000292	XXYL1	54	-0.08784	0.028509	LAIR2
5	0.120612	0.000292	cg24119516	55	0.106873	0.029466	IQCF3
6	0.113871	0.000298	cg08821715	56	0.076872	0.029466	DENND4A
7	-0.12415	0.000298	cg11974480	57	0.076261	0.030178	CARD18
8	0.128902	0.000438	cg23087546	58	0.09	0.031172	CCDC54
9	0.109316	0.000527	FER	59	-0.07952	0.036895	PUS7
10	-0.11472	0.001002	cg04772776	60	-0.10496	0.036895	CSNK1G3
11	0.099793	0.001002	cg16532912	61	0.090023	0.036895	CASC18
12	0.098038	0.001028	cg13169253	62	0.075776	0.03798	CDC42BPA
13	-0.109	0.00128	SNORD116-27	63	0.075249	0.038194	TWIST1
14	-0.1066	0.001357	cg11488897	64	-0.12693	0.038385	cg05565979
15	-0.11103	0.001557	cg23990529	65	-0.0813	0.038646	cg13061124
16	0.093686	0.001717	DENND2A	66	-0.07106	0.038646	PCBP2
17	-0.09675	0.001717	cg26772934	67	0.090481	0.038723	INTS1
18	-0.09797	0.002461	cg10501976	68	0.074601	0.039504	ATF7IP
19	0.150171	0.002461	ZC3H3	69	-0.08738	0.039742	KYNU
20	0.10153	0.002511	RXFP1	70	-0.08598	0.039742	PTPRE
21	-0.12526	0.002936	C14orf39	71	0.085658	0.040289	AGO3
22	0.122415	0.003061	PFKP	72	-0.07954	0.040289	MYO5A
23	-0.19459	0.003108	cg09746869	73	0.085769	0.041619	PPP1R12A
24	-0.08768	0.003402	AGFG1	74	0.080246	0.045785	OTOG
25	0.090135	0.004565	BCL9	75	0.081378	0.048179	cg11725581
26	-0.13964	0.004742	KIF21A	76	0.078769	0.049255	LRCH3
27	0.096873	0.007129	KIF26B	77	-0.07583	0.049255	CNNM2
28	0.083768	0.007129	ANKRD28	78	-0.08245	0.04933	CMTM8
29	0.085823	0.00887	SERTAD4	79	0.083361	0.050102	cg07882021
30	-0.11015	0.009133	TEX10	80	0.076579	0.050102	cg13826454
31	0.089337	0.009289	IFNG	81	-0.10221	0.050102	cg15124820
32	0.087245	0.010983	cg01409736	82	0.076543	0.050102	LIN28B
33	-0.0915	0.011088	RRM1	83	0.071702	0.050102	cg22862619
34	0.115252	0.011699	ATP1A1	84	0.092779	0.050175	TMED10
35	0.08781	0.01184	cg20007255	85	0.072091	0.050175	POLK
36	0.088089	0.012139	cg03498309	86	0.078752	0.050794	MAST3
37	0.103564	0.013322	cg09412344	87	0.072178	0.054865	SPOPL
38	0.091749	0.013322	TGFBR2	88	0.080187	0.054865	cg17508108
39	0.08153	0.013919	PPP1CC	89	0.077326	0.054865	INADL
40	0.092112	0.014907	cg09667243	90	-0.07654	0.054865	cg24145276
41	0.107743	0.01958	RBFOX1	91	0.07505	0.056804	cg04209173
42	0.09168	0.019856	MYO10	92	0.080741	0.056804	USH2A
43	0.096955	0.019856	cg17112492	93	0.073124	0.057118	LINC00616
44	0.08092	0.020359	CBFA2T3	94	0.070581	0.057118	CADPS
45	0.088529	0.023125	HTR1D	95	0.072653	0.057118	ANKS1B
46	0.088694	0.023125	ARMS2	96	-0.08884	0.057229	cg02680901
47	-0.08569	0.023385	LRRC2	97	0.083357	0.058895	cg01933774
48	0.078489	0.023879	cg14816421	98	0.078447	0.058895	ARID5B
49	0.084401	0.023879	cg21453690	99	0.076456	0.058895	SGMS1
50	-0.08381	0.027169	GULP1	100	-0.07626	0.058982	PAPPA

9.4.7.1.2 Cytoscape pathway enrichment analysis of the top 100 dmCpGs associated the short-term 1,25(OH)₂D treatment of ATCC UC-MSCs

Table 78. The full ontology results for the Cytoscape pathway enrichment analysis conducted with the top 100 dmCpGs associated with the 24-hour treatment of ATCC UC-MSCs with 1,25(OH)₂D

GO-ID	Corrected <i>p</i> -value	Description	Genes in test set
6091	7.65E-04	generation of precursor metabolites and energy	PPP1CC NDUFA5 NDUFA10 PFKM PFKP
15980	1.19E-02	energy derivation by oxidation of organic compounds	PPP1CC NDUFA5 NDUFA10
6006	1.19E-02	glucose metabolic process	PPP1CC PFKM PFKP
6066	1.55E-02	alcohol metabolic process	PPP1CC DDC PFKM PFKP
19318	1.55E-02	hexose metabolic process	PPP1CC PFKM PFKP
5996	1.55E-02	monosaccharide metabolic process	PPP1CC PFKM PFKP
43234	1.55E-02	protein complex	NCOR2 PPP1CC NDUFA5 TEX10 KIF26B NDUFA10 PFKM PFKP
16773	2.93E-02	phosphotransferase activity, alcohol group as acceptor	CSNK1G3 NDUFA10 PFKM PFKP
32991	3.43E-02	macromolecular complex	NCOR2 PPP1CC NDUFA5 TEX10 KIF26B NDUFA10 PFKM PFKP
9987	3.85E-02	cellular process	CSNK1G3 DDC TMED10 DTNA NDUFA5 NDUFA10 CADPS ARID5B NCOR2 PPP1CC MDFIC KIF26B PFKM PFKP
44262	3.96E-02	cellular carbohydrate metabolic process	PPP1CC PFKM PFKP
16310	3.96E-02	phosphorylation	CSNK1G3 NDUFA5 MDFIC NDUFA10
46907	4.74E-02	intracellular transport	IFNG TMX1 MYO5A AGFG1

