UNIVERSITY OF SOUTHAMPTON

Faculty of Medicine

Human Development and Health

Vitamin D transport and the effects on placental function and fetal growth

by

Claire Louise Simner

Thesis for the degree of Doctor of Philosophy

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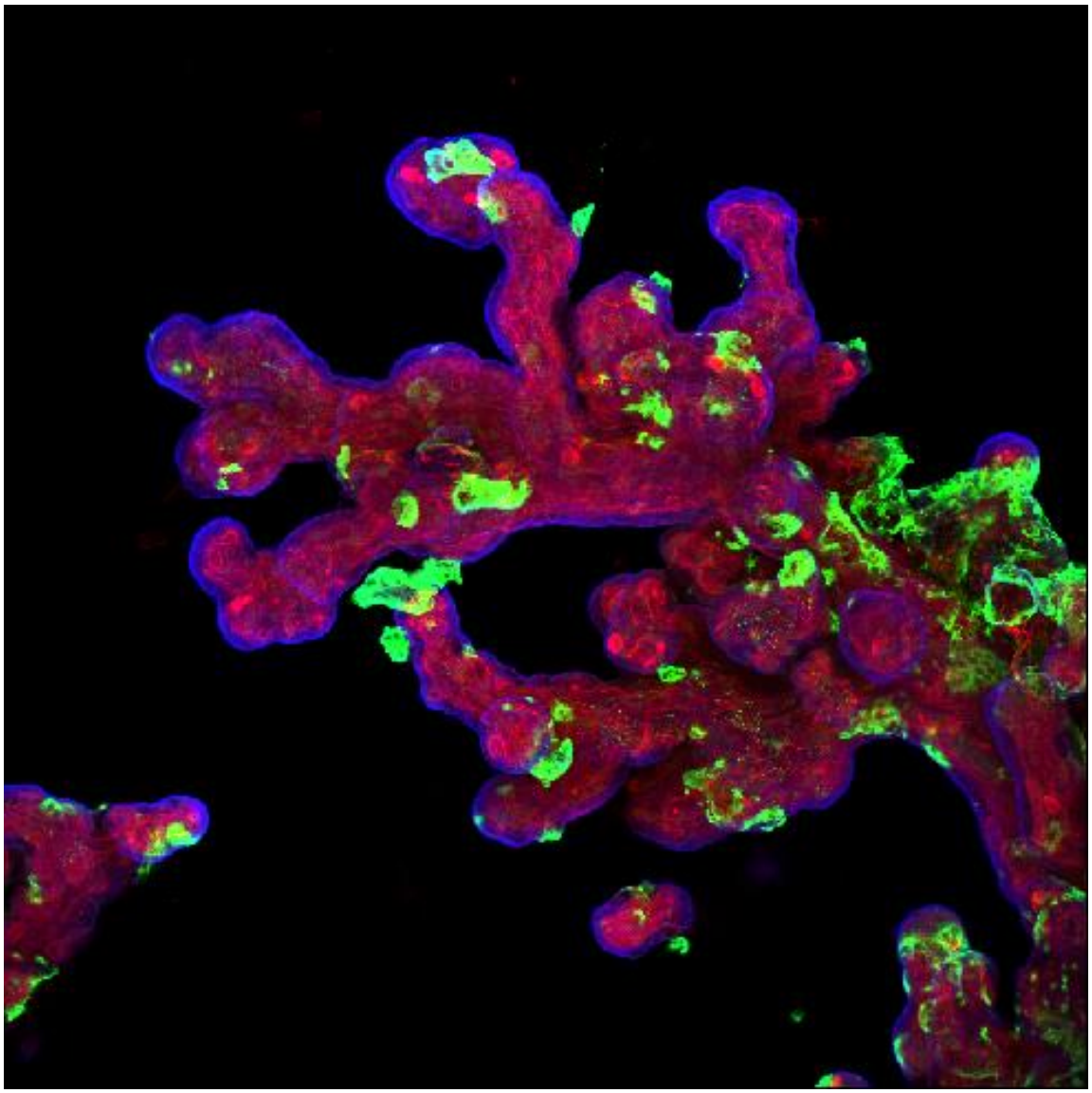

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ABSTRACT

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VITAMIN D TRANSPORT AND THE EFFECTS ON PLACENTAL FUNCTION AND FETAL GROWTH

Claire Louise Simner

Reduced fetal growth has been linked to an increased risk of chronic disease in later life, highlighting the importance of the *in utero* period. Maternal vitamin D levels have been identified as one specific factor linked to suboptimal fetal growth in human epidemiological studies and have specifically been linked to infant bone development. Vitamin D can only be obtained from the maternal diet or dermal synthesis from sunlight, therefore all vitamin D must be transferred across the placenta to reach the fetus. The effects of vitamin D on fetal growth may be mediated by effects on the developing fetus or by effects on the placenta. There are two major forms of vitamin D within the circulation, the inactive 25-hydroxyvitamin D (25(OH)D) and the active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). It is currently thought that 25(OH)D is transported by the placenta but whether $1,25(OH)_2D$ is transported by the placenta is unclear. However, this is based on associations between maternal and cord serum vitamin D levels and the mechanism of vitamin D transport has not been clearly defined.

Bone development is dependent on both calcium and amino acids. Indeed, reduced placental transport of amino acids and calcium to the fetus has been associated with reduced fetal bone growth and impaired bone development. As vitamin D acts via the vitamin D receptor as a transcription factor it is hypothesised that vitamin D may affect fetal growth through altering placental gene expression of amino acid and calcium transporters. This thesis investigates the effects of vitamin D on placental gene expression of nutrient transporters and the uptake of vitamin D into the placenta.

Gene expression of amino acid and calcium transporters was measured in placentas from the Southampton Women's Survey using quantitative reverse transcription polymerase chain reaction. The mRNA levels of placental amino acid transporters including *4F2hc*, *LAT1*, *LAT3* and *SNAT1* were positively associated with maternal 25(OH)D and vitamin D binding protein (DBP) levels measured at 34 weeks of gestation. The mRNA levels of specific placental amino acid and calcium transporters, including *PMCA1*, *ASCT2* and *LAT1* were also positively associated with placental mRNA expression

of *CYP24A1*, a known vitamin D responsive gene, suggesting that placental exposure to active $1,25(OH)_2D$ is important. The associations between placental gene expression and DBP suggest that this binding protein may play an important role in delivery of vitamin D to the placenta.

Ex vivo studies using human placental villous fragment culture demonstrated increased mRNA expression of a vitamin D responsive gene upon exposure to 25(OH)D with albumin compared to 25(OH)D alone. This suggests that albumin, a vitamin D carrier protein, may aid uptake of 25(OH)D into the placenta. Placental uptake of $1,25(OH)_2D$ was also observed, without any dependence on a carrier protein. Furthermore, uptake of fluorescently labelled albumin into placental villous fragments was reduced when incubated at 4°C compared to 37°C, suggesting that carrier protein-mediated uptake of vitamin D occurs via a carrier-protein mediated mechanism. These *ex vivo* studies also show that inactive 25(OH)D increased placental gene expression, indicating conversion into $1,25(OH)_2D$ within the placenta. RNA sequencing on placental fragments cultured with 25(OH)D demonstrated increased mRNA expression of the calcium transporters *NCX2* and *CaT1*, and the amino acid transporter *xCT*.

Placental mRNA expression was investigated in samples from a second human cohort, Maternal Vitamin D Osteoporosis Study, a randomised placebo controlled trial of vitamin D supplementation during pregnancy. Vitamin D supplementation resulted in altered expression of one amino acid transporter, *ASCT2*, but this was dependent on the season of supplementation. The differing effects observed in these placentas compared with placental fragment culture could result from the differences between chronic and acute response to vitamin D. However, mRNA expression of specific amino acid and calcium transporters, *PMCA4*, *ASCT2* and *TAT1,* was associated with placental *CYP24A1* expression, again suggesting that the exposure of the placenta to $1,25(OH)_2D$ is important.

These findings suggest that both 25(OH)D and $1,25(OH)_2D$ are transported into the human placenta, and that 25(OH)D is hydroxylated into the active $1,25(OH)_2D$ within the placenta. Placental 25(OH)D uptake may be facilitated by the presence of a carrier protein. In addition, placental exposure to vitamin D may regulate expression of specific amino acid and calcium transporters within the placenta. This has implications for the development of the fetus and suggests that the associations observed in human epidemiological studies between maternal vitamin D and fetal growth may arise partly through the effects of vitamin D on placental nutrient transport.

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DECLARATION OF AUTHORSHIP

I, Claire Louise Simner,

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

'Vitamin D transport and the effects on placental function and fetal growth'

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

Cleal, J.K., Day, P.E., **Simner, C.L.,** Barton, S.J., Mahon, P.A., Inskip, H.M., Godfrey, K.M., Hanson, M.A., Cooper, C., Lewis, R.M., Harvey, N.C., and the SWS Study Group (2015). Placental amino acid transport may be regulated by maternal vitamin D and vitamin D-binding protein: results from the Southampton Women's Survey. British Journal of Nutrition *113*(12), 1903-10.

Signed: .....

Date: .....

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Definitions and Abbreviations

Abbreviation	Definition
25(OH)D	25-hydroxyvitamin D
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
ACTB	β-actin
AGA	Appropriate for gestational age
Ala	L-alanine
APS	Adenosine 5'phosphate
ANOVA	Analysis of variance
Arg	L-arginine
ASCT	Alanine/serine/cysteine/threonine transporter
Asn	L-asparagine
Asp	L-aspartate
ATP	Adenosine triphosphate
ATP5B	ATP synthase
AZA	5-Aza-2'deoxycytidine
BA	Bone area
BCA	Bicinchoninic acid
BM	Basement membrane
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CaT	Calcium transporter
cDNA	Complementary DNA
cp	Crossing point
CI	Confidence interval
CV	Coefficient of variation
CYC1	Cytochrome C-1
CYP	Cytochrome P450
Cys	L-cysteine
DAPI	4',6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
DBP	Vitamin D binding protein
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT	DNA methyltransferase

dNTP	Deoxynucleotide triphopshate
DOHaD	Developmental Origins of Health and Disease
DPBS	Dulbecco's phosphate buffered saline
DSL	*Datura Stramonium* Lectin
dTTP	Deoxythymidine triphosphate
DTT	Dithiothreitol
dUTP	Deoxyuridine triphosphate
DXA	Dual X-ray absorptiometry
EAAT	Excitatory amino acid transporter
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FcRn	Neonatal Fc receptor
FGR	Fetal growth restriction
FITC	Fluorescein isiothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
Glu	L-glutamate
Gln	L-glutamine
GLUT	Glucose transporter
Gly	Glycine
hCG	Human chorionic gonadotropin
HEK293	Human embryonic kidney 293
His	L-histidine
HKG	Housekeeping gene
hPL	Human placental lactogen
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
ICM	Inner cell mass
IGF	Insulin-like growth factor
Iso	L-isoleucine
IUGR	Intrauterine growth restriction
IVF	*In vitro* fertilsation
LAT	L-type amino acid transporter
LB	Luria Bertani
Leu	L-leucine
LGA	Large for gestational age
LPL	Lipoprotein lipase
Lys	L-lysine
M-MLV	Moloney Murine Leukaemia Virus
MAVIDOS	Maternal Vitamin D Osteoporosis Study
MCT	Monocarboxylate transporter
MeCP2	Methyl CpG-binding protein 2
Met	L-methionine

miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MVM	Microvillous membrane
NCX	Sodium-calcium exchanger
NEC	No enzyme control
NTC	No template control
nVDRE	Negative vitamin D response element
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	PBS-Tween
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Phe	L-phenylalanine
PMCA	Plasma membrane calcium ATPase
PPi	Pyrophosphate
Pro	L-proline
PSA	*Pisum Sativum* Agglutinin
PTH	Parathyroid hormone
PTH1R	Parathyroid hormone 1 receptor
PTHrP	Parathyroid hormone-related peptide
PVDF	Polyvinylidine fluoride
qrt-PCR	Quantitative reverse transcription polymerase chain reaction
RAP	Receptor-associated protein
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay
RNase	Ribonuclease
RPL13A	Ribosomal protein L13A
rRNA	Ribosomal RNA
RT	Room temperature
rt	Reverse transcription
rt-PCR	Reverse transcription polymerase chain reaction
RXRα	Retinoid X receptor α
SAM	S-adenosylmethionine
SD	Standard deviation
SDHA	Succinate dehydrogenase complex, subunit A
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Ser	L-serine
SGA	Small for gestational age
SNAT	Sodium dependent neutral amino acid transporter
SNP	Single nucleotide polymorphism
SWS	Southampton Women's Survey
TAT	T-type amino acid transporter
TBE	Tris borate EDTA
TDE	2,2'thiodiethanol

Thr	L-threonine
TOP1	Topoisomerase 1
Trp	L-tryptophan
TXNIP	Thioredoxin-interacting protein
Tyr	L-tyrosine
u	Units
UBC	Ubiquitin C
UPL	Universal probe library
UV	Ultraviolet
Val	L-valine
VDIR	VDR-interacting repressor
VDR	Vitamin D receptor
VDRE	Vitamin D response element
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Chapter 1:

Introduction

1.1. Introduction

Poor fetal growth results in an increased risk of perinatal morbidity and mortality as well as an increased risk of chronic disease in later life (Barker, 1998). Fetal growth is reliant on an adequate supply of vitamin D (Harvey *et al.*, 2008; Bowyer *et al.*, 2009; Mahon *et al.*, 2010; Ioannou *et al.*, 2012), which must come from the maternal circulation as vitamin D can only be obtained from the diet or dermal synthesis from sunlight. Vitamin D deficiency is common in women of childbearing age (Dawodu *et al.*, 2001; Gale *et al.*, 2008), therefore many infants may be at risk from altered fetal growth due to maternal vitamin D deficiency. All vitamin D must be transferred across the placenta meaning that placental function may influence the fetal supply and subsequent growth. The placenta forms a barrier between the mother and fetus and mediates transfer of nutrients to the fetus. Changes in placental transport of other nutrients have been associated with changes in fetal growth (Cleal *et al.*, 2011). Yet despite being a fundamental biological process, the mechanism of placental vitamin D transfer and actions of vitamin D within the placenta are not fully understood. Vitamin D circulates bound to a binding protein and this could allow a specific receptor-mediated uptake mechanism within the placenta. Changes to this mechanism could therefore influence supply to the fetus; metabolism or breakdown within the placenta could also influence fetal supply. Vitamin D acts as a transcription factor (Carlberg, 2014), therefore vitamin D could also influence fetal growth by altering gene expression of placental nutrient transporters. This could modify nutrient levels reaching the fetal circulation, and result in altered fetal growth. This thesis therefore aims to investigate how vitamin D is transported into the human placenta, whether it is metabolised within the placenta and whether vitamin D alters expression of nutrient transporter genes within the placenta.

1.2. Fetal Programming

1.2.1. Developmental Origins of Health and Disease

Studies in human populations show associations between birth weight and the risk of developing non-communicable diseases in adult life, including cardiovascular disease (Barker, 1990), type 2 diabetes (Hales *et al.*, 1991) and osteoporosis (Cooper, 1997, 2001). These associations are continuous across the range of birth weights with maximal effect at the extremes (Barker, 1998) and have been replicated in diverse populations in both developed and developing countries (Huxley *et al.*, 2000). The resultant Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that the *in utero* environment programs physiological fetal adaptations, aimed at immediate survival, which persist into adulthood and influence the development of chronic disease (Gluckman and Hanson, 2004).

The placenta has also been implicated in fetal programming. Individuals who were smaller babies with larger placentas, suggesting failure to reach growth potential, had the highest risk of developing hypertension at 46-54 years of age (Barker *et al.*, 1990). This shows that placental development during fetal life also has the potential to cause lifelong alterations to health. The finding that a disproportionately large placenta in comparison to fetal growth is associated with adverse outcomes has been described in recent studies in relation to adverse perinatal outcome (Shehata *et al.*, 2011), higher systolic blood pressure at 7 years of age (Wen *et al.*, 2011) and increased risk of coronary heart disease in adulthood (Risnes *et al.*, 2009). These studies suggest that a reduced placental efficiency may prevent optimal fetal growth resulting in an increased risk of chronic disease. The shape of the placenta has also been associated with postnatal health outcomes; in offspring from short mothers the risk of hypertension was related to placental surface area (Barker *et al.*, 2010).