9.4.7.1.3 *TGFβ-R2* and *TWIST1* plots



Figure 88. The methylation state of the differentially methylated cg24719910 and cg27334919 CpGs, mapped to the *TGFB2* and *TWIST1* genes respectively according to treatment group

9.5 Extracted DNA quality and concentration data

9.5.1 MAVIDOS DNA

Table 79. Example of nanodrop assessment of DNA (n=70) post-phenol/chloroform extraction from crushed umbilical cord tissue taken from births within the MAVIDOS RCT for the purpose of EPIC array interrogation

Below, 70 of a total of 244 DNA samples which were all extracted from crushed umbilical cord tissue taken from births within the MAVIDOS RCT where mothers were supplemented with either cholecalciferol or a placebo during pregnancy. DNA extractions were performed using the phenol-chloroform technique as described in section 2.3.2. Once extracted, DNA quantity and quality was assessed using nanodrop spectrophotometer and 500ng assessed using agarose gel electrophoresis as described in section **Error! Reference source not found.**

Sample ID	Treatment	ng/ul	A260	A280	260/280	260/230	Sample ID	Treatment	ng/ul	A260	A280	260/280	260/230
9	Cholecalciferol	124.07	2.481	1.571	1.58	1.75	195	Cholecalciferol	45.5	0.91	0.576	1.58	1.77
21	Placebo	72.12	1.442	0.936	1.54	1.71	201	Placebo	169.21	3.384	2.091	1.62	1.84
25	Cholecalciferol	90.13	1.803	1.141	1.58	1.8	203	Cholecalciferol	121.15	2.423	1.517	1.6	1.86
27	Placebo	82.57	1.651	1.092	1.51	1.6	207	Placebo	136.97	2.739	1.717	1.6	1.86
34	Placebo	125.15	2.503	1.567	1.6	1.68	208	Cholecalciferol	131.39	2.628	1.657	1.59	1.88
35	Placebo	141.1	2.822	1.795	1.57	1.68	210	Placebo	208.7	4.174	2.561	1.63	1.9
48	Placebo	147.73	2.955	1.868	1.58	1.77	211	Placebo	88.17	1.763	1.119	1.58	1.82
51	Placebo	98.38	1.968	1.263	1.56	1.68	215	Placebo	56.47	1.129	0.711	1.59	1.96
75	Placebo	320.99	6.42	3.768	1.7	1.92	223	Placebo	178.49	3.57	2.202	1.62	1.76
76	Placebo	89.16	1.783	1.175	1.52	1.75	229	Placebo	77.72	1.554	0.983	1.58	1.96
82	Placebo	116.92	2.338	1.476	1.58	1.76	230	Placebo	176.06	3.521	2.179	1.62	1.91
86	Placebo	176.99	3.54	2.206	1.6	1.9	241	Cholecalciferol	121.23	2.425	1.526	1.59	1.82
90	Cholecalciferol	165.96	3.319	2.066	1.61	1.81	246	Cholecalciferol	105.87	2.117	1.351	1.57	1.83
96	Placebo	128.25	2.565	1.602	1.6	1.78	252	Cholecalciferol	150.84	3.017	1.912	1.58	1.79
97	Placebo	175.74	3.515	2.126	1.65	1.88	254	Placebo	90.26	1.805	1.145	1.58	1.83
98	Cholecalciferol	79.81	1.596	1.012	1.58	1.68	255	Cholecalciferol	115.23	2.305	1.436	1.61	1.86
100	Cholecalciferol	111.88	2.238	1.407	1.59	1.89	264	Placebo	105.55	2.111	1.356	1.56	1.79
119	Placebo	62.16	1.243	0.805	1.55	1.89	274	Placebo	113.66	2.273	1.427	1.59	1.95
120	Placebo	59.09	1.182	0.765	1.54	1.84	279	Placebo	65.66	1.313	0.85	1.55	1.91
121	Cholecalciferol	104.49	2.09	1.33	1.57	1.88	289	Cholecalciferol	147.12	2.942	1.845	1.59	1.85
123	Placebo	56.52	1.13	0.696	1.62	1.84	292	Placebo	102.03	2.041	1.286	1.59	1.93
125	Cholecalciferol	259.54	5.191	3.088	1.68	1.97	294	Placebo	95.06	1.901	1.167	1.63	1.78
129	Placebo	143.57	2.871	1.774	1.62	1.97	295	Cholecalciferol	148.35	2.967	1.83	1.62	2
130	Cholecalciferol	215.99	4.32	2.585	1.67	2.02	301	Cholecalciferol	140.96	2.819	1.738	1.62	1.95
144	Cholecalciferol	214.14	4.283	2.589	1.65	2.02	333	Cholecalciferol	136.6	2.732	1.696	1.61	1.91
148	Placebo	84.46	1.689	1.074	1.57	1.81	334	Cholecalciferol	141.71	2.834	1.758	1.61	1.9
152	Cholecalciferol	90.42	1.808	1.126	1.61	1.91	342	Cholecalciferol	86.88	1.738	1.129	1.54	1.85
156	Cholecalciferol	78.29	1.566	0.98	1.6	1.95	343	Cholecalciferol	113.68	2.274	1.437	1.58	1.91
161	Cholecalciferol	128.59	2.572	1.599	1.61	1.95	351	Placebo	140.55	2.811	1.761	1.6	1.88
163	Placebo	104.77	2.095	1.311	1.6	1.93	354	Placebo	130.89	2.618	1.651	1.59	1.91
166	Cholecalciferol	164.72	3.294	2.045	1.61	1.88	366	Placebo	122.53	2.451	1.533	1.6	1.86
167	Cholecalciferol	97.42	1.948	1.215	1.6	1.99	372	Cholecalciferol	114.14	2.283	1.455	1.57	1.9
169	Placebo	109.43	2.189	1.37	1.6	1.92	409	Placebo	134.5	2.69	1.672	1.61	1.87
182	Placebo	162.24	3.245	2.042	1.59	1.82	420	Cholecalciferol	94.76	1.895	1.206	1.57	1.82
189	Placebo	415.38	8.308	4.695	1.77	1.91	444	Cholecalciferol	133.31	2.666	1.644	1.62	1.88

9.5.2 ATCC UC-MSC

Table 80. Nanodrop assessment of DNA (n=36) post-extraction from ATCC-UCMSC cell pellets for the purpose of EPIC array interrogation

ATCC UC-MSC cells were grown at 6x10⁵ cells per flask in T175 flasks and treated with either 50nm 1,25(OH)₂D (n=6) or vehicle (n=6) for 24 hours. Cell were trypsinised from flasks as described in section 2.5.2, pelleted, and all excess media removed. DNA was extracted from cell pellets using the Sigma GeneElute™ Mammalian Genomic DNA Miniprep Kit according to the manufacturer's protocol. DNA was eluted in three sequential elutions of 50 µl DNase free water. Quality and quantity of the extracted DNA was assessed using a NanoDrop ND-1000 (NanoDrop technologies) and agarose gel electrophoresis as described in section **Error! Reference source not found.**

Sample ID	Treatment	ng/ul	A260	A280	260/280	260/230
V1E1	Placebo	88.16	1.763	0.932	1.89	2.35
V1E2	Placebo	40.13	0.803	0.454	1.77	2.42
V1E3	Placebo	19.99	0.4	0.271	1.47	1.98
V2E1	Placebo	69.12	1.382	0.763	1.81	1.94
V2E2	Placebo	36.53	0.731	0.423	1.73	2.35
V2E3	Placebo	27.81	0.556	0.317	1.75	2.55
V3E1	Placebo	78.16	1.563	0.862	1.81	1.86
V3E2	Placebo	29.83	0.597	0.344	1.74	3.05
V3E3	Placebo	25.74	0.515	0.322	1.6	2.19
V4E1	Placebo	82.94	1.659	0.898	1.85	1.98
V4E2	Placebo	38.13	0.763	0.433	1.76	1.98
V4E3	Placebo	17.83	0.357	0.23	1.55	1.61
V5E1	Placebo	77.38	1.548	0.834	1.86	2.03
V5E2	Placebo	42.13	0.843	0.506	1.67	2.09
V5E3	Placebo	16.39	0.328	0.216	1.51	2.04
V6E1	Placebo	98.98	1.98	1.079	1.83	1.88
V6E2	Placebo	44.26	0.885	0.503	1.76	2
V6E3	Placebo	14.69	0.294	0.191	1.54	1.57
D1E1	Calcitriol	76.82	1.536	0.869	1.77	1.72
D1E2	Calcitriol	38.07	0.761	0.45	1.69	2.2
D1E3	Calcitriol	14.54	0.291	0.168	1.73	2.15
D2E1	Calcitriol	69.19	1.384	0.762	1.82	2.25
D2E2	Calcitriol	35.66	0.713	0.39	1.83	2.47
D2E3	Calcitriol	27.04	0.541	0.309	1.75	2.13
D3E1	Calcitriol	80.42	1.608	0.884	1.82	2
D3E2	Calcitriol	45.27	0.905	0.509	1.78	2.48
D3E3	Calcitriol	16.45	0.329	0.219	1.5	1.93
D4E1	Calcitriol	80.35	1.607	0.877	1.83	2.03
D4E2	Calcitriol	40.34	0.807	0.472	1.71	1.8
D4E3	Calcitriol	26.28	0.526	0.309	1.7	1.76
D5E1	Calcitriol	68.91	1.378	0.756	1.82	1.94
D5E2	Calcitriol	36.45	0.729	0.405	1.8	2.14
D5E3	Calcitriol	32.68	0.654	0.382	1.71	1.78
D6E1	Calcitriol	69.99	1.4	0.745	1.88	2.2
D6E2	Calcitriol	35.37	0.707	0.412	1.72	2.19
D6E3	Calcitriol	33.89	0.678	0.391	1.74	1.97

Figure 89. A beadchip plan for the EPIC array interrogation DNA extracted from ATCC UC-MSC cells treated short term *in vitro* with either vehicle (V) or 1,25(OH)₂D

ATCC UC-MSC cells (n=12) were cultured and treated for 24 hours *in vitro* with a vehicle (V) or 1,25(OH)₂D, after which the DNA was extracted from each culture as in section 2.3.3. Samples were randomised across chips, alternating by treatment group. Both 0%(0/2) and 100% (100% 1/2) methylation inter-chip and intra-chip controls were included on the beadchips to ensure concordant methylation readings both within and across chips.

9.7 IPA third-party database list

- Entrez Gene GTEx Isoform-level Tissue Expression Orphanet
- RefSeq HumanCyc metabolic pathway information Hazardous Substance Database (HSDB)
- Conserved Domain Database (CDD) BIND, DIP, MIPS, BioGRID, IntAct, Cognia
- protein-protein interactions TargetScan
- OMIM clinicaltrials.gov miRBase
- ClinVar Drugs@FDA miRecords
- GWAS Database European Medicines Agency OncoTree
- Gene Ontology (GO) Pharmaceuticals and Medical Devices Agency TarBase
- Human Metabolome Database (HMDB) Mosby's Drug Consult COSMIC
- Human Phenotype Ontology (HPO) Goodman & Gilman's Pharmacological Basis of
- Therapeutics
- Chemical Carcinogenesis Research Information
- System Database (CCRIS)
- GNF Tissue Expression Body Atlas DrugBank The Mouse Genome Database (MGD) from The
- Jackson Laboratory (JAX)
- NCI-60 Cell Line Expression Atlas Obesity Gene Map Database