Maternal size and nutritional status may alter placental size. Maternal fat mass, a marker of current nutritional status, was associated with larger placental surface. While placental efficiency was positively associated with increased maternal fat mass alongside higher maternal birth weight and head circumference, which are markers of fetal and infant growth of the mother. This illustrates that the mothers past and current nutritional status can affect placental structure and development and therefore efficiency (Winder *et al.*, 2011).

In addition, the relationship between hypertension and placental weight can be sexually dimorphic. In men, a large placental lesser diameter was associated with reduced birth weight and increased hypertension risk. While, in women, a small placental area and diameters were associated with a decreased birth weight and increased hypertension risk. These associations were explored further in a Finnish cohort which underwent food shortages in World War Two. In men, the relationship between birth weight and placental breadth was restricted to those with middle class mothers who would have been better nourished at the time of conception but underwent nutritional deprivation during pregnancy. In women, the relationship between placental size and diameter and birth weight was stronger in those whose mothers were shorter, indicating a stronger link with lifetime nutrition. As girls invest more in placental development, they appear to have less capacity for placental expansion in response to adverse nutritional stimuli. Female *in utero* development is more dependent on the lifetime nutrition of the mother as the larger placental size in relation to birth weight means that there is already some capacity to deal with unexpected nutritional challenge. In males, placental expansion occurs more readily in response to stimuli, however if the maternal nutrients are not available for transport across the newly enlarged placenta more precious resources will be required to maintain the expanded placenta leaving the male fetus with further nutritional deprivation (Eriksson *et al.*, 2010).

The fetus is malleable in terms of environmental stimuli and this plasticity makes the *in utero* period a promising window for intervention to reduce the incidence of non-communicable disease (Figure 1.1). The DOHaD theory suggests that non-communicable disease could be addressed by optimising fetal growth and development (Barker *et al.*, 2013), through improving maternal health and nutrition.

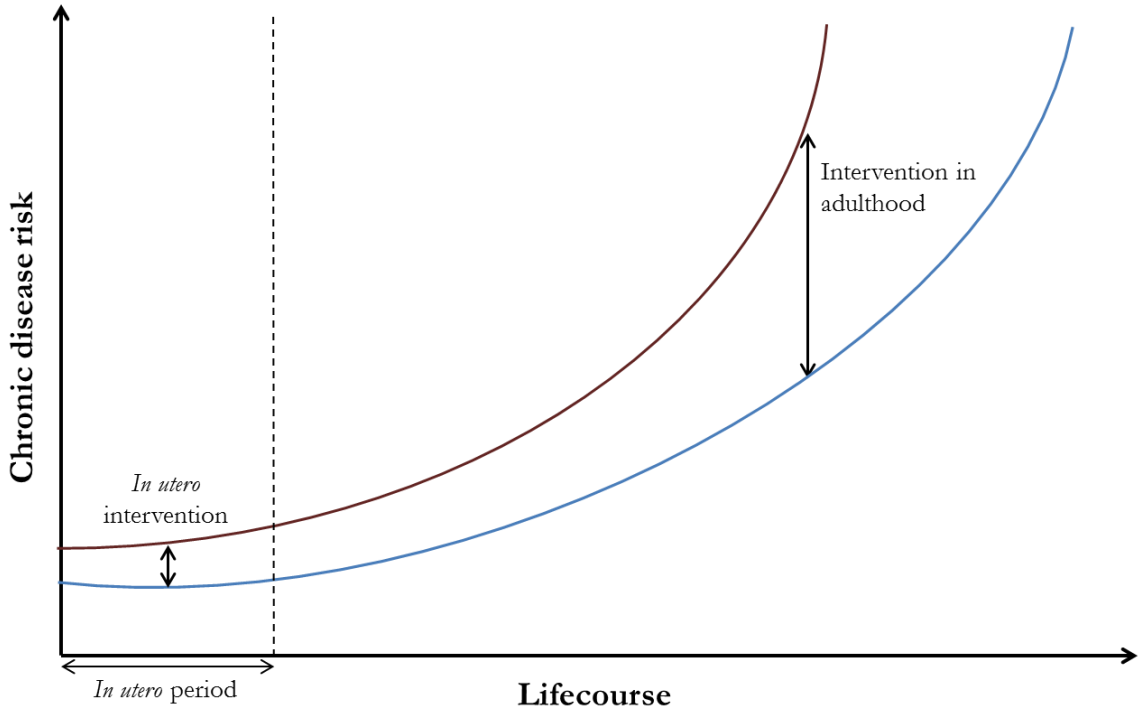

Figure 1.1: The lifecourse approach to chronic disease risk. Intervention during the *in utero* period may reduce the risk of chronic disease development throughout the lifecourse. During the *in utero* period the fetus is more plastic than in adulthood and therefore more responsive to intervention.

- **The association of low birth weight with outcomes in later life is thought to be a marker of adaptations to changes in the *in utero* environment.**

1.2.2. The *in utero* environment and fetal programming

The fetus responds to signals from the mother. Levels of nutrients and hormones reaching the fetus via the placenta result in physiological adaptations in fetal growth and development, which can result in effects that persist into adulthood.

Human studies

The Dutch Hunger Winter

The Dutch Hunger Winter (November 1944-April 1945), resulted from a German embargo on food transport to the Netherlands and an early and severe winter and meant that rations decreased from ~1800 to 400-800 calories a day. During the famine antenatal and birth records were maintained which

contributed to studies into DOHaD (Painter *et al.*, 2005). Glucose intolerance in later life was observed in individuals exposed to the famine *in utero* (Roseboom *et al.*, 2006). Effects on birth weight and size depended on the timing of famine exposure. Mid- and late-gestation exposure resulted in smaller, thinner babies with smaller placentas (Roseboom *et al.*, 2001; Stein *et al.*, 2004). In contrast, exposure in early gestation resulted in babies who were larger and heavier than those not exposed to famine (Painter *et al.*, 2005).

While, famine in early gestation did not reduce birth weight, it did result in poor lifelong health including an atherogenic lipid profile, altered blood coagulation, obesity and coronary heart disease. The incidence of heart disease in these individuals was not only increased but the onset of the disease occurred earlier in life than those not exposed to famine during gestation (Painter *et al.*, 2005; Roseboom *et al.*, 2006). The Dutch Hunger Winter showed that the nutrient supply to the fetus is important for fetal development and subsequent disease risk and also highlighted two important concepts. Firstly, the timing of an insult during gestation determines the impact on later health, due to different organ systems growing and developing at different stages of gestation. Secondly, birth weight is a crude and sometimes unrepresentative measure of suboptimal *in utero* conditions. Individuals exposed to famine during early gestation did not exhibit reduced birth weight but in adult life suffered a higher number of health problems compared to those exposed to famine later in gestation.

The Southampton Women's Survey

The Southampton Women's Survey (SWS) was established to investigate pre-pregnancy and *in utero* factors affecting fetal growth and subsequent childhood development (Inskip *et al.*, 2006). Specific influences on neonatal and infant body composition have been demonstrated in the SWS. Specifically, excessive maternal weight gain during pregnancy correlated with increased infant fat mass during the neonatal period and at 4 and 6 years of age (Crozier *et al.*, 2010). Other environmental factors found to be associated with infant adiposity include increased maternal plasma n-6 polyunsaturated fatty acid (Moon *et al.*, 2013). The SWS has also revealed intrauterine factors which impact neonatal and infant skeletal development. Maternal pre-pregnancy body mass index (BMI) positively associated with neonatal whole body bone mineral content (BMC; (Harvey *et al.*, 2011). Fetal growth measures have also been related to skeletal development. For example, fetal abdominal circumference growth in late pregnancy was associated with bone mass at birth, while the same measure in early pregnancy was associated with bone mass at 4 years (Harvey *et al.*, 2012b). Moreover, fetal femur growth in late gestation was associated with an index of femoral neck strength at 6 years of age (Harvey *et al.*, 2013). Smoking was identified as another factor which may impact fetal skeletal development. Infants whose mothers smoked during pregnancy had a reduced BMC at birth compared to those whose mothers did not smoke (Harvey *et al.*, 2011).

Measurement of fetal blood flow revealed that increased shunting of blood away from the liver and towards the brain was associated with a reduced neonatal sum of skinfold thickness. The shunting of blood away from the liver and towards the brain is indicative of brain-sparing which can occur with fetal stress. Interestingly, this brain-sparing effect was observed more in male fetuses and also in those with lower placental weights, which could indicate increased stress due to inadequate nutrient transfer from the placenta resulting in reduced deposition of fetal fat reserves (Godfrey *et al.*, 2012).

Further associations between the placenta and fetal development have been revealed in the SWS. Placental volume at 19 weeks of gestation was positively associated with neonatal percentage fat mass and negatively with percentage lean mass. Placental volume was also positively associated with the neonatal skeletal parameters, bone area (BA), BMC and bone mineral density (BMD). Furthermore, placental volume was positively associated with maternal height, pre-pregnancy body fat and age at child's birth (Holroyd et al., 2012). This suggests that maternal factors impact upon the placenta, which consequently impacts fetal growth and development. In support of this, molecular changes within the placenta have been linked to changes in fetal development as well as maternal lifestyle factors. Mothers who reported undertaking strenuous exercise at the time of study recruitment had higher placental expression of the imprinted gene *Pleckstrin homology-like domain family A member 2* compared to those who did not. In addition, higher placental expression of this gene was associated with a lower femur growth velocity between 19-34 weeks gestation and reduced BMC, BA, and BMD at 4 years of age (Lewis et al., 2012).

The findings from the SWS show that maternal nutrition and body composition relate to fetal growth and skeletal development. Furthermore, they have revealed specific placental adaptations to maternal lifestyle which appear to subsequently impact fetal growth and development. These changes to the fetus are still evident during childhood.

Other human cohorts

Findings from the Dutch Hunger Winter and the SWS have been replicated in other human cohorts. For example, placental weight and placental to birth weight ratio were lower from women fasting during Ramadan compared to those not *in utero* during Ramadan (Alwasel *et al.*, 2010). While, in the New Delhi birth cohort, BMI at birth was associated with BMC in adulthood (Tandon *et al.*, 2012) and increased circulating pro-inflammatory markers, which could link size at birth with cardiovascular disease (Lakshmy *et al.*, 2011).

Animal studies

Animal models of nutrient restriction during gestation support findings from epidemiological studies. 30% nutrient restriction during pregnancy in rats and guinea pigs resulted in reduced offspring birth

weight (Woodall *et al.*, 1996; Ozaki *et al.*, 2001; Kind *et al.*, 2003). Furthermore, these studies show physiological effects including increased blood pressure (Woodall *et al.*, 1996; Ozaki *et al.*, 2001) and reduced glucose tolerance (Kind *et al.*, 2003), which persist or manifest in later life of the offspring. In addition, while a sheep model of 15% nutrient restriction during early pregnancy did not affect offspring birth weight, altered stress responses with blunted cortisol and adrenocorticotrophic hormone responses were observed in offspring (Hawkins *et al.*, 2000). Protein restriction during gestation is also used as an *in utero* nutritional challenge. Offspring from protein restricted rats exhibit numerous features of metabolic disease, including hypertension (Langley-Evans *et al.*, 1994), vascular dysfunction (Torrens *et al.*, 2009) and increased glucose levels (Burdge *et al.*, 2008). These studies support the DOHaD theory and show that the *in utero* environment can program adaptations which result in an altered susceptibility to chronic disease in later life.

As well as general nutrient restriction, reduction in specific nutrients as well as alterations in maternal hormones also have programming effects on offspring. Maternal iron restriction in rats throughout gestation resulted in offspring with higher blood pressure, lower serum triacylglycerol and reduced body weight at 18 months of age (Lewis *et al.*, 2002). In terms of hormonal disturbances, glucocorticoids are growth inhibitory and have been shown to affect fetal development. Administration of glucocorticoids to pregnant rats at specific time points throughout gestation has been shown to result in hypertension (Levitt *et al.*, 1996) and insulin resistance (Nyirenda *et al.*, 1999) in offspring as well as increased sensitivity to postnatal stress (Welberg *et al.*, 2001). Furthermore, glucocorticoids may also be responsible for mediating some of the effects of maternal nutritional challenges on fetal development (Fowden *et al.*, 2006a).

- **These studies suggest that events *in utero* can influence lifelong health. Maternal nutrition and body composition have been implicated as important factors regulating fetal development and underlying these associations.**

1.3. Fetal growth and development

Human gestation lasts for 38 weeks and consists of three stages; pre-implantation, embryonic and fetal. Size and weight of the fetus increase with advancing gestation and throughout gestation tissues and organ systems develop (Kaplan and Bolender, 1998).

1.3.1. The pre-implantation period

The first two weeks following conception are classed as the pre-implantation period. Following fertilisation the zygote undergoes a number of cell divisions, and at the 16 cell stage is compacted to

form a morula. At the 32 cell stage the blastocyst is formed (Johnson and Everitt, 2007) and the extraembryonic and embryonic lineages are differentiated. The outer trophectoderm layer forms the placenta, while the inner cell mass (ICM) forms the embryo proper (Cross, 1998; Figure 1.2). Around day 7 post-conception the blastocyst attaches to and implants into the uterine wall (Bernirschke *et al.*, 2006). This signals the end of the pre-implantation period of fetal development. Adverse stimuli during the pre-implantation period can alter the proportions of cells in the trophectoderm and ICM of the blastocyst, which can result in alterations to numerous cell lineages (Kwong *et al.*, 2000).

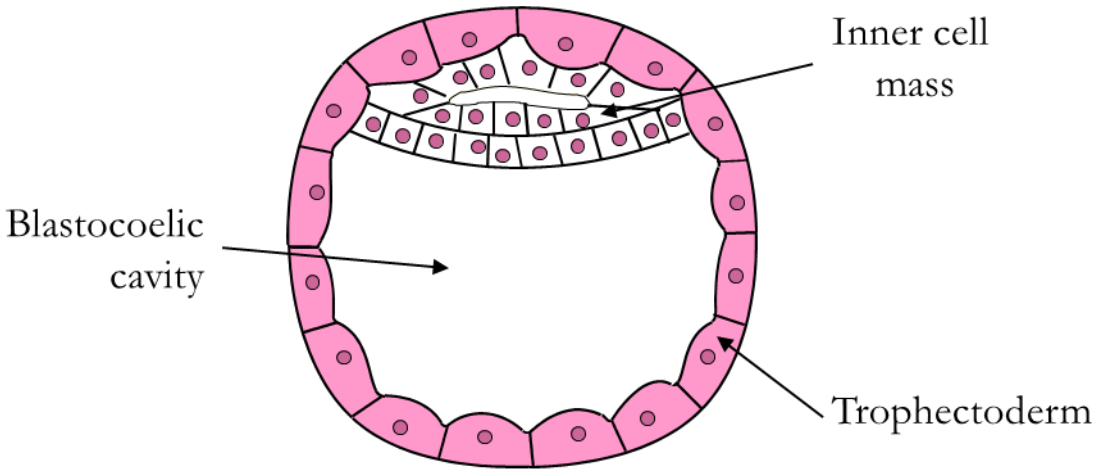

Figure 1.2: The blastocyst.

1.3.2. The embryonic period

The embryonic period lasts from the end of the pre-implantation period until the eighth week of gestation. This is the stage when morphogenesis of organs begins and morphogenesis of many organs is complete by the end of the embryonic period. Unlike most organs systems, the heart and blood vessels are functional and from the fourth week of gestation blood circulates around the developing embryo. By the end of the embryonic period all organ systems are present (Kaplan and Bolender, 1998; Larsen, 2001). Environmental insults during this period of development can result in discrete changes in the development of specific organs. Each organ undergoes critical periods of development at different times throughout gestation; therefore the timing of the insult will determine which organ(s) are affected (Fowden *et al.*, 2006a).

1.3.3. The fetal period

The fetal period follows the embryonic period and lasts until birth. This period is characterised by rapid fetal growth and maturation of the organ systems laid down during the embryonic period. During the fetal period the fetus undergoes a large increase in size and weight from ~8 g at the end of the embryonic period to ~3400 g at birth. The majority of this weight gain occurs during the final 2

months of gestation, whilst during months 4-6 of gestation the fetus undergoes the majority of its linear growth. The proportions of the fetus also undergo major changes during this stage of development. At week 9 of gestation the head of the fetus represents half of the crown-rump length, whereas by birth the body of the fetus has grown considerably and the head represents a quarter of the crown-rump length. The organ systems laid down during the embryonic period undergo expansion and differentiation of cell populations, while for organ systems which did not complete morphogenesis during the embryonic period, such as the ear and palate, morphogenesis is completed (Kaplan and Bolender, 1998; Larsen, 2001). As the fetal period involves rapid growth, it is susceptible to alterations in nutrient supply. Inadequate nutrient supply during this stage can result in decreased cell proliferation with a consequent reduction in total cell number (Fowden *et al.*, 1998).

1.3.4. Abnormal fetal growth

Environmental insult during any stage of fetal development can result in abnormal fetal growth, including intrauterine growth restriction (IUGR), or large for gestational age (LGA).

Intrauterine growth restriction

IUGR babies are those who have not reached their full growth potential and are classified as infants whose birth weight or length is below the tenth percentile for their gestational age. IUGR can be divided into two types; symmetrical and asymmetrical. Symmetrical IUGR is characterised by a reduction in size of both the fetal body and head and accounts for ~25% of IUGR cases. Symmetrical IUGR occurs as a result of insult during the earlier stages of gestation (first and second trimester), when cell growth and differentiation are occurring. Asymmetrical IUGR is characterised by a reduction in fetal body size with no reduction in head size, and indicates a brain-sparing mechanism at the expense of body size. It occurs as a result of insult in the third trimester when cells are proliferating to drive organ maturation and increased fetal weight (Neumann and Carroll, 1984; Wollmann, 1998). IUGR is important as reduced infant size is associated with an increased risk of neonatal morbidity and mortality; IUGR babies account for 30% of new born deaths (Neumann and Carroll, 1984). Furthermore, IUGR infants have an increased risk of developing chronic disease in later life (see section 1.2).

Large for gestational age

LGA or fetal macrosomia is classified as babies with a weight in or above the ninetieth percentile and a birth weight of 4000 g or above. Fetal macrosomia can result in adverse consequences for both the baby and the mother. One of the major consequences for the fetus is shoulder dystocia, which can result in damage to the nerves of the face or arms as well as fractured bones of the infant. LGA increases the likelihood that a caesarean section will be required for safe delivery of the infant. The

most common cause of LGA is maternal diabetes, which doubles the risk of fetal macrosomia (Sparks *et al.*, 1998; Zamorski and Biggs, 2001). Maternal diabetes is thought to result in LGA infants as a result of increased placental weight (Lao *et al.*, 1997) and increased glucose transfer to the developing fetus (Osmond *et al.*, 2001; Bibee *et al.*, 2011). Fetal macrosomia is associated with increased risk of developing obesity and diabetes in later life (Lewis *et al.*, 2013b).

1.3.5. Regulation of fetal growth

Fetal growth is regulated by factors including maternal nutrition, oxygen and endocrine status (Fowden *et al.*, 2006a). Hormone levels change across gestation and towards term endocrine changes signal the final maturation stages of fetal development. Different hormones have differing effects on fetal growth, for example insulin is growth stimulatory and insulin levels are positively associated with birth weight, while glucocorticoids are growth inhibitory. Insulin deficiency in the fetus leads to symmetric IUGR, whereas glucocorticoid levels are elevated in IUGR. Glucocorticoids play an important role in tissue maturation prior to birth, but exposure to excess glucocorticoids at inappropriate times in gestation may result in maladaptation in fetal development and can also result in functional changes in organ systems including the kidney, liver and lungs (Fowden *et al.*, 1998). Adequate oxygen supply to the fetus is also crucial for development, and in rats hypoxia resulted in asymmetrical IUGR (de Grauw *et al.*, 1986). Maternal nutrient levels are also important for fetal growth, and reduced fetal nutrition results in down-regulation of fetal growth. Furthermore, if there is a sudden reduction in the nutrient supply to the fetus the timing during gestation in which this occurs will determine the impact of the insult on fetal growth as evidenced by the Dutch Hunger Winter (See section 1.2.2; Roseboom *et al.*, 2001; Stein *et al.*, 2004). Alongside the impact of global nutrient restriction on fetal growth, many studies have shown that restriction of a specific nutrient, for example, protein impacts fetal growth. Specifically, in both rats and mice, a maternal low protein diet resulted in reduced nephron number in offspring (Woods *et al.*, 2004; Hoppe *et al.*, 2007), while in rats a reduction in cardiomyocyte number in response to maternal protein restriction has also been described (Corstius *et al.*, 2005). Restriction of micronutrients, such as vitamin D, may also impact fetal growth as maternal vitamin D levels have been shown to associate positively with birth weight (Harvey *et al.*, 2014a; discussed further in section 1.5.4). Other factors, such as maternal constraint also play a role in fetal development, as a baby's size at birth is strongly related to its mothers size (Godfrey and Barker, 1995).

The placenta plays a key role in regulation of fetal growth as placental weight and infant birth weight are positively correlated (Neumann and Carroll, 1984). Placental factors including size, blood flow, concentration gradients of nutrients, transport capacity and endocrine function will all impact fetal growth, for example, the size of the placenta will determine the surface area for nutrient transport (Fowden *et al.*, 2006b; Tarrade *et al.*, 2015). The placenta mediates transport of substances including

hormones and nutrients from the mother to the fetus, therefore if the placenta fails to deliver adequate nutrition to the developing fetus, then fetal growth will be compromised (Neumann and Carroll, 1984). This is evidenced by the fact that umbilical cord amino acid levels were lower in IUGR compared to appropriate for gestational age (AGA) infants (Cetin, 1996). Furthermore, in a rat model reduced placental transport of amino acids preceded the development of IUGR suggesting the reduced transport was a cause of IUGR rather than a consequence (Jansson *et al.*, 2006). Deletion of the placental *insulin-like growth factor* (*IGF*)*2* transcript (P0) resulted in reduced placental growth and fetal IUGR (Constancia *et al.*, 2002), alongside alterations in placental transport of glucose, calcium and methylaminoisobutyric acid, a system A substrate (see section 1.4.4; Constancia *et al.*, 2002; Constancia *et al.*, 2005; Dilworth *et al.*, 2010). This suggests that this gene is particularly important in regulation of placental nutrient transport to the fetus. Furthermore, it is possible that regulation of IGF2 via maternal nutritional signals, such as vitamin D levels, could play a role in mediating nutrient transport to the fetus.

- **Throughout gestation the fetus grows and development of all organ systems occurs. Environmental insults during this period can result in reduced birth weight and irreversible changes to organ functionality. One of the key determinants of fetal growth is the placenta.**

1.4. Placental development and function

The placenta forms a barrier between the maternal and fetal circulations, and is involved in protecting the fetus from potentially harmful substances in the maternal circulation as well as nutrient transport to the fetus. The transfer capabilities of the placenta are crucial in fetal growth and development and the placenta transfers nutrients, oxygen and hormones from the mother to the fetus, as well as waste products from the fetus to the mother. The substances transferred and the amount transferred provides information about the maternal environment to the placenta and fetus. These cues may cause adaptive changes in both the placenta and fetus which could alter the fetal growth trajectory (Gluckman, 2008). Furthermore, the placenta produces and secretes a variety of hormones, cytokines and signalling molecules. These can be released into both the fetal and maternal circulations, and regulate fetal and maternal physiology. For example, they are involved in regulation of fetal growth and development and also the maternal physiological adaptations to pregnancy (Cleal and Lewis, 2008).

1.4.1. Placental structure and development

The human placenta is a haemomonochorial placenta, characterised by its large surface area for exchange; a highly developed vasculature; and distinct maternal and fetal circulations, which lie close enough for rapid and efficient transfer of substances.

First trimester placental development (0–12 weeks)

The placenta develops from the trophectoderm of the blastocyst (Figure 1.2) and the main structure is established by 3-4 weeks of pregnancy. Around day 7 post-conception the prelacunar stage of placental development begins, with the ICM pole of the blastocyst attaching to the uterine epithelium (Figure 1.3a). Following attachment, the trophoblast cells invade into the endometrial epithelium. Invasion is aided by proteolytic enzymes secreted from the trophoblasts which degrade the epithelium. (Figure 1.3b). As invasion proceeds the trophoblast cells differentiate in mononuclear cytotrophoblasts (Figure 1.3c) which proliferate and fuse to form a double layer. The outer layer forms the multinucleated syncytiotrophoblast, which makes direct contact with the maternal blood (Bernirschke *et al.*, 2006; Figure 1.3d). Throughout gestation the cytotrophoblast cells provide a proliferative source of cells to fuse with and maintain the syncytiotrophoblast (Kliman *et al.*, 1986).

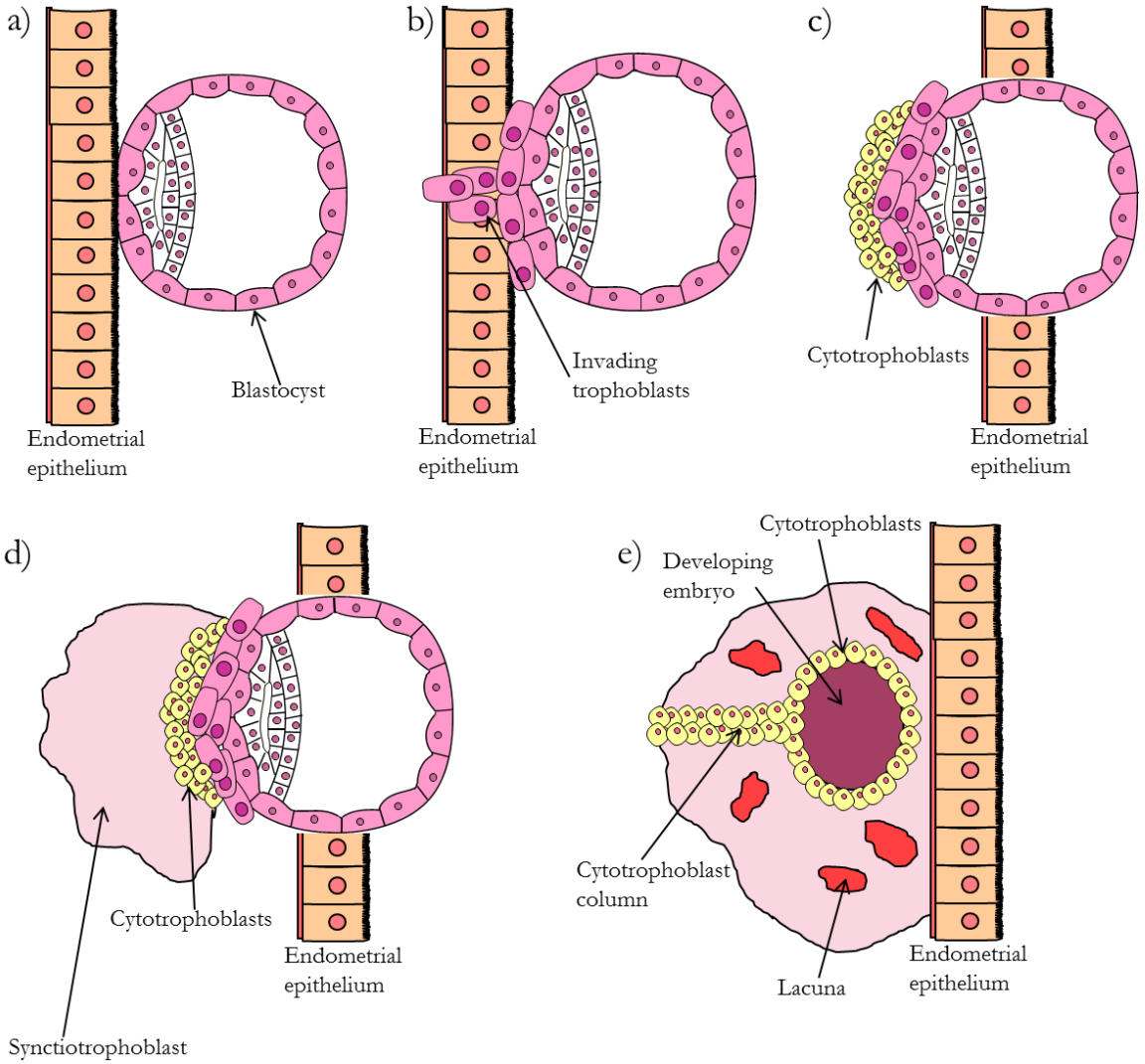

Figure 1.3: Attachment and invasion of the blastocyst into the maternal endometrium. a) Attachment. b) Invasion into the endometrium. c) During invasion mononucleated cytotrophoblasts differentiate. d) A multinucleated syncytiotrophoblast differentiates from the cytotrophoblast population. e) Lacuna develop within the syncytiotrophoblast layer. A layer of mononucleated cytotrophoblasts surrounds the developing embryo and forms columns which project through the syncytiotrophoblast. Images adapted from Dr J. Cleal.

Around day 8 post-conception the lacunar stage of placental development begins (Figure 1.3e). Lacuna are formed within the syncytiotrophoblast from small intrasyncytial vacuoles and grow to form a system of lacunae. The syncytiotrophoblast continues to expand and by the end of the lacunar phase the entire blastocystic surface is covered by syncytiotrophoblast. By day 13 post-conception the blastocyst is fully implanted into the maternal endometrium and the implantation site is closed. The thicker syncytiotrophoblast layer at the implantation site is where syncytiotrophoblast formation began and eventually forms the term placenta, while the thinner trophoblast layer at the opposite pole forms the membranes (Bernirschke *et al.*, 2006).

Lacunae formation results in three sections of trophoblast; the primary chorionic plate, the lacunar system and the trophoblastic shell. The primary chorionic plate consists of cytotrophoblast facing the developing embryo with a syncytiotrophoblast layer facing towards the lacunae. The lacunar system

consists of lacunae separated by pillars of syncytiotrophoblast called trabeculae. The trabeculae fuse at the periphery forming the trophoblastic shell facing the endometrium (Figure 1.4a). The syncytiotrophoblast trabeculae also branch into the lacunae and towards the end of the lacunar stage are invaded by cytotrophoblasts. The cytotrophoblasts form columns penetrating the length of the trabeculae. This forms the primary villus and is the first step in placental villi development. By day 15 post-conception, cytotrophoblasts reach and form part of the trophoblastic shell creating the anchoring villi (Figure 1.3e; Figure 1.4b). Extravillous cytotrophoblasts penetrate into the maternal tissue invading the maternal spiral arteries and eroding the smooth muscle to prevent arterial constriction (Cross *et al.*, 2002). During the first trimester the extravillous cytotrophoblasts also occlude the maternal spiral arteries to prevent blood flow to the placenta and fetus. The lacunae are now defined as the intervillous space, where maternal blood from the spiral arteries will be delivered later in gestation (Bernirschke *et al.*, 2006; Johnson and Everitt, 2007).

Secondary villi form when primary villi are invaded by mesenchyme from the extraembryonic tissue (Figure 1.4c). The secondary villus becomes a tertiary or mesenchymal villus upon invasion by fetal blood vessels and *de novo* blood vessel formation within the mesenchymal core of the villi. From 5 weeks post-conception the tertiary villus then differentiates into four villus types; immature intermediate villi, stem villi, terminal villi and mature intermediate villi. During the first trimester, mesenchymal villi differentiate into immature intermediate villi. The immature intermediate villi begin to form around 8 weeks of gestation and consist of a thick trophoblast layer, fluid filled channels and fetal vasculature. These will form the growth centre for the differentiation of stem villi and provide sites for nutrient exchange between the maternal and fetal circulations prior to development of the more specialised villus types. Immature intermediate villi develop into stem villi, which are characterised by dense stroma, a thick trophoblast layer and low degree of fetal capillarisation. Vessels within the stem villi differentiate into arteries and veins. These villi provide mechanical support for the villous trees which will develop in the second trimester and due to the low level of fetal capillarisation are not thought to play a large role in nutrient exchange (Bernirschke *et al.*, 2006).