9.8 pGL4.23 osteocalcin VDRE insert sequencing results

9.8.1 pGL4.23-Osteocalcin VDRE positive clone sequences

CLUSTAL O(1.2.4) multiple sequence alignment

```

PGL4.23          -----GGCCTAACTGGCCGGTACCTATGGGTGAACGGGGGCAAATGGGTGAACGGGGG
SequencedFWRDgel -TtTCTCTGGCctACTGGCCGGtAccTATGGgTGAACGGGGGCAAATGGGTGAACGGGGG
SequencedFWRDHI  TtnCTCTGGCCTAaCTGGCCGGtaCcTATGGgTGAACGGGGGCAAATGGGTGAACGGGGG
SequencedFWRDPCR TtnCTCTGGCCTAaCTGGCCGGtaCcTATGGgTGAACGGGGGCAAATGGGTGAACGGGGG
                  * *  *****

PGL4.23          CAATAGGGTGAACGGGGGCAAGAGCTCGCTAGCCTCGAGGATATCAAGATCTGGCCTCGG
SequencedFWRDgel CAATAGGGTGAACGGGGGCAAGAGCTCGCTAGCCTCGAGGATATCAAGATCTGGCCTCGG
SequencedFWRDHI  CAATAGGGTGAACGGGGGCAAGAGCTCGCTAGCCTCGAGGATATCAAGATCTGGCCTCGG
SequencedFWRDPCR CAATAGGGTGAACGGGGGCAAGAGCTCGCTAGCCTCGAGGATATCAAGATCTGGCCTCGG
                  *****

PGL4.23          CGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAGCTTGGCAATCC
SequencedFWRDgel CGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAGCTTGGCAATCC
SequencedFWRDHI  CGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAGCTTGGCAATCC
SequencedFWRDPCR CGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAGCTTGGCAATCC
                  *****

PGL4.23          GGTACTGTTGGTAAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCA
SequencedFWRDgel GGTACTGTTGGTAAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCA
SequencedFWRDHI  GGTACTGTTGGTAAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCA
SequencedFWRDPCR GGTACTGTTGGTAAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCA
                  *****

PGL4.23          TTCTACCCACTCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCATGAAGCGCTAC
SequencedFWRDgel TTCTACCCACTCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCATGAAGCGCTAC
SequencedFWRDHI  TTCTACCCACTCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCATGAAGCGCTAC
SequencedFWRDPCR TTCTACCCACTCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCATGAAGCGCTAC
                  *****

PGL4.23          GCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTAC
SequencedFWRDgel GCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTAC
SequencedFWRDHI  GCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTAC
SequencedFWRDPCR GCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTAC
                  *****

PGL4.23          GCCGAGTACTTCGAGATGAGCGTTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAAT
SequencedFWRDgel GCCGAGTACTTCGAGATGAGCGTTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAAT
SequencedFWRDHI  GCCGAGTACTTCGAGATGAGCGTTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAAT
SequencedFWRDPCR GCCGAGTACTTCGAGATGAGCGTTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAAT
                  *****

PGL4.23          ACAAACCATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTGTG
SequencedFWRDgel ACAAACCATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTGTG
SequencedFWRDHI  ACAAACCATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTGTG
SequencedFWRDPCR ACAAACCATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTGTG
                  *****

PGL4.23          GGTGCCCTGTTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAG
SequencedFWRDgel GGTGCCCTGTTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAG
SequencedFWRDHI  GGTGCCCTGTTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAG
SequencedFWRDPCR GGTGCCCTGTTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAG
                  *****

PGL4.23          CTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCTGTGAGCAAGAAAGGGCTG
SequencedFWRDgel CTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCTGTGAGCAAGAAAGGGCTG
SequencedFWRDHI  CTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCTGTGAGCAAGAAAGGGCTG
SequencedFWRDPCR CTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCTGTGAGCAAGAAAGGGCTG
                  *****

PGL4.23          CAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGAT
SequencedFWRDgel CAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGAT
SequencedFWRDHI  CAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGAT
SequencedFWRDPCR CAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGAT
                  *****

PGL4.23          AGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTGGCCA
SequencedFWRDgel AGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTGGCCA
SequencedFWRDHI  AGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTGGCCA
SequencedFWRDPCR AGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTGGCCA
                  *****

PGL4.23          CCCGGCTTCAACGAGTACGACTTCGTGCCCCGAGAGCTTCGACCGGGACAAAACCATCGCC
SequencedFWRDgel CCCGGCTTCAACGAGTACGACTTCGTGCCCCGAGAGCTTCGACCGGGACAAAACCATCGCC
SequencedFWRDHI  CCCGGCTTCAACGAGTACGACTTCGTGCCCCGAGAGCTTCGACCGGGACAAAACCATCGCC
SequencedFWRDPCR CCCGGCTTCAACGAGTACGACTTCGTGCCCCGagAGCTTCGACCGGGACAAAACCATCGCC