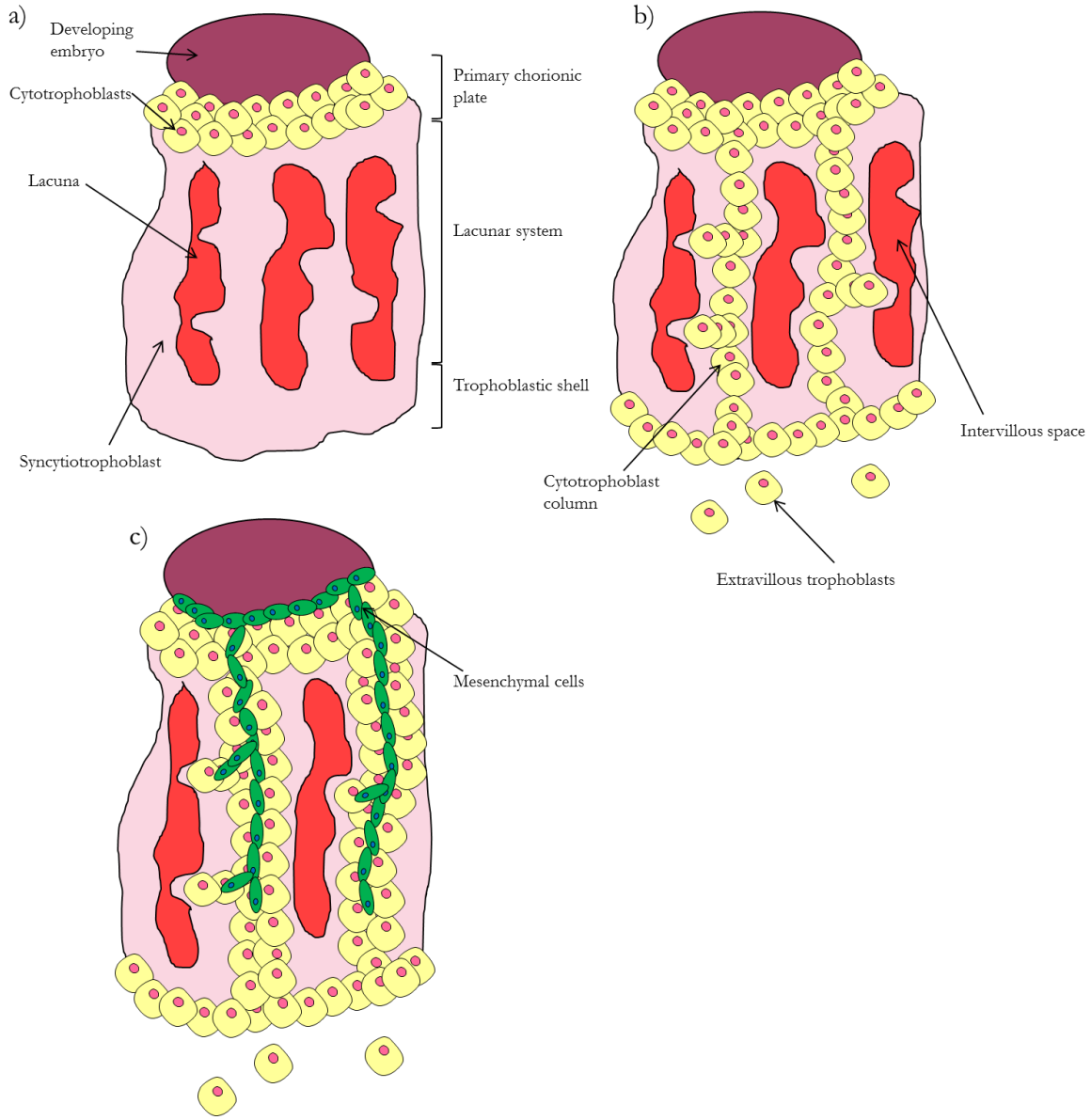

Figure 1.4: The beginning of placental villi development. a) Lacunae are separated by pillars of syncytiotrophoblast, which also project into the lacunae. b) Cytotrophoblast columns invade the syncytiotrophoblast and reach the trophoblastic shell. Cytotrophoblasts past the trophoblastic shell are defined as extravillous cytotrophoblasts. c) Mesenchymal cells of embryonic origin line the primary chorionic plate and also invade the cytotrophoblast column between the intervillous spaces.

During the first 10-12 weeks of gestation the maternal blood supply to the placenta is prevented by occlusion of the maternal spiral arteries by extravillous cytotrophoblasts. This means that initial fetal development occurs under conditions of low oxygen, possibly to protect the fetus from reactive oxygen species. During this period histiotrophic nutrition from the uterine glands secreting nutrients into the intervillous space supports the fetus. After 10-12 weeks the trophoblast plugs are dislodged, and the maternal blood supply to the placenta is fully established. Now haemotrophic support from the maternal vasculature nourishes the fetus. This is more efficient for nutrient transport which is essential for the growth and maturation of the organ systems laid down in the first trimester (Johnson and Everitt, 2007).

Second trimester placental development (13-27 weeks)

During the second trimester placental villi development continues with the formation of new villi and extensive branching of existing villi. Capillaries within the villi further develop with branching angiogenesis. At the end of the second trimester the development of new immature intermediate villi stops. From this point onwards newly formed mesenchymal villi develop into mature intermediate villi (Bernirschke *et al.*, 2006), to prepare for the increased nutrient transport required for the fetus during the final trimester of pregnancy.

Third trimester placental development (28 weeks-term)

At the start of the third trimester mature intermediate villi develop; these are long slender villi with a thin syncytiotrophoblast layer and fewer cytotrophoblast cells. The bends of their zigzag shape are where terminal villi will differentiate soon after mature intermediate villi development begins. Terminal villi have a more developed fetal vasculature with arterioles, venules and capillaries. As capillary growth exceeds longitudinal villous growth the capillaries within the terminal villi form loops resulting in grape-like projections into the intervillous space. The terminal villi are the major areas of nutrient exchange in the third trimester with the thinner syncytiotrophoblast layer in these villi providing a reduced distance, of about 3.7 μm, for nutrient exchange between the maternal and fetal circulations (Figure 1.5; Bernirschke *et al.*, 2006).

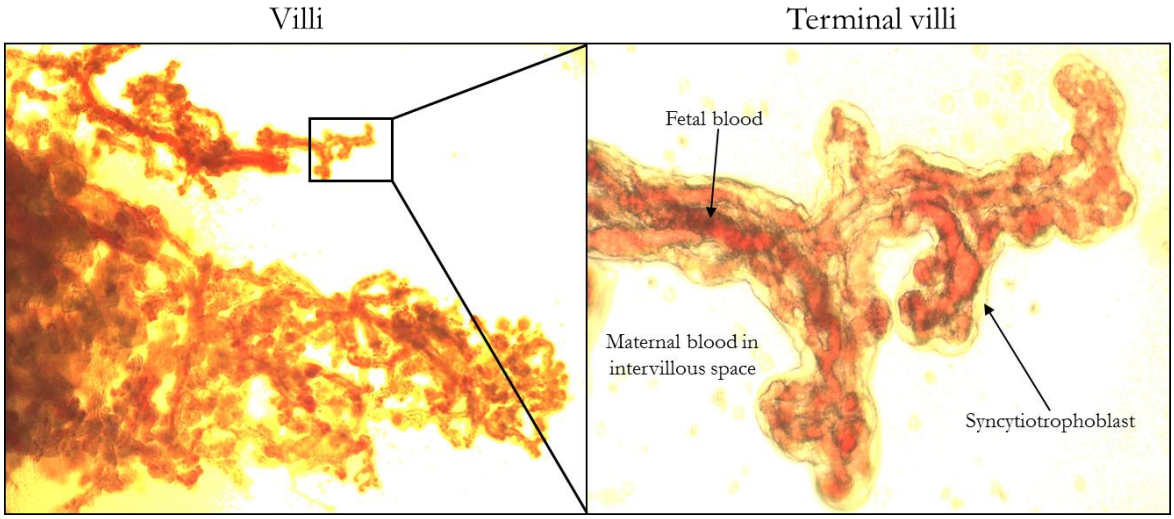

Figure 1.5: The placental villi and terminal villi. Image from Dr R. Lewis.

While development of new immature intermediate villi ceases at the end of the second trimester, their differentiation into stem villi continues, therefore at term the number of immature intermediate villi declines. In the term placenta, immature intermediate villi account for 0-5% of the total placental volume, whilst stem villi constitute 20-25% of the volume. Mature intermediate villi account for 25% of the total placental volume, and terminal villi comprise the largest volume at 40% of the total placental volume. The term placenta is composed of 20-40 lobules (Figure 1.6). These are the

functional units of the placenta, and each has their own maternal and fetal blood supplies with at least one villous tree (Johnson and Everitt, 2007).

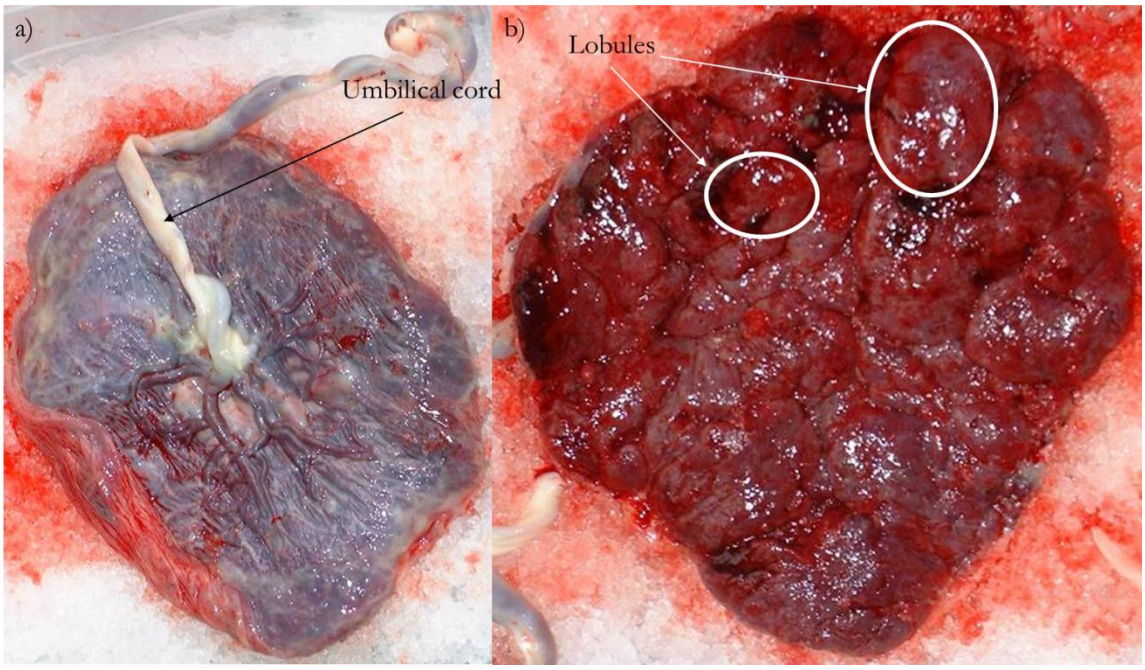

Figure 1.6: The human term placenta. a) The fetal side of the placenta with the umbilical cord. b) The maternal side of the placenta with placental lobules highlighted.

1.4.2. Placental function

The placenta forms the barrier between the maternal and fetal circulations and is crucial for both support and protection of the fetus. The placental barrier protects the fetus from potentially harmful substances in the maternal blood, for example placental 11β-hydroxysteroid dehydrogenase 2 inactivates glucocorticoids, therefore protecting the fetus from their growth inhibitory effects (Fowden and Forhead, 2004). The placenta also provides the route by which nutrients, ions, gases and hormones are transferred to the fetus and by which waste products are removed. Placental transfer of nutrients is actively regulated and the placenta is not merely a passive structure in this transfer. The placenta also metabolises nutrients, including vitamin D and amino acids, thereby altering the metabolites which reach the fetal circulation (Diaz *et al.*, 2000; Belkacemi *et al.*, 2011; Wright and Sibley, 2011). Alongside its barrier and transport functions the placenta is also an important endocrine organ, producing a range of hormones which have roles in regulation of both fetal and maternal physiology. Placentally produced human chorionic gonadotropin (hCG), human placental lactogen (hPL) and progesterone have effects on the mother; hCG supports the corpus luteum, while progesterone stimulates endometrial decidualization and hPL promotes maternal weight gain. In contrast, IGFs regulate placental and fetal growth and parathyroid hormone-related peptide (PTHrP) may play a role in regulation of placental calcium transport (see 1.4.7; McNamara and Kay, 2001). Through these

functions the placenta impacts fetal growth and development, determining the health of the fetus *in utero* and in later life.

1.4.3. Placental nutrient transport

For normal fetal growth, optimum nutrient transport via the placenta is crucial and as gestation progresses increased placental nutrient transport is required to match the increasing fetal demands (Battaglia, 2001). Nutrient transport across the placenta requires three steps (Figure 1.7). Nutrients must be transported from the maternal blood into the placental syncytiotrophoblast across the maternal-facing microvillous membrane (MVM). Once inside the syncytiotrophoblast nutrients must travel through the cytoplasm. Finally, to reach the fetal blood, nutrients must be transported across the fetal-facing basement membrane (BM). Following transport across the BM, diffusion through connective tissues and the fetal capillary endothelium allows nutrients to reach the fetal blood. Two transport systems are required for substrates to reach the fetal circulation, i.e. one for each placental membrane (Battaglia, 2001).

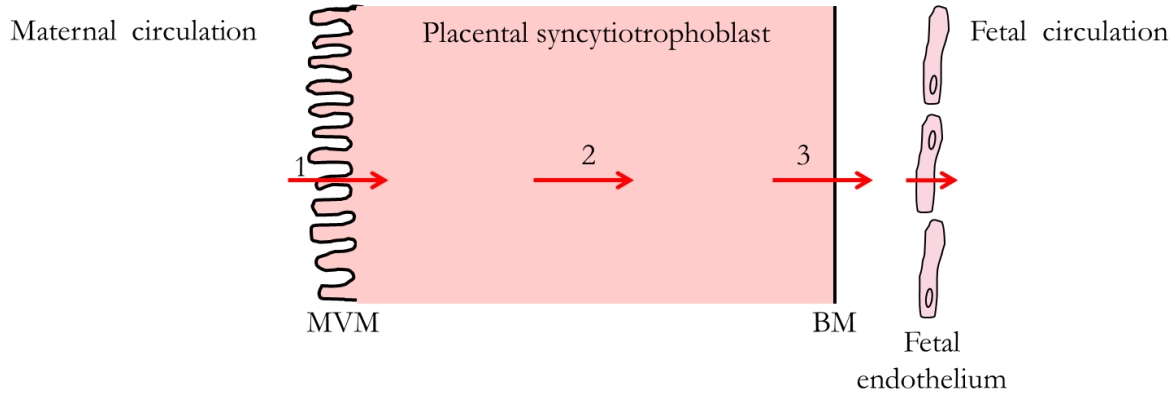

Figure 1.7: Placental nutrient transport. 1) Transport across the MVM. 2) Transport through the multinucleated placental syncytiotrophoblast. 3) Transport across the BM, through the fetal endothelium and into the fetal blood.

The placenta transports all the nutrients, ions and gases that the fetus requires. Transport of oxygen to the fetus occurs via passive diffusion. The capacity of the placenta for passive diffusion is related to the surface area for exchange, the diffusional distance and placental blood flow. With advancing gestation, placental surface area increases with formation of the terminal villi and increasing numbers of microvilli on the MVM, and the diffusional distance reduces. The maternal blood flow is also important for delivery of substances and reductions in blood flow reduce delivery of oxygen and nutrition to the fetus. Hydrophilic solutes can cross the placenta via paracellular or transcellular routes. Paracellular diffusion occurs via extracellular, fluid-filled channels within the syncytiotrophoblast. This diffusion occurs passively with no specific transport proteins required, and has been shown to occur for creatinine transfer. The transcellular route is the major transport route for a number of important nutrients, including calcium, amino acids, vitamin D, lipids and glucose. The transcellular route

involves transport across the placental syncytiotrophoblast and in a lot of cases is mediated via specific transporters. This method of transport allows tight regulation of nutrient delivery to the fetus. Transcellular transport of glucose across the placenta occurs via facilitated diffusion down a concentration gradient and is mediated by specific glucose transporters (GLUTs). Lipoproteins are transported into the placenta via lipoprotein receptors, while lipoprotein triglycerides can be hydrolysed through the action of lipoprotein lipase (LPL) in the MVM, this enzyme then releases free fatty acids which can then enter the placenta via diffusion or through receptor-mediated endocytosis (Hammad, 2000; van der Vusse, 2009; Wright and Sibley, 2011). This thesis will focus on placental transport of vitamin D (see 1.6.4), amino acids and calcium.

- **The placenta is critical for fetal growth and development. Amongst its functions, nutrient transport to the fetus is key in the placentas support of fetal growth.**

1.4.4. Placental amino acid transport

Amino acids are critical for fetal growth through their incorporation into proteins and as precursors for vital non-protein substances including, nucleotides, neurotransmitters and nitric oxide (Grillo *et al.*, 2008). Specific amino acids, including the essential amino acids which the fetus cannot synthesise, will be required in greater amounts. Therefore, adequate amino acid transport will be determined not only by the level of placental transfer but also the composition transferred to the fetal circulation (Cleal and Lewis, 2008). Furthermore, the placenta metabolises amino acids which will alter the amount and types of amino acids available for transport into the fetal circulation. In the sheep placenta maternal and fetal serine are converted to glycine and this glycine, rather than maternal glycine, is transported into the fetal circulation (Geddie *et al.*, 1996; Chung *et al.*, 1998), while in the human placenta glutamate from both the maternal and fetal circulations is metabolised into glutamine within the placenta (Day *et al.*, 2013).

Amino acid transport is a determinant of fetal growth. Inadequate amino acid transport has been implicated in IUGR with reduced fetal plasma amino acid concentrations observed in growth restricted pregnancies (Cetin, 1996). Furthermore, a reduction in amino acid transporter expression and activity has been found in human IUGR placentas (Paolini *et al.*, 2001; Jansson *et al.*, 2002; Mando *et al.*, 2013). Animal models have revealed similar findings with placentas from a maternal low protein rat model displaying reduced placental System A amino acid transport before the onset of IUGR. Thus supporting the notion that down-regulation of amino acid transport may be a cause of IUGR (Jansson *et al.*, 2006).

Amino acid concentrations are higher in the fetal than the maternal circulation, indicating that active transport is required for amino acids to reach the fetus (Cetin *et al.*, 2005). There are three main classes of amino acid transporters; accumulative transporters, amino acid exchangers and facilitated efflux transporters (Figure 1.8). Accumulative transporters mediate influx of amino acids, therefore they bring amino acids into the syncytiotrophoblast layer, but are unable to mediate efflux into the fetal circulation (Figure 1.8). Exchangers switch the cellular positions of two amino acids, therefore can switch an amino acid in the syncytiotrophoblast layer with one in the fetal circulation. This enables amino acid composition to be altered without any osmotic effects, but does not increase overall fetal amino acid levels. Facilitated efflux transporters mediate efflux of amino acids from the cell down their concentration gradient (Figure 1.8). The amino acid transport systems expressed within the human placenta are presented in Table 1.1.

Table 1.1: Amino acid transport systems in the placenta.

Transporter (gene name)	Membrane localisation	Mechanism of transport	Substrates
Accumulative transporters			
EAAT1 (*SLC1A3*)	Activity: MVM, BM	Accumulative transporter $3Na^+/1H^+/AA$-cotransport/$1K^+$-exchange	Glu, Asp
EAAT2 (*SLC1A2*)	Activity: MVM, BM	Accumulative transporter $3Na^+/1H^+/AA$-cotransport/$1K^+$-exchange	Glu, Asp
EAAT3 (*SLC1A1*)	Activity: MVM, BM	Accumulative transporter $3Na^+/1H^+/AA$-cotransport/$1K^+$-exchange	Glu, Asp, Cys
SNAT1 (*SLC38A1*)	mRNA Activity: MVM, BM	Accumulative transporter 1 Na^+/AA cotransporter	Ala, Ser, Cys, Gln, Asn, His
SNAT2 (*SLC38A2*)	mRNA Activity: MVM, BM	Accumulative transporter 1 Na^+/AA cotransporter	Gly, Pro, Ala, Ser, Cys, Gln, Asn, His, Met
SNAT3 (SLC38A3)	mRNA Activity: MVM		Gln, His, Ala, Asn
SNAT4 (*SLC38A4*)	mRNA Activity: MVM, BM	Accumulative transporter 1 Na^+/AA cotransporter	Gly, Ala, Ser, Cys, Asn
SNAT5 (SLC38A5)	mRNA Activity: MVM Protein: MVM		Gln, Asn, His, Ser
Exchangers			
ASCT1 (*SLC1A4*)	Activity: BM	Na^+-dependent exchanger	Ala, Ser, Cys
ASCT2 (*SLC1A5*)	Activity: BM	Na^+-dependent exchanger	Ala, Ser, Cys, Thr, Gln
LAT1/4F2hc (*SLC7A5*)	Activity: MVM, BM	Exchanger	His, Met, Leu, Iso, Val, Phe, Tyr, Trp
LAT2/4F2hc (*SLC7A8*)	Activity: MVM, BM	Exchanger	Ala, Ser, Cys, Thr, Asn, Gln, His, Met, Leu, Iso, Val, Phe, Tyr, Trp
xCT/4F2hc (*SLC7A11*)	mRNA Activity: MVM	Exchanger	Cys, Glu
y^+LAT1 (*SLC7A7*)	mRNA Activity: MVM, BM	Na^+-dependent exchanger	Lys, Arg, Gln, His, Met, Leu
y^+LAT2 (*SLC7A6*)	mRNA Activity: MVM, BM	Na^+-dependent exchanger	Lys, Arg, Gln, His, Met, Leu, Ala, Cys
Facilitated transporters			
LAT3 (*SLC43A2*)	mRNA Activity: BM	Na^+-independent facilitated diffusion	Phe, Leu, Iso, Met, Val
LAT4 (*SLC43A2*)	mRNA Activity: BM	Na^+-independent facilitated diffusion	Phe, Leu, Iso, Met
TAT1 (*SLC16A10*)	mRNA Activity: BM	Na^+-independent facilitated diffusion	Phe, Trp, Tyr

Modified from Cleal and Lewis (2008). System xc^- (unpublished observations by Dr E. Lofthouse). SNAT3 and SNAT5 from Day *et al.* (2013). Messenger ribonucleic acid (mRNA). Alanine/serine/cysteine/threonine transporter (ASCT); excitatory amino acid transporter (EAAT); L-type amino acid transporter (LAT); sodium dependent neutral amino acid transporter (SNAT); T-type amino acid transporter (TAT). L-alanine (Ala), L-arginine (Arg), L-asparagine (Asn), L-aspartate (Asp), L-cysteine (Cys), L-glutamate (Glu), L-glutamine (Gln), glycine (Gly), L-histidine (His), L-isoleucine (Iso), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser), L-threonine (Thr), L-tryptophan (Trp), L-tyrosine (Tyr), L-valine (Val).

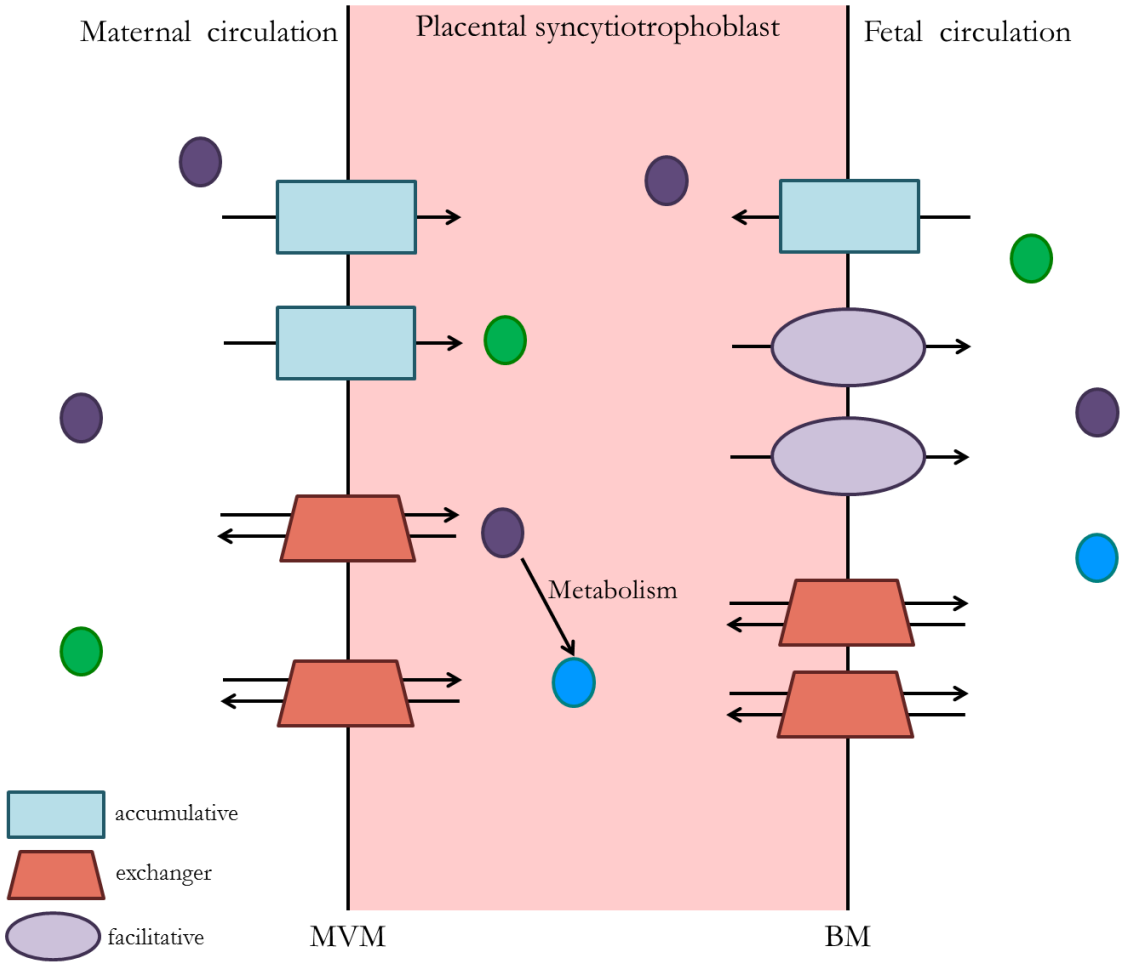

Figure 1.8: Placental amino acid transport. Accumulative transporters bring amino acids into the placental syncytiotrophoblast. Inside the syncytiotrophoblast these amino acids can be metabolised. On the BM accumulative transporters bring amino acids from the fetal circulation back into the placental syncytiotrophoblast, while exchangers on the BM and MVM switch amino acids. Facilitated transporters mediate efflux of amino acids from the syncytiotrophoblast into the fetal circulation.

Transport across the MVM

Transport of amino acids from the maternal circulation into the placenta requires active transport across the MVM, as amino acid concentrations are higher within the placenta than in the maternal blood. Accumulative transport by system X^{AG} provides glutamate and aspartate, while system A mediates uptake of small neutral amino acids. Activity of system A (SNAT1, SNAT2 and SNAT4) is likely to be important as this provides substrates for the exchangers. Activities of the exchange systems L (LAT1 and LAT2) and y^+L (y^+LAT1 and y^+LAT2) can then alter the composition of amino acids within the placental syncytiotrophoblast using the small neutral amino acids supplied by system A as substrates (Cleal and Lewis, 2008; Figure 1.9).

Transport across the BM

Net efflux of amino acids from the placenta into the fetal circulation is mediated via the facilitated transporters LAT3, LAT4 and TAT1 (Cleal *et al.*, 2011). These mediate transport of substrates down their concentration gradient, and as amino acid concentrations are lower in the fetal circulation than in

the placenta, they result in efflux of alanine, leucine, phenylalanine, isoleucine, tyrosine, valine and methionine into the fetal circulation. While these transporters cannot supply the fetus with all of the amino acids it requires, these amino acids within the fetal circulation can then serve as substrates for exchangers on the placental BM. Using this mechanism of facilitated transporters and exchangers the fetus can obtain all the essential amino acids from placental transport. Functional evidence using human placental perfusion has shown activity of the exchangers LAT1, ASCT2 and y^+LAT on the placental BM (Cleal *et al.*, 2007). The function of accumulative transporters on the BM of the placenta is unclear as they will result in transport of amino acids from the fetus and into the placenta. However, system X^{AG} mediates uptake of fetal glutamate for metabolism in the placenta (Day *et al.*, 2013; Lewis *et al.*, 2013a; Figure 1.9).

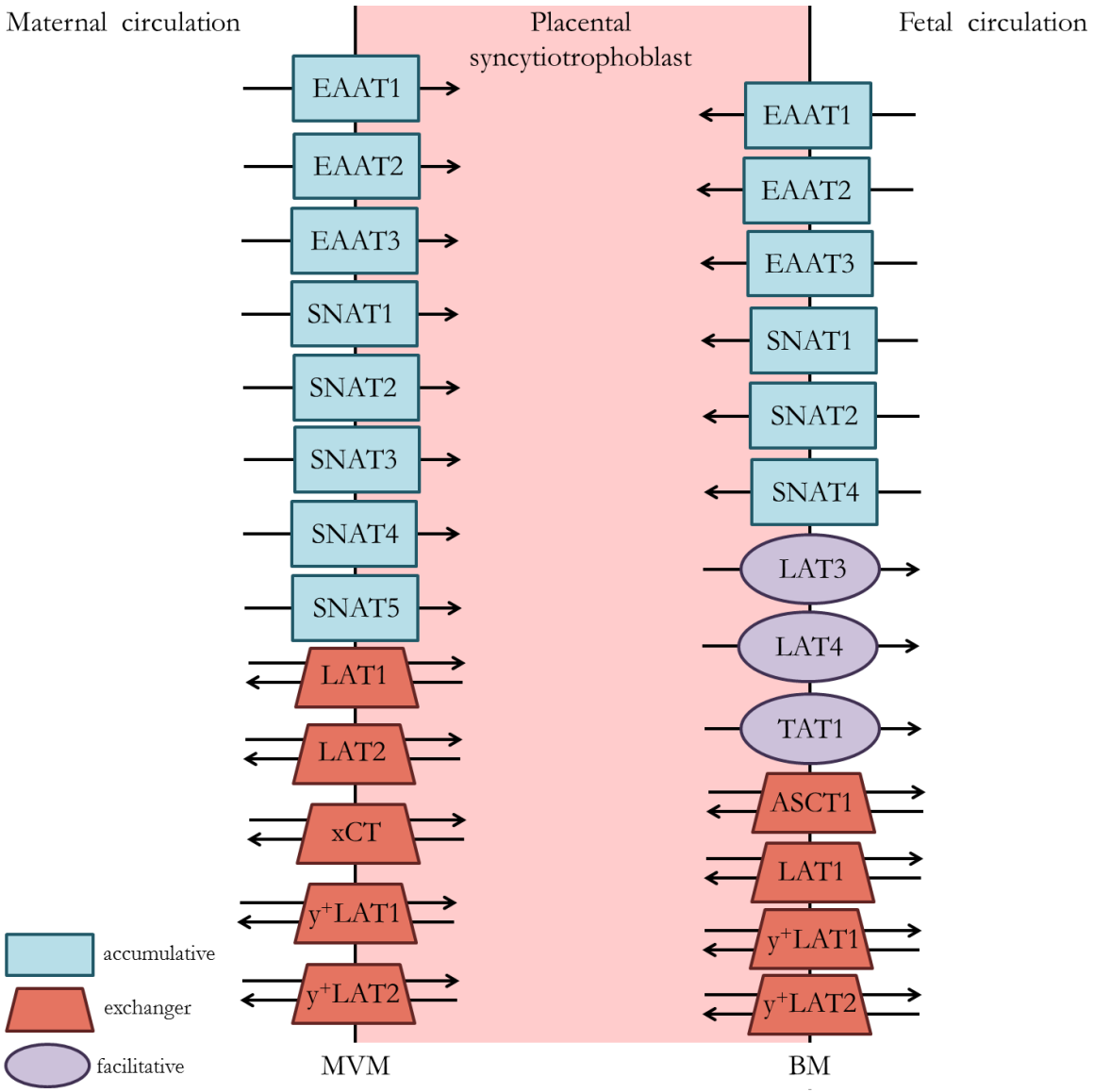

Figure 1.9: Amino acid transporter localisation on the MVM and BM of the placental syncytiotrophoblast (Cleal *et al.*, 2007; Cleal and Lewis, 2008; Cleal *et al.*, 2011; Day *et al.*, 2013).