```

SequencedFWRDPCR	CCCGGCTTCAACGAGTACGACTTCGTGCCCCGAGAGCTTCGACCGGGACAAAACCATCGCC *****
PGL4.23	CTGATCATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGC
SequencedFWRDgel	CTGATCATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGC
SequencedFWRDHI	CTGATCATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTagCCCTACCGCACCGC
SequencedFWRDPCR	CTGATCATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGC *****
PGL4.23	ACCGCTTGTGTCCGATTTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCC
SequencedFWRDgel	ACCGCTTGTGTCCGATTTCAGTCATGCCCGCGACCCcATCTTCGGCAACCAGATCATCCCC
SequencedFWRDHI	ACCGCTTGTGTCCGATTTCAGTCATGCCCGCGACCCcATCTTCGGCAACCAGATCATCCCC
SequencedFWRDPCR	ACCGCTTGTGTCCGATTTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCC *****
PGL4.23	GACACCGCTATCCTCAGCGTGGTGCCATTTACCACGGCTTCGGCATGTTACCACGCTG
SequencedFWRDgel	GACACCGCTATCCTCAGCGTGGTGCCATTTACCACGGCTTCGGCATGTTACCACGctg
SequencedFWRDHI	GACACCGCTATCCTCAGCGTGGTGCCATTTACCACGGCTTCGGCATGTTACCACGctg
SequencedFWRDPCR	GACACCGCTATCCTCAGCGTGGTGCCATTTACCACGGCTTCGGCATGTTACCACGCTG *****
PGL4.23	GGCTACTTGA-TCTGC-GGCTTTCGGGTCGTGCTCATGTACCGCTTCGAGGAGGAGCTAT
SequencedFWRDgel	GggCTACTTGATCTGCGGCTTTCGGGTCGTGCTCATGTACCGcTttnnggAggaGCTAT
SequencedFWRDHI	gggCTACTtGATCTGCGgctTtTCGGGTCGTGCTCatGTACcGctt-----
SequencedFWRDPCR	GGCTACTTG-ATCTGCGG-CTTTCGGGTCGTGCTCATGTACCGCTTnnnggaggancTaT ** * ***** * ** ** * **
PGL4.23	TCTTGCGCAGCTTGCAAGACTATAAGATTCAATCTGCCCTGCTGGTGCCACACTATTTA
SequencedFWRDgel	TcttGCGCAGCTTGCAAGACTanangaTTCaaTctgCCctGCTggngCCcACACTatt--
SequencedFWRDHI	-----
SequencedFWRDPCR	tCTTGCGCAGCTTGcaagACTATAAnaTTCaATCTGCCcTGCTGGTGCCcAcaCTATTTA
PGL4.23	GCTTCTTCGCTAAGAGCACTCTCATCGACAAGTACGACCTAAGCAACTTGCACGAGATCG
SequencedFWRDgel	-----
SequencedFWRDHI	-----
SequencedFWRDPCR	GCTTCTTcncTAanaGcActntcATCGaCAAGTACGACCTAaGcAACttgcncgag---
PGL4.23	CCAGCGGCGGGGCGCCGCTCAGCAAGGAGGTAGGTGAGGCCGTGGCCAAACGCTTCCACC
SequencedFWRDgel	-----
SequencedFWRDHI	-----
SequencedFWRDPCR	-----

Figure 90. The aligned pGL4.23 plasmid with OC-VDRE inserts purified in a number by gelatin purification, heat inactivation or PCR purification kit.

A section of the aligned sequences of the pGL4.23 plasmid without insert (PGL4.23), the pGL4.23 plasmid with a gelatin purified oligonucleotide insert (SequencesFWRDgel), the pGL4.23 plasmid with a heat inactivated oligonucleotide insert (SequencesFWRDHI) and the pGL4.23 plasmid with a PCR purification kit purified oligonucleotide insert (SequencesFWRDPCR). The oligonucleotide insert is highlighted in yellow.

Chapter 9

9.8.2 pGL4.23 CYP24A1 VDRE positive clone sequences

CLUSTAL O(1.2.4) multiple sequence alignment

PGL4.23	-----GGCCTAACTGGCCGGTACCTATCGCAAGTGGCCACAAATCGCAAGTGGCCCA	53
CYP24A1.COL3-pGL3	TTTCTCTGGCCTAaCTGGCCGGTACCTATCGCAAGTGGCCACAAATCGCAAGTGGCCCA	60

PGL4.23	CAATACGCAAGTGGCCCAACAAGAGCTCGCTAGCCTCGAGGATATCAAGATCTGGCCTCGG	113
CYP24A1.COL3-pGL3	CAATACGCAAGTGGCCCAACAAGAGCTCGCTAGCCTCGAGGATATCAAGATCTGGCCTCGG	120

PGL4.23	CGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAGCTTGGCAATCC	173
CYP24A1.COL3-pGL3	CGGCCAAGCTTAGACACTAGAGGGTATATAATGGAAGCTCGACTTCCAGCTTGGCAATCC	180

PGL4.23	GGTACTGTGTGTAAGCCACCATGGAAGATGCCAAAACATTAAGAAGGGCCAGCGCCA	233
CYP24A1.COL3-pGL3	GGTACTGTGTGTAAGCCACCATGGAAGATGCCAAAACATTAAGAAGGGCCAGCGCCA	240

PGL4.23	TTCTACCCACTCGAAGACGGGACCGCGGCGAGCAGCTGCACAAAGCCATGAAGCGCTAC	293
CYP24A1.COL3-pGL3	TTCTACCCACTCGAAGACGGGACCGCGGCGAGCAGCTGCACAAAGCCATGAAGCGCTAC	300

PGL4.23	GCCCTGGTGGCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTAC	353
CYP24A1.COL3-pGL3	GCCCTGGTGGCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTAC	360

PGL4.23	GCCGAGTACTTCGAGATGAGCGTTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGTGAAT	413
CYP24A1.COL3-pGL3	GCCGAGTACTTCGAGATGAGCGTTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGTGAAT	420

PGL4.23	ACAAACCATCGGATCGTGGTGTGTCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTG	473
CYP24A1.COL3-pGL3	ACAAACCATCGGATCGTGGTGTGTCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTG	480

PGL4.23	GGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAG	533
CYP24A1.COL3-pGL3	GGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAG	540

PGL4.23	CTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCTGTGAGCAAGAAAGGGCTG	593
CYP24A1.COL3-pGL3	CTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCTGTGAGCAAGAAAGGGCTG	600

PGL4.23	CAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAGATCATCATCATGGAT	653
CYP24A1.COL3-pGL3	CAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAGATCATCATCATGGAT	660

PGL4.23	AGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTTGCCA	713
CYP24A1.COL3-pGL3	AGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTTGCCA	720

PGL4.23	CCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAGCTTCGACCGGGACAAAACCATCGCC	773
CYP24A1.COL3-pGL3	CCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAGCTTCGACCGGGACAAAACCATCGCC	780

PGL4.23	CTGATCATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGC	833
CYP24A1.COL3-pGL3	CTGATCATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGC	840

PGL4.23	ACCGCTTGTGTCCGATTCACTCATGCCCGGACCCCATCTTCGGCAACCAGATCATCCCC	893
CYP24A1.COL3-pGL3	ACCGCTTgtGTCCGATTCACTCATGCCCGGACCCCATCTTCGGCAACCAGATCATCCCC	900

PGL4.23	GACACCGCTATCCTCAGCGTGGTGCCATTTACCACGGCTTCGGCATGTTACCACGCTG	953
CYP24A1.COL3-pGL3	GACACCGCTATCCTCAGCGTGGTGCCATTTACCACGGCTTCGGCATGTTACCACGCTG	960