1.4.5. Placental calcium transport

Calcium is crucial for development of the fetal skeleton. The human fetus accumulates a total of 30 g of calcium throughout gestation, 80% of which is accrued during the third trimester when skeletal mineralisation occurs. To meet fetal calcium demands maternal bone resorption and intestinal calcium absorption are increased (Goodfellow *et al.*, 2011). Calcium transport increases when placental cytotrophoblasts differentiate into syncytiotrophoblasts, highlighting the importance of the placental syncytiotrophoblast in calcium transport to the fetus (Belkacemi *et al.*, 2003). In addition, placental calcium transport is thought to increase in the third trimester to meet the increased fetal demand for calcium (Husain and Mughal, 1992). Reduced calcium transport to the fetus has been associated with impaired bone development. Specifically, lower cord blood calcium levels were associated with reduced BMC of both the whole body and lumbar spine, reduced whole body BA and reduced lumbar spine BMD of children at 9 years of age (Javaid *et al.*, 2006). Furthermore, supplementation of pregnant women with low calcium intake resulted in infants with a higher BMC than those who were not supplemented (Koo *et al.*, 1999).

Transport across the MVM

Transport of calcium from the maternal circulation across the MVM is mediated via the calcium transporters, calcium transporter (CaT)1 and CaT2, also known as TRPV6 and TRPV5, respectively. These transporters mediate transport of calcium down its concentration gradient. As calcium levels are lower within the placental syncytiotrophoblast compared to the maternal circulation this results in transport of calcium into the placenta. CaT1 and CaT2 therefore constitute the rate limiting step in calcium absorption into the placenta. *CaT1* and *CaT2* mRNA has been detected in placental syncytiotrophoblasts with expression of *CaT1* higher than that of *CaT2* (Moreau, 2002b, a). In addition, immunohistochemistry and western blotting has localised CaT1 and CaT2 mainly to the placental MVM (Bernucci *et al.*, 2006; Figure 1.10). Dose-dependent inhibition of calcium uptake was observed in human placental trophoblasts in response to specific inhibitors of CaTs (Moreau, 2002b), providing support for the importance of these transporters in calcium influx into the placenta.

Transport through the syncytiotrophoblast

While amino acids are thought to diffuse freely across the placental syncytiotrophoblast, the movement of calcium across this layer is thought to be facilitated by calcium binding proteins. Calbindin-D9K and calbindin-D28K may be particularly important for transfer of calcium across the placental syncytiotrophoblast (Figure 1.10). These calcium binding proteins are thought to act both as shuttles for calcium and in a buffering capacity. In the human syncytiotrophoblast the movement of large quantities of calcium would result in calcium fluxes that would disrupt cellular processes. Transport of calcium associated with binding proteins prevents such disturbances. Calbindin-D9K and calbindin-

D28K can bind more than one calcium molecule at a time, creating a high capacity system for shuttling of calcium across the syncytiotrophoblast (Belkacemi *et al.*, 2002). Expression of both calbindin-D9K and calbindin-D28K has been demonstrated at the mRNA and protein level in human placental cells (Belkacemi *et al.*, 2003, 2004). In addition, calbindin-D28K associates with CaT1 and CaT2 to create an effective transport system for calcium. When the association between CaT and calbindin-D28K is disrupted, calcium buffering and transcellular transport is impaired. This association is also thought to prevent inactivation of CaTs so that a high level of calcium entry to the cell is maintained (Lambers *et al.*, 2006). The importance of these calcium binding proteins in placental calcium transfer is highlighted by the fact that in rat and sheep placenta, increased expression of calbindin-D9K is observed alongside the increased fetal demand for calcium in late gestation (Glazier *et al.*, 1992; Morgan *et al.*, 1997).

Transport across the BM

Calcium concentrations are higher in the fetal blood compared to the placental syncytiotrophoblast, therefore extrusion of calcium into the fetal blood requires active transport. The primary mechanism of calcium transport across the placental BM is likely to occur via plasma membrane calcium ATPases (PMCAs; Figure 1.10). PMCAs mediate adenosine triphosphate (ATP) driven active transport of calcium out of the cell. Four isoforms of PMCA are known, PMCA1-4 (Belkacemi *et al.*, 2005a). Of these isoforms, PMCA1 and PMCA4 have been shown to be expressed within the placenta (Santiago-Garcia *et al.*, 1996; Moreau *et al.*, 2003a; Moreau *et al.*, 2003b; Martin *et al.*, 2007), while PMCA2 has been detected at low levels (Moreau *et al.*, 2003a; Martin *et al.*, 2007). *PMCA3* mRNA expression has been detected in some studies (Moreau *et al.*, 2003a; Martin *et al.*, 2007), but not in others (Santiago-Garcia *et al.*, 1996). PMCA expression has been localised to the BM of the placenta (Borke *et al.*, 1989; Strid and Powell, 2000), although one study showed higher expression of PMCA on the MVM compared to the BM (Marin *et al.*, 2008). Human trophoblast cells exhibit dose-dependent reduction in calcium efflux in response to increasing concentrations of erythrosine B, a PMCA inhibitor (Moreau *et al.*, 2003a), demonstrating the importance of these transporters for calcium efflux from the placenta.

A second type of transporter also plays a role in efflux of calcium across the placental BM into the fetal circulation; the sodium-calcium exchangers (NCXs; Figure 1.10). These transporters mediate the exchange of sodium and calcium across the membrane, and expression has been demonstrated on the BM and not the MVM of the human placenta (Kamath and Smith, 1994). There are three isoforms of the NCX transporters, NCX1-3. Expression of NCX1 and NCX3 has previously been demonstrated in human placenta (Moreau *et al.*, 2003a; Moreau *et al.*, 2003b). There is currently no evidence for the presence or absence of NCX2 within the human placenta. The significance of the contribution of NCX on the placental BM is unclear as when human placental trophoblasts were cultured in sodium free medium there was no difference in calcium efflux compared to those cultured in sodium containing medium (Moreau *et al.*, 2003a). NCXs are a low affinity but high capacity system for calcium

transport that can respond rapidly to changes in cellular calcium levels, so it is probable that under basal conditions PMCAs mediate the bulk of calcium transport to the fetus while NCXs mediate calcium efflux in response to large changes in intracellular calcium (Khananshvili, 2013).

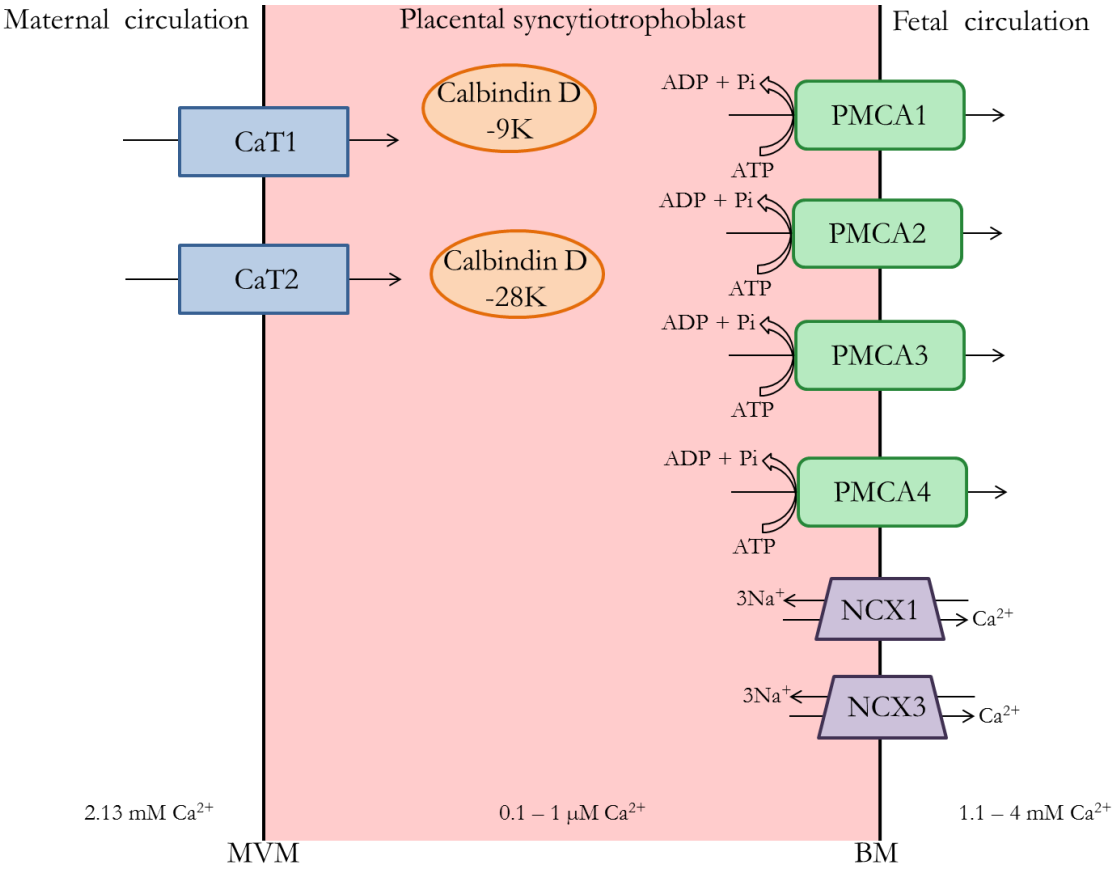

Figure 1.10: Calcium transporter expression in the placental syncytiotrophoblast. Modified from Belkacemi *et al.* (2002).

1.4.6. Placental nutrient transport and fetal growth

Placental amino acid transport and fetal growth

Placental system A amino acid transport was positively correlated with pre-pregnancy upper arm muscle area, and was lower in mothers who reported undertaking strenuous exercise. This is suggestive of nutrient conservation in favour of the mother. Placental system A activity was also negatively correlated with birth to placental weight ratio (Lewis et al., 2010a). Placental mRNA expression levels of facilitated transporters were associated with various fetal outcomes in samples from the SWS. Specifically, placental *TAT1* mRNA levels were correlated with increased birth weight, neonatal lean mass and head circumference. While placental *LAT3* mRNA levels were correlated with increased head circumference (Cleal *et al.*, 2011). In the same cohort, lean mass at 4 years of age was positively associated with placental mRNA expression of *LAT3*, *TAT1* and *y+LAT1*, whilst BMD was positively associated with *LAT4* and negatively associated with *ASCT2* and *EAAT3* (Cleal et al., 2015). In support of the relationship between these transporters and fetal growth, phenylalanine, a substrate of

both TAT1, LAT3 and LAT4, and leucine, a LAT3 and LAT4 substrate showed reduced placental transfer *in vivo* in pregnancies complicated by IUGR (Paolini *et al.*, 2001), implicating a potential role for these transporters in fetal growth restriction. Furthermore, *TAT1* mRNA levels were significantly reduced in early third trimester IUGR placental samples compared to AGA samples (Loubiere *et al.*, 2010). Placental transport of amino acids across the MVM was also altered in placentas from IUGR infants. Uptake of methylaminoisobutyric acid, a system A substrate, was significantly reduced in MVM from IUGR placentas compared to MVM from AGA infants (Mahendran *et al.*, 1993; Jansson *et al.*, 2002). These studies show that placental amino acid transport to the fetus is vital for optimal fetal growth.

Placental calcium transport and fetal growth

Placental calcium transporter expression has also been positively associated with fetal growth. In SWS placentas, mRNA expression of the calcium efflux transporter, *PMCA3*, was positively associated with BA, BMC, birth weight and lean mass of the neonate at birth (Martin *et al.*, 2007). Further support for the importance of placental calcium transport comes from a study which showed a reduction in PMCA protein on BM preparations from IUGR placentas compared to placentas from AGA infants (Strid *et al.*, 2003). Calcium transport was also reduced in primary syncytiotrophoblasts from pre-eclamptic placentas. Pre-eclampsia is a pregnancy-specific condition characterised by hypertension and proteinuria, alongside the complications for the mother pre-eclampsia can also result in fetal growth restriction (FGR). Pre-eclampsia is proposed to occur due to abnormal implantation and is associated with abnormal placental development and function, with pathophysiologies including shallow trophoblast invasion, inadequate spiral artery remodelling, reduced placental perfusion, oxidative stress and increased inflammation (Bodnar *et al.*, 2007b; Acharya *et al.*, 2014). Calcium transport was 60% lower in syncytiotrophoblasts from pre-eclamptic placentas compared to placentas from normal pregnancies. In addition to this reduction in calcium transport there was reduced mRNA and protein expression of CaT1 and CaT2, calbindin-D9K, calbindin-D28K, PMCA1 and PMCA4 (Hache *et al.*, 2011).

1.4.7. Regulation of placental nutrient transport

As altered expression of placental nutrient transporters has been associated with alterations in fetal growth and bone development, this raises the question of what causes the alterations in transporter expression within the placenta. The placenta is responsive to numerous stimuli including, hormones, glucocorticoids, nutrients and factors such as leptin and IGF1. Self-regulation of placental nutrient transport is also proposed as the placenta may act as a 'nutrient sensor', perceiving nutrient availability in the syncytiotrophoblast, maternal and fetal circulations, and adjusting transport accordingly. In cases of low maternal nutrition, this can result in a decrease in placental nutrient transfer to a level more

sustainable from the maternal perspective, but results in nutrient, and therefore growth, restriction in the fetus (Jansson and Powell, 2006).

Regulation of placental amino acid transport

The regulation of placental amino acid transport could occur through endocrine mechanisms. The placenta produces and also responds to hormones and placental amino acid uptake has been shown to be regulated by IGF1, growth hormone, leptin and cortisol (Kniss *et al.*, 1994; Jansson, 2003; Ericsson *et al.*, 2005; Jones *et al.*, 2006). Furthermore, a maternal low protein rat model which showed reduced system A transporter activity also showed reduced maternal insulin and IGF1 levels, implicating this as a possible mechanism through which transporter activity may be regulated (Jansson *et al.*, 2006). Similar regulatory mechanisms may occur in human placenta as lower umbilical cord levels of IGF1 were present in fetuses with a lower placental weight (Godfrey, 2002). However, *in vitro* studies in cultured human primary trophoblast and BeWo, a choriocarcinoma cell line, have shown that amino acid uptake was enhanced by physiological levels of IGF1 (Karl, 1995; Fang *et al.*, 2006). It is possible that placental amino acid transport is only down-regulated when IGF1 levels are elevated above a threshold. Glucocorticoids may also regulate placental amino acid transport. 24 h exposure to glucocorticoids increased system A activity in BeWo cells (Jones *et al.*, 2006). However, this finding was not replicated in human first trimester placental villous fragments (Ericsson *et al.*, 2005) and animal studies have shown that administration of synthetic glucocorticoids inhibits fetal growth (Sloboda *et al.*, 2000).