PGL4.23	G-GCTACTTGATCTGCGGCTTTTCGGGTCGTGCTCATGTACCGCTTCGAGGAGGAGCTATT	1012
CYP24A1.COL3-pGL3	GggCTACTTGATCTGCGgcTTTCGGGTCGTGCTCATGTACCGCTtc-----	1006
	* *****	

Figure 91 The aligned pGL4.23 plasmid with CYP24A1-VDRE inserts purified in a number by gelatin purification, heat inactivation or PCR purification kit

A section of the aligned sequences of the pGL4.23 plasmid without insert (pGL4.23) and one with CYP24A1-VDRE oligonucleotide fused into multiple cloning region of pGL4.23 (CYP24A1.COL3-pGL3). CYP24A1.COL3-pGL3 was purified plasmid DNA grown from one of three colonies (others not shown) sent for sequencing. The oligonucleotide insert is highlighted in yellow.

9.9 RXR and VDR expression in ATCC UC-MSCs

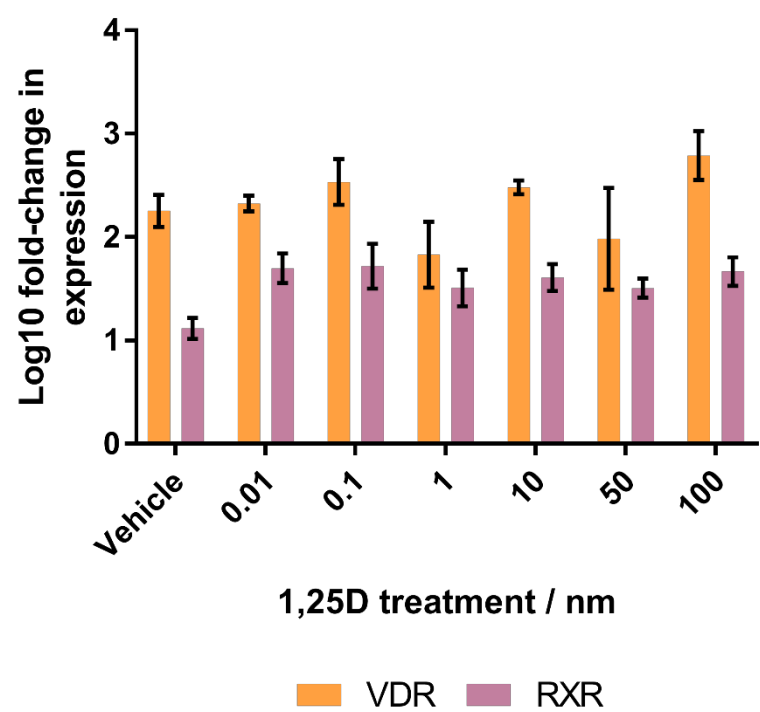


Figure 92. The relative expression of VDR and RXR mRNA in ATCC UC-MSCs in response to 24h treatment with varying concentrations of 1,25(OH)₂D

9.10 MSC Tri-lineage differentiation protocol

The differentiation protocols for the osteogenic, adipogenic and chondrogenic differentiation of UC-MSC cells would initially be carried out under the conditions as described below. The protocols would all be subject to further optimisation and therefore as currently written may not determine the finalised procedure to be used for future work described in this dissertation. Cells would initially be seeded at 5×10^3 cells/cm² for both osteogenic and adipogenic differentiation protocols and optimised further prior to future work being carried out. Cells would be cultured in triplicate and duplicated across plates to allow for DNA to be harvested for the RT-qPCR assessment of differentiation lineage specific gene expression. All protocols would include the growth of control, undifferentiated MSCs in a standard UC-MSC medium (section 2.5.1.1). Initially cells would be grown to 80% confluence, trypsinised as in section 2.5.2 and protocols carried out as below.

9.10.1 Osteogenic differentiation

Seed UC-MSCs in 6-well culture dishes in the UC-MSC culture medium (section 2.5.1.1) and incubate (37°C, humidified environment, 5% CO₂) until 60-70% confluent. Remove the medium, wash with PBS and replaced with an osteogenic medium consisting of DMEM, 10% FBS, 100 nM dexamethasone, 50 µM ascorbic acid, and 10 mM β-glycerolphosphate. Replace medium every 48 hours days being careful not to disturb the cell monolayer and between days 14-21 (subject to optimisation) assess the matrix mineralisation (calcium phosphate deposition) with Alizarin Red S staining. To do so, wash the cell or matrix layer with PBS, fix with 10% formalin (RT, 30 minutes), remove the formalin, wash twice with distilled water. Add sufficient 1% Alizarin Red S (5 minutes) to cover the cell monolayer and incubate (RT, 30 minutes). Wash with distilled water until supernatant runs clear and visualise using a light microscope. Mineralised matrix will stain intense red-orange when compared to control.

9.10.2 Adipogenic differentiation

Seed UC-MSCs in 6-well culture dishes in the basal culture medium (section 2.5.1.1) and incubate (37°C, humidified environment, 5% CO₂) until 70-80% confluent. Remove the culture medium and replace with adipogenic induction medium consisting of a basal medium consisting of DMEM supplemented with 10% FBS, 500 nM dexamethasone, 0.5 mM isobutylmethylxanthine, 50 µM indomethacin, and 10 mg/ml of insulin. Culture for 14/21 days (subject to optimisation), replacing the culture medium every 3 days. At day 14/21, remove the culture medium, wash the cell monolayer three times with PBS and fix with 10% formalin (RT, 30 minutes). Aspirate the formalin, wash with distilled water and add sufficient 0.5% Oil Red O solution to cover the monolayer and incubate (RT, 10 minutes). Remove Oil Red O solution and wash cells three times with distilled water until clear and visualise red intracellular lipid vacuoles using a light microscope.