It should be noted, that many studies on regulation of placental amino acid transport report altered placental amino acid uptake. Little is known about the regulation of amino acid efflux from the placenta via TAT1, LAT3 and LAT4. However, there is some evidence to suggest they may be regulated by hormonal and nutritional factors. Treatment of rabbits with thyroid hormone resulted in a down-regulation of *TAT1* mRNA in skeletal muscle (Mebis *et al.*, 2009), whilst *LAT3* mRNA increased in murine liver and skeletal muscle in response to 24 h starvation, which was proposed to occur due to increased levels of branched chain amino acids (Fukuhara *et al.*, 2007).

There is currently little evidence to suggest whether or not vitamin D may play a role in the regulation of placental amino acid transport, however there are some clues that vitamin D could play a role. Links between vitamin D and amino acid transport were observed in the 1950s when children with vitamin D deficient rickets were also shown to suffer from aminoaciduria, involving increased excretion of a specific selection of amino acids, namely, threonine, serine, glycine, alanine, histidine, lysine and glutamate. Furthermore, aminoaciduria reduced following vitamin D supplementation (Jonxis *et al.*, 1952; Jonxis and Huisman, 1953). This phenomenon was also observed in a model of vitamin D deficiency, where increased urinary excretion of taurine and proline occurred. This was shown to be a result of reduced renal absorption of the amino acids and was corrected upon administration of

vitamin D supplementation (Dabbagh *et al.*, 1990). Furthermore, administration of vitamin D and retinoic acid to a porcine kidney cell line resulted in up-regulation of a taurine transporter, TauT, reporter construct. The up-regulation of TauT was also observed within the cell at the protein level following exposure to vitamin D and retinoic acid. This response was not seen in a human breast cancer cell line, suggesting that the response may be tissue-specific (Chesney and Han, 2013). Additional support for the potential regulation of placental amino acid transport by vitamin D comes from the fact that vitamin D response elements (VDREs) are present within the promoter regions of transporters such as *LAT3* and *ASCT1* (Wang *et al.*, 2005).

Regulation of placental calcium transport

Several factors have been proposed to play a role in the regulation of placental calcium transfer including PTHrP, oestrogen, calcitonin and vitamin D (Kovacs and Kronenberg, 1997). In the non-pregnant state parathyroid hormone (PTH) plays a key role in calcium homeostasis. However, in pregnancy PTH levels fall and PTHrP levels rise (Ardawi *et al.*, 1997) suggesting that PTHrP plays a more dominant role in calcium homeostasis during pregnancy. In sheep removal of PTHrP by thyroparathyroidectomy resulted in loss of the placental calcium gradient, furthermore injection of PTHrP into hypothyroidectomised sheep increased placental calcium transport (Care *et al.*, 1986). In support of the role of fetal PTHrP, PTHrP null mice fetuses showed significantly reduced levels of calcium as well as reversal of the maternofetal calcium gradient. Furthermore placental transfer of radiolabelled calcium was significantly reduced. This reduction in placental calcium transfer was reversed upon administration of PTHrP or specific PTHrP fragments to the fetus (Kovacs *et al.*, 1996). PTHrP has also specifically been shown to dose-dependently increase PMCA activity in human placental BM (Strid *et al.*, 2002), suggesting that PTHrP may regulate placental calcium transport by increasing calcium efflux from the placental syncytiotrophoblast and into the fetal circulation. Both PTH and PTHrP act via the same receptor, PTH 1 receptor (PTH1R). However, in PTH1R null fetuses, placental calcium transfer was increased despite the apparent inability of the placenta to respond to PTHrP (Kovacs *et al.*, 1996), suggesting that PTHrP may also act via another as yet unidentified receptor.

As with PTHrP, the concomitant rise in maternal active vitamin D with placental calcium transport across gestation has implicated vitamin D as having a role in regulation of placental calcium transport. In sheep fetal nephrectomy resulted in reduced vitamin D levels and consequently the fetus became hypocalcemic following reduced placental calcium transport (Belkacemi *et al.*, 2002). In contrast, in rats fetal nephrectomy had no impact on the maternofetal calcium gradient (Chalon and Garel, 1985). Calcium uptake was increased in JEG-3 cells, a placental choriocarcinoma cell line, upon administration of active vitamin D (Tuan *et al.*, 1991). Furthermore, transfer of radiolabelled calcium was positively associated with placental vitamin D receptor (VDR) protein expression in humans

(Young *et al.*, 2014). Both calbindin D-9K and CaT1 possess VDREs within their promoter regions (Jeung *et al.*, 1994; Wang *et al.*, 2005), suggesting regulation by vitamin D.

Ex vivo studies using human duodenal biopsies have shown increased mRNA expression of *CaT1* and *PMCA1* measured by quantitative reverse transcription polymerase chain reaction (qrt-PCR) in response to active vitamin D (Balesaria *et al.*, 2009), while *CaT1* mRNA in human duodenal samples was positively correlated with patients active vitamin D level (Walters *et al.*, 2007). *Calbindin-D9K* showed no change in mRNA expression in response to active vitamin D in these studies, however, in an intestinal cell line both *CaT1* and *calbindin-D9K* showed increased mRNA expression in response to vitamin D treatment (Cotter and Cashman, 2006). Human placental cells cultured with active vitamin D also showed increased mRNA expression of both *calbindin-D9K* and *calbindin-D28K* (Halhali *et al.*, 2010). Furthermore, JEG-3 cells showed increased expression of *calbindin-D28K* in response to treatment with active vitamin D. This increased expression has inhibited by actinomycin D, an RNA synthesis inhibitor, and by a VDR antagonist, demonstrating that vitamin D actions on mRNA expression were VDR-mediated and occurred at the level of gene transcription (Belkacemi *et al.*, 2005b). PMCA1 and PMCA4 protein expression has also been shown to be increased by vitamin D in a canine kidney cell line. This increase in protein expression manifested as an increased calcium flux from the apical to the basolateral compartment of vitamin D treated cells (Kip and Strehler, 2004).

However, some studies suggest there may be no link between placental calcium transport and vitamin D. For example, supplementation of pregnant women with vitamin D did not result in changes to cord blood calcium concentrations (Dawodu *et al.*, 2013). In addition, a meta-analysis which looked at the effect of maternal vitamin D supplementation found no overall effect of supplementation on cord blood calcium levels. Although, when data were separated into daily and weekly/monthly dosing regimens, a positive effect of weekly/monthly supplementation on cord blood calcium was observed (Yang *et al.*, 2015). In addition, *VDR*$^{-/-}$ mouse fetuses showed no difference in placental calcium transfer or fetal calcium levels compared to *VDR*$^{+/+}$ fetuses (Kovacs *et al.*, 2005), suggesting that fetal and placental *VDR* expression and therefore vitamin D action is not paramount for placental calcium transfer to the fetus.

The above studies have shown potential effects of vitamin D on expression of genes involved in transport of calcium across both membranes of the placenta as well on calcium binding proteins important for translocation of calcium across the placental syncytiotrophoblast. A number of these studies have used intestinal and renal cell lines therefore it is unclear whether the same effects will be observed within the human placenta. In addition, a number of the studies used reverse-transcription PCR (rt-PCR) followed by gel electrophoresis to quantify mRNA expression, whereas changes to mRNA expression can be more accurately assessed by qrt-PCR.

- **Placental calcium and amino acid transport to the fetus is crucial for fetal growth and development. Human epidemiology studies as well as data from animal studies and *ex vivo* cell culture studies support the notion that vitamin D may play a role in regulation of nutrient transport in the placenta. As actions of vitamin D may be tissue-specific we need to establish whether these effects also occur in the human placenta.**

1.5. Vitamin D

1.5.1. Vitamin D activation and metabolism

Maternal vitamin D is derived from two sources (Figure 1.11); vitamin D_2 (ergocalciferol) is derived solely from dietary sources while vitamin D_3 (cholecalciferol) is obtained from both ultraviolet (UV) exposure and dietary sources. In humans vitamin D_3 is three times as potent as D_2 (Shin *et al.*, 2010). Vitamin D_3 is formed from the action of UVB on 7-dehydrocholesterol in the skin. Vitamin D is then hydroxylated in the liver to form 25-hydroxyvitamin D (25(OH)D). A number of 25-hydroxylase enzymes have been identified; cytochrome P450 (CYP)27A1, CYP2R1, CYP2J2 and CYP3A4 (Zhu and DeLuca, 2012). Further hydroxylation in the kidneys through 1α-hydroxylase (CYP27B1) forms the biologically active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)2D; Figure 1.12). While the kidney is regarded as the major site of this hydroxylation, CYP27B1 is present in other tissues including the colon (Tangpricha *et al.*, 2001), prostate, skin (Schwartz *et al.*, 1998) and placenta (Tanaka *et al.*, 1979). 24-hydroxylase (CYP24A1) acts on both 25(OH)D and $1,25(OH)_2D$ and catalyses the formation of the less active metabolites, $24,25(OH)_2D$ and $1,24,25(OH)_3D$, respectively (Shin *et al.*, 2010).

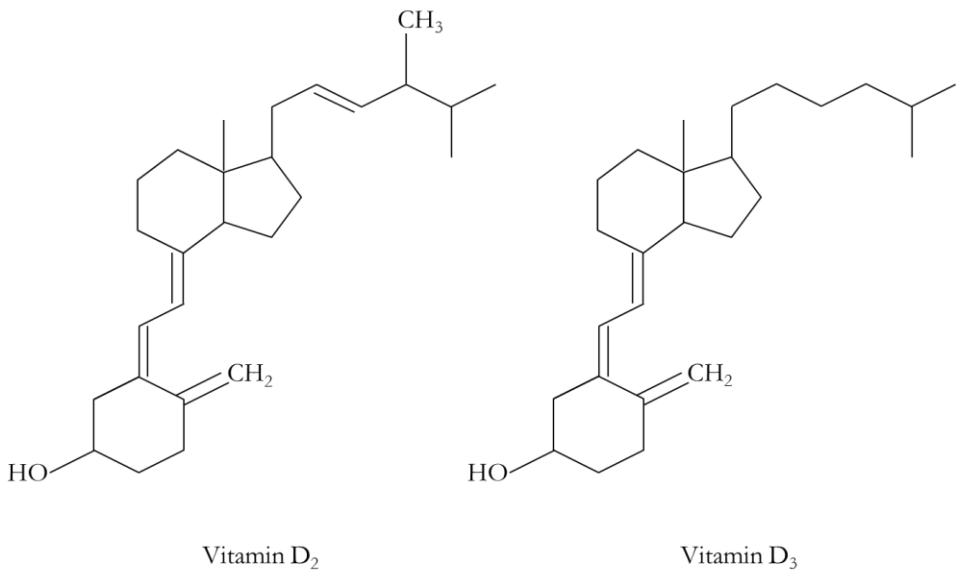

Figure 1.11: Chemical structures of vitamins D_2 and D_3.

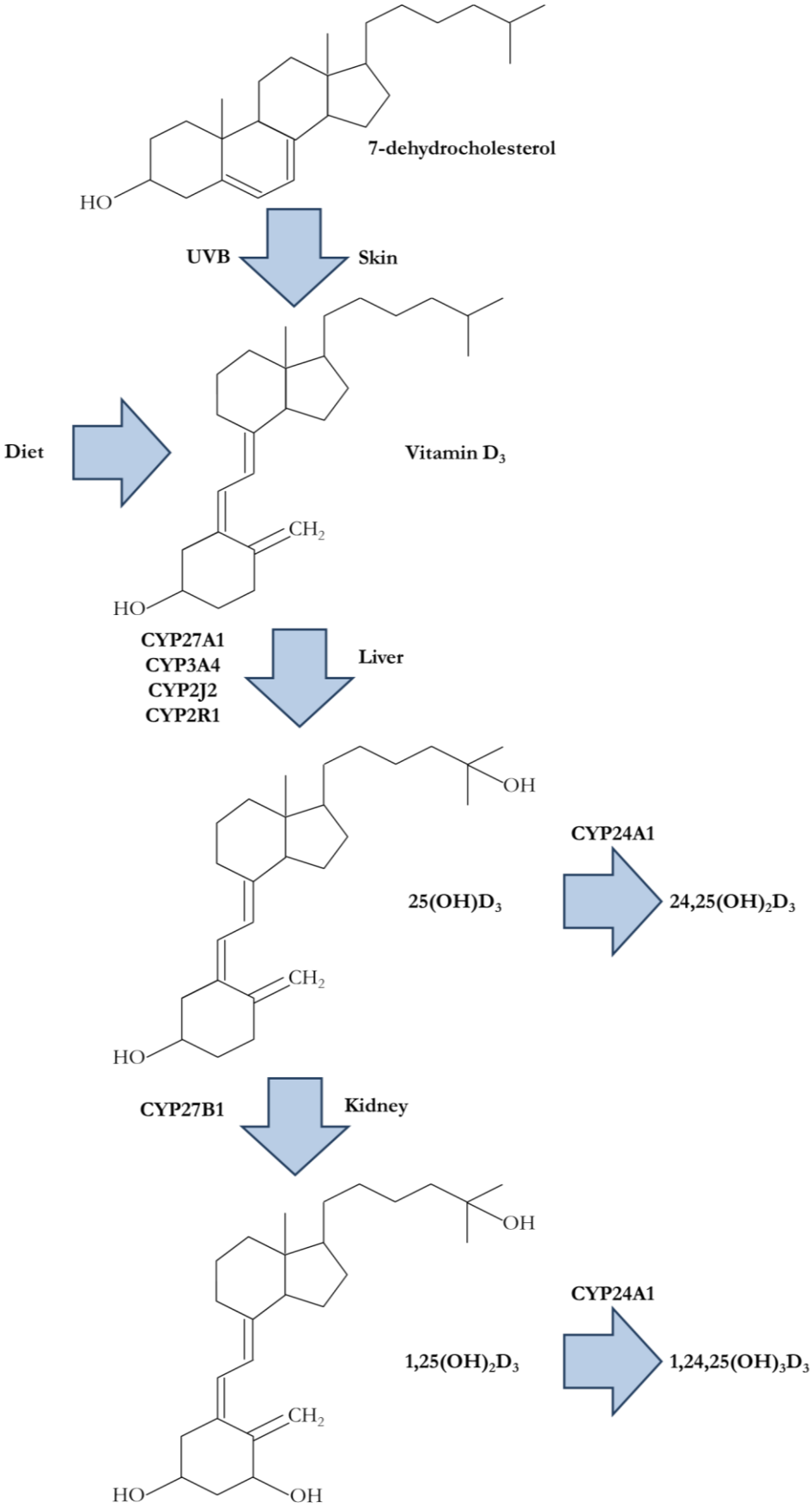

Figure 1.12: Vitamin D metabolism. This diagram shows the metabolism of vitamin D_3, however the same hydroxylation steps also apply to vitamin D_2. Adapted from Shin *et al.* (2010).

25(OH)D is the major circulating form of vitamin D and is used to monitor Vitamin D status as it has a much longer half-life of 2-3 weeks, compared to that of $1,25(OH)_2D$ (DeLuca, 2004; Holick *et al.*, 2011). Furthermore, while $1,25(OH)_2D$ is more biologically potent than 25(OH)D it circulates at concentrations 1000 times lower and the blood level is highly regulated. Thus levels do not reflect that of vitamin D reserves (Holick *et al.*, 2011). In all of the human epidemiological studies reported in section 1.2, 25(OH)D was used as the measure of maternal vitamin D status.