9.10.3 Chondrogenic differentiation

Centrifuge cells (RT, 300 g, 5 minutes) and resuspend cells at a concentration of 1.25×10^6 cells/ml in chondrogenic medium consisting of DMEM high glucose supplemented with 1 % penicillin/streptomycin, 100 nM dexamethasone, 10 % insulin-transferrin-selenium (ITS), 170 μ M ascorbic acid, 100 μ M sodium pyruvate, 350 μ M proline and 10 ng/ml Human Transforming Growth Factor β 3. Add 200 μ l (2.5×10^5 cells/well) to each well of a U-bottom 96-well polypropylene plate. Centrifuge the plates (500 g, 5 minutes) and incubate (37°C, humidified environment, 5% CO₂) for 21 days, changing the medium daily, taking care not to aspirate the forming cellular aggregates. To assess stage of chondrogenic differentiation and cartilage formation use Alcian blue staining to stain for the presence of glycosaminoglycans which are a constituent of aggrecan, the major proteoglycan cartilage. To do so, aspirate the medium and wash aggregates twice with PBS, incubate with 10% formalin (RT, 30 minutes). Remove the formalin, wash twice with distilled water and add sufficient Alcian blue to cover the aggregates and incubate in the dark (overnight, RT). Aspirate Alcian blue solution, wash twice with de-staining solution (1x acetic acid:1.5x absolute ethanol), aspirate de-staining solution and add 1x PBS. Cartilage aggregates will stain dark blue when compared to control.

9.10.4 RT-qPCR assessment of differentiation lineage specific markers

At the end of the UC-MSC differentiation treatment, aspirate differentiation medium from each well of the 6-well plate. Wash twice with PBS (1 ml), aspirate PBS, add 0.25% trypsin-EDTA (1 ml) (ThermoFisher) and incubate for a minimum of three minutes. Once cells are detached, add four volumes of UC-MSC medium, transfer to a 50 ml centrifuge tube and centrifuge (1000 g, 5 minutes). Remove all medium from the pellet and extract DNA from cell pellets using the Sigma GeneElute™ Mammalian Genomic DNA Miniprep Kit as in section 2.3.3.1. Extracted DNA can then be used to interrogate the expression of differentiation markers of interest using the RT-qPCR protocols as described in section 2.6.2.

9.11 ATCC certificate of analysis for primary, umbilical-cord derived mesenchymal stem cells



CERTIFICATE OF ANALYSIS

ATCC® Number: PCS-500-010™
Lot Number: 64310874
Name: Primary Umbilical Cord-Derived Mesenchymal Stem Cells; Normal, Human
Species: Human (*Homo sapiens*)
Manufacture Date: 21MAY2016
Fill Volume: 1 mL
Passage Number: 1
Donor Tissue Source: Umbilical cord
Donor Gender: Male
Donor Age: Newborn
Donor Race: Caucasian
Product Format: Cells cryopreserved in the appropriate cryopreservation medium
Expiration Date: Not applicable
Storage Conditions: Store at less than –130°C, preferably in liquid nitrogen vapor. Cells shipped on dry ice should be immediately transferred to liquid nitrogen or liquid nitrogen vapor-phase storage. Do *not* store ATCC Primary Cell Solutions™ cells at –70°C or any other temperature above –130°C.

Test / Method	Specification	Result
Post-freeze viability (Trypan blue exclusion)	≥ 70%	84%
Viable cell count	≥ 5.0 × 10 ⁵ viable cells/ampule	6.5 × 10 ⁵ viable cells/ampule
Morphology	Normal or Pass	Pass
Mycoplasma contamination test	Negative or None detected	None detected
Population doubling capacity	≥ 10 doublings post-freeze	12 doublings post-freeze
Sterility test (bacteria, yeast, fungi)	Negative or No growth	No growth
Virus testing		
Hepatitis B	Negative or Not detected	Negative
Hepatitis C	Negative or Not detected	Negative
HIV-1 & HIV-2	Negative or Not detected	Negative

ATCC
 10801 University Boulevard
 Manassas, VA 20110-2209 USA
 www.atcc.org

800-638-6597 or 703-365-2700
 Fax: 703-365-2750
 E-mail: tech@atcc.org
 or contact your local distributor

- Page 1 of 2 -

Template Doc ID: 31194

Template Revision: 4

Template Effective Date: 12/31/2014

Figure 93. Page 1 of a 2-page report detailing the characteristics of human umbilical cord mesenchymal stem cells purchased from American Type Culture Collections



CERTIFICATE OF ANALYSIS

ATCC® Number: PCS-500-010™
 Lot Number: 64310874

Characterization / Cell-specific staining	CD29: Positive, > 90% expression of marker CD44: Positive, > 90% expression of marker CD73: Positive, > 90% expression of marker CD90: Positive, > 90% expression of marker CD105: Positive, > 90% expression of marker CD166: Positive, > 90% expression of marker CD14: Negative, < 5% expression of marker CD19: Negative, < 5% expression of marker CD31: Negative, < 5% expression of marker CD34: Negative, < 5% expression of marker CD45: Negative, < 5% expression of marker	CD29: Positive, 100.0% expression of marker CD44: Positive, 99.9% expression of marker CD73: Positive, 100.0% expression of marker CD90: Positive, 99.4% expression of marker CD105: Positive, 100.0% expression of marker CD166: Positive, 100.0% expression of marker CD14: Negative, 1.67% expression of marker CD19: Negative, 1.38% expression of marker CD31: Negative, 1.38% expression of marker CD34: Negative, 1.93% expression of marker CD45: Negative, 2.04% expression of marker
Differentiation test	Chondrocyte differentiation: Pass Adipocyte differentiation: Pass Osteocyte differentiation: Pass	Chondrocyte differentiation: Pass Adipocyte differentiation: Pass Osteocyte differentiation: Pass

Kim Ellis

Digitally signed by Kim Ellis
 DN: cn=Kim Ellis, o=ATCC, ou=Quality Assurance, email=kellis@atcc.org, c=US
 Date: 2017.02.03 19:00:07 -05'00'

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Figure 94. Page 2 of a 2-page report detailing the characteristics of human umbilical cord mesenchymal stem cells purchased from American Type Culture Collections

9.12 Vector construct plasmid and normalisation plasmid values for the transfection of SaOS-2 and UC-MSC cells with pGL4-CYP and OC plasmids

22/08/2018	pGL4.23 VDRE	OC						CYP						SaOS-2
		1	2	3	4	5	6	7	8	9	10	11	12	
		A	24500	29260	27870	27620	31340	28080	26410	26610	27140	24790	24970	25990
		B	27830	32440	29710	24370	26950	21170	58670	63260	59750	53130	58400	56060
		C	26450	24830	27090	24060	26800	25470	23770	25380	25570	25840	24370	23790
		D	35580	40220	36080	35540	36690	33910	51880	49850	55510	49300	51890	57240
		E	1745	1307	5193	3129	2143	1120	1678	3291	2252	1832	1668	1630
		F	2056	3785	5526	2646	2471	5634	4377	6136	4518	5159	6126	4075
		G	1556	2240	2629	3388	1536	3290	1788	2068	3030	1925	2342	1747
		H	3147	2337	2612	6367	2962	2673	4271	3822	3314	4703	4372	3517
	Normalisation CMV	OC						CYP						SaOS-2
		1	2	3	4	5	6	7	8	9	10	11	12	
		A	860100	895900	871200	809200	860500	818900	799200	988300	858900	780900	761300	790800
		B	965800	836700	791500	785900	878100	764000	1525000	1566000	1248000	1254000	1361000	1303000
		C	618500	726100	787100	749700	777900	647800	741700	676900	678500	682500	629500	680700
		D	647500	792900	689200	671900	658200	713300	997700	975600	1164000	1121000	1075000	1251000
		E	1858000	1889000	3016000	3138000	1854000	1554000	1888000	2306000	1999000	1992000	1548000	2133000
		F	3222000	2848000	3106000	2952000	2666000	2454000	3481000	2916000	3139000	2796000	3057000	4487000
		G	2168000	2457000	2465000	2306000	1689000	2436000	1789000	2280000	1612000	1879000	1973000	1947000
		H	3119000	2194000	2655000	2956000	2367000	2321000	3388000	2955000	3124000	2956000	3396000	3141000
	SAOS	OC						CYP						UC-MSC
		Vehicle	No insert	0.0284851	0.0326599	0.0319904	0.0341325	0.0364207	0.0342899	0.0330455	0.026925	0.0315986	0.0317454	0.0327992
			Insert	0.0288155	0.0387714	0.0375363	0.031009	0.0306913	0.0277094	0.0384721	0.0403959	0.0478766	0.0423684	0.0429096
		1,25D	No insert	0.0427648	0.0341964	0.0344175	0.0320928	0.0344517	0.0393177	0.032048	0.0374945	0.0376861	0.0378608	0.0387133
			Insert	0.0549498	0.0507252	0.0523506	0.0528948	0.0557429	0.0475396	0.0519996	0.0510968	0.047689	0.0439786	0.0482698
		Vehicle	No insert	0.0009392	0.0006919	0.0017218	0.0009971	0.0011559	0.0007207	0.0008888	0.0014271	0.0011266	0.0009197	0.0010775
			Insert	0.0006381	0.001329	0.0017791	0.0008963	0.0009269	0.0022958	0.0012574	0.0021043	0.0014393	0.0018451	0.0020039
		1,25D	No insert	0.0007177	0.0009117	0.0010665	0.0014692	0.0009094	0.0013506	0.0009994	0.000907	0.0018797	0.0010245	0.001187
			Insert	0.001009	0.0010652	0.0009838	0.0021539	0.0012514	0.0011517	0.0012606	0.0012934	0.0010608	0.001591	0.0012874

Figure 95. A plate map indicating the relative fluorescence values for transfected pGL4.23-OC and pGL4.23-CYP plasmids and accompanying Renilla plasmids into both SaOS-2 and ATCC UC-MSCs

9.13 The association between methylation of cg18397653 in SWS umbilical cord blood DNA and BMC at four years of age in the SWS cohort

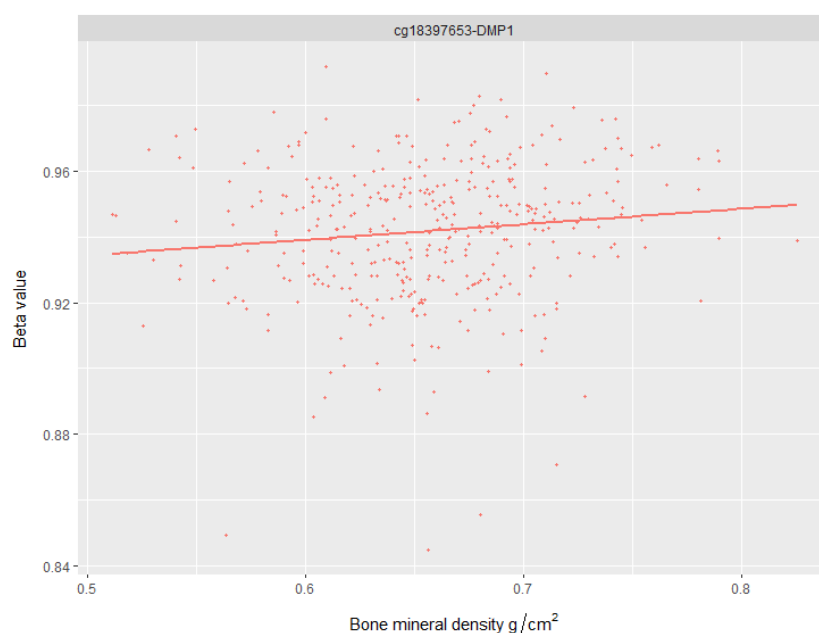


Figure 96. The association between cg18397653 methylation in UCB DNA and BMC at four years of age in the SWS cohort

The differential methylation analysis assessing the association between the methylation of 339 samples of UCB DNA and BMC at four years of age in the SWS cohort found significant differential methylation of CpG cg18397653, mapped to the DMP-1 gene. The methylation of the dmCpG showed a positive association with BMC at four years of age.

10 Chapter 10 – Bibliography

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