Numerous lifestyle factors can impact upon circulating 25(OH)D levels. Obesity, increased BMI and being a smoker have been linked with lower circulating 25(OH)D in a number of studies (Bodnar et al., 2007a; Brock et al., 2010; Zhao et al., 2012; Bjorn Jensen et al., 2013; Vimaleswaran et al., 2013; Gill et al., 2014). Moreover, one study revealed that 25(OH)D status was associated with specific genetic variants indicative of BMI, whilst BMI showed no association with genetic variants indicative of vitamin D status (Vimaleswaran et al., 2013). This shows that BMI is likely the causative factor in terms of low 25(OH)D, rather than low 25(OH)D resulting in obesity. This is supported by work showing that obese subjects showed attenuated increases in vitamin D levels upon exposure to oral supplementation or UVB exposure. This was thought to occur due to sequestration of the fat-soluble vitamin within the larger pool of body fat resulting in decreased bioavailability of the vitamin in obese individuals (Wortsman et al., 2000). Higher levels of physical activity have been shown to be associated with higher circulating 25(OH)D levels (Brock et al., 2010; Gill et al., 2014). This may be an indicator of a healthier lifestyle with increased time spent outdoors. Alternatively, a direct relationship between exercise and vitamin D status may exist, as following adjustment for sun exposure one study still reported an association between physical activity and vitamin D levels (Brock et al., 2007).

1.5.2. Vitamin D actions on gene expression

$1,25(OH)_2D$ acts on mRNA expression as both a transcriptional activator and repressor. The actions of $1,25(OH)_2D$ are mainly exerted through activation of VDR. VDR is a nuclear receptor, which forms a heterodimer with retinoid X receptor α (RXRα). The formation of the heterodimer is crucial for the activation of downstream transcriptional effects (Lin and White, 2004). $1,25(OH)_2D$ binding induces a conformational change in VDR, transforming it into a transcriptional activator. The activated VDR-RXRα heterodimer binds to VDREs within the promoter region of specific genes and activates mRNA transcription (Carlberg, 2014; Figure 1.13). Furthermore, $1,25(OH)_2D$-mediated mRNA transcription can be enhanced through the action of co-activators, specifically steroid receptor activating complex 1-3, and VDR activating complex (Shin *et al.*, 2010). VDREs have been reported in large numbers within the human genome (Ramagopalan *et al.*, 2010) leading to the notion that $1,25(OH)_2D$ could affect transcription of a large number of genes. VDR expression has been detected in a wide range of tissues including, intestine, kidney and placenta (Pospechova *et al.*, 2009; Wang *et al.*, 2012b), suggesting that a

wide range of organ systems are susceptible to $1,25(OH)_2D$-mediated effects. $1,25(OH)_2D$ may also mediate changes in mRNA expression through epigenetic mechanisms, including histone acetylation which creates an open chromatin state more accessible for mRNA transcription (Karlic and Varga, 2011; Fetahu *et al.*, 2014; section 1.7.4).

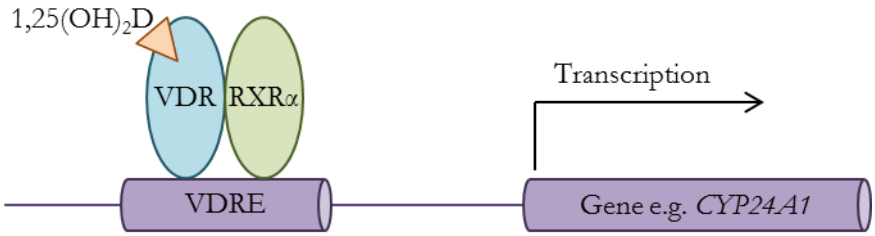

Figure 1.13: $1,25(OH)_2D$-mediated mRNA transcription. Binding of $1,25(OH)_2D$ to the VDR activates the VDR-RXRα heterodimer. This promotes its binding to VDREs within the promoter regions of genes such as *CYP24A1*, and results in increased transcription.

$1,25(OH)_2D$ can also act as a transcriptional repressor through negative VDREs (nVDREs) within the promoter region of genes. $1,25(OH)_2D$ bound VDR-RXRα does not directly to the nVDRE. Instead the ligand bound receptor heterodimer binds VDR-interacting repressor (VDIR), which itself is directly bound to the nVDRE. In the absence of ligand activated VDR, VDIR is bound to nVDREs and promotes mRNA transcription. Association of ligand bound VDR to the VDIR results in transrepression of mRNA expression (Figure 1.14). In addition the association between activated VDR and VDIR mediates the epigenetic responses of increased deoxyribonucleic acid (DNA) methylation and histone deacetylation which promote the formation of an inactive chromatin state that is prohibitive for mRNA transcription (Kim *et al.*, 2007a).

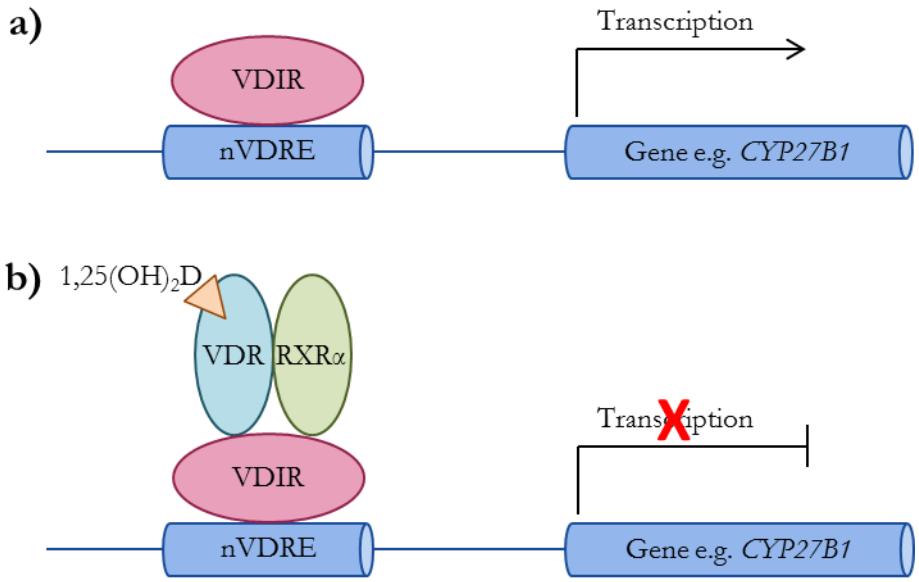

Figure 1.14: $1,25(OH)_2D$-mediated transrepression of mRNA transcription. a) In the absence of activated VDR, VDIR binds nVDREs within the promoter region of genes and activates mRNA transcription. b) Activated VDR associates with VDIR bound to nVDREs and this association results in repression of mRNA transcription.

$1,25(OH)_2D$ mediates a classical negative feedback loop upon VDR activation. This involves enhanced transcription of the vitamin D catabolism enzyme, *CYP24A1* and reduced transcription of the vitamin D activation enzyme, *CYP27B1* (Turunen *et al.*, 2007; Shin *et al.*, 2010). This feedback loop serves to increase catabolism of vitamin D and reduce further activation of 25(OH)D into $1,25(OH)_2D$, allowing tight regulation of active vitamin D levels. The fact that *CYP24A1* expression is increased in the presence of $1,25(OH)_2D$ raises the possibility that mRNA expression of this gene could be used as a biomarker for active vitamin D levels.

1.5.3. Transport of vitamin D

Vitamin D is largely present in the circulation bound to the carrier proteins, vitamin D binding protein (DBP) and albumin. Vitamin D was thought to act in accordance with the free hormone hypothesis whereby only the unbound vitamin D is available for uptake into the cell. The free hormone hypothesis proposes that due to the lipophilic nature of vitamin D, passive diffusion of free 25(OH)D and $1,25(OH)_2D$ is the major route of entry into the cell (Mendel, 1989). However, due to the large molar excess of the vitamin D carrier proteins only a small fraction of vitamin D is free within the circulation. The binding affinities of each carrier protein for 25(OH)D and $1,25(OH)_2D$ are displayed in Table 1.2. 88% of 25(OH)D is thought to be bound to DBP in the circulation, while 11.97% is bound to albumin. This leaves 0.03% of circulating 25(OH)D free (Bikle *et al.*, 1986). A slightly higher percentage, 0.4%, of $1,25(OH)_2D$ is free within the circulation, while 85% is bound to DBP and 14.6% is bound to albumin (Bikle *et al.*, 1985).

Table 1.2: Binding affinities of 25(OH)D and $1,25(OH)_2D$ for the carrier proteins DBP and albumin.

	DBP	Albumin
25(OH)D	70 nmol/l	60 μmol/l
$1,25(OH)_2D$	370 nmol/l	540 μmol/l

(Bikle *et al.*, 1985; Bikle *et al.*, 1986).

With such low proportions of free 25(OH)D and $1,25(OH)_2D$ in the circulation it seems unlikely that vitamin D actions do follow the free hormone hypothesis. Indeed, in kidney cells, transport of 25(OH)D has been shown to occur via DBP-mediated uptake via the endocytic receptors megalin and cubilin (Figure 1.15). The importance of megalin for renal uptake of 25(OH)D was identified following the discovery that megalin knockout mice had significantly increased urinary excretion of DBP. Furthermore, when radiolabelled DBP or 25(OH)D were infused into the proximal convoluted tubules of rats in the presence of receptor-associated protein (RAP), a megalin inhibitor, there was a significant reduction in absorption of the compounds into the blood and kidney and a significant increase in radiolabelled 25(OH)D and DBP in the urine. Megalin knockout mice also exhibit reduced growth and reduced bone density, and a 70% reduction in plasma levels of 25(OH)D and $1,25(OH)_2D$ (Nykjaer *et*

al., 1999; Nykjaer *et al.*, 2001) suggesting they suffer from vitamin D deficiency due to the reduced reabsorption of 25(OH)D in the kidneys.

Following the discovery of megalin in renal uptake of 25(OH)D, a second receptor, cubilin, was also shown to play an important role in renal uptake of DBP bound 25(OH)D. Cubilin lacks transmembrane and cytoplasmic domains, and once it has bound a ligand association with megalin is required for the receptor ligand complex to be endocytosed into the cell. Administration of anti-cubilin antibodies to a yolk sac cell line resulted in a 70% reduction in DBP and 25(OH)D uptake, while simultaneous exposure to anti-cubilin and anti–megalin antibodies only reduced DBP and 25(OH)D uptake by an additional 10% suggesting that the majority of DBP and 25(OH)D uptake is mediated via the interaction between megalin and cubilin. Furthermore cubilin, megalin and DBP showed co-localisation in canine kidneys (Hammad, 2000; Nykjaer *et al.*, 2001; Willnow and Nykjaer, 2002; Figure 1.15b).

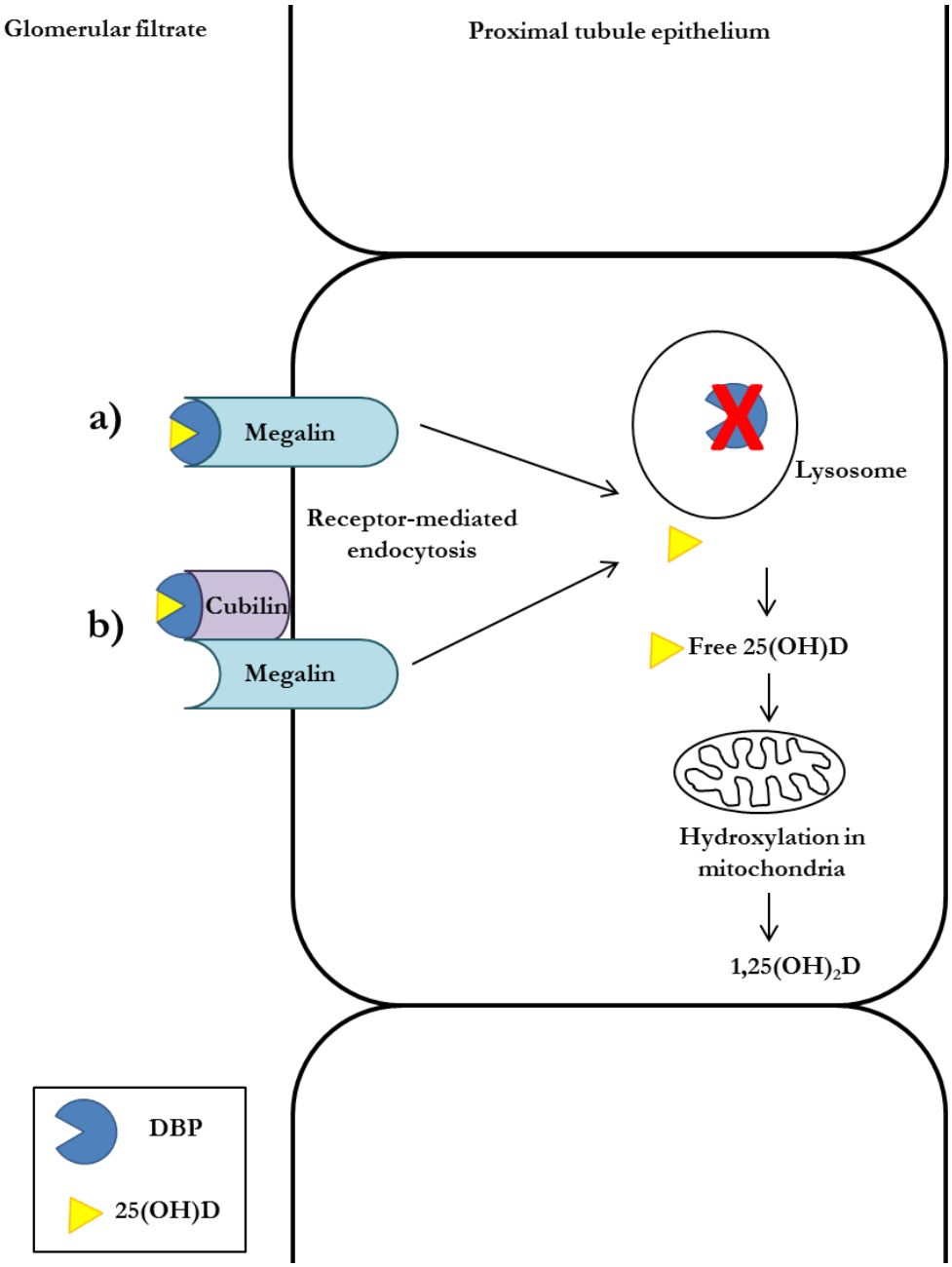

Figure 1.15: Receptor-mediated endocytosis of 25(OH)D and DBP by megalin and cubilin in the kidney. a) Megalin can bind and internalise the 25(OH)D-DBP complex. b) Cubilin requires interaction with megalin to internalise the 25(OH)D-DBP complex. Once the 25(OH)D-DBP complex has been endocytosed into the cell, the DBP is directed down the lysosomal pathway for degradation while the 25(OH)D is released into the cell. Once free within the cell, CYP27B1 can then act on the 25(OH)D converting it to the active $1,25(OH)_2D$.

Once DBP bound 25(OH)D has been transported into the cell, the 25(OH)D needs to be released for CYP27B1 to act upon it. 25(OH)D can be released from its carrier protein via degradation within the lysosome. Co-localisation experiments have revealed the presence of DBP and albumin within the lysosome while 25(OH)D was observed in the cytosol (Nykjaer *et al.*, 2001), suggesting that 25(OH)D is released within the endosomal pathway. Specifically, the lysosomal protease, legumain, has been shown to degrade both albumin and DBP (Chen *et al.*, 1997; Chen *et al.*, 1998; Yamane *et al.*, 2002). While this section has focussed on uptake of DBP bound 25(OH)D, due to the high homology

between DBP and albumin, uptake of albumin bound 25(OH)D occurs via the same mechanism (Birn *et al.*, 2000; Amsellem *et al.*, 2010).

- **The transport and metabolism of vitamin D is crucial for its function. It is possible that alterations in vitamin D metabolism and transport within the placenta may have a role in fetal programming.**

1.5.4. Vitamin D and fetal programming

Vitamin D insufficiency in women of childbearing age is relatively common (Dawodu *et al.*, 2001; Bodnar *et al.*, 2007c; Hypponen, 2007; Gale *et al.*, 2008). As the fetus is entirely reliant on its mother for vitamin D this means large numbers of fetuses are at risk of being born with vitamin D deficiency. In support of this, it has been established that offspring of women with low vitamin D levels are born with low vitamin D stores (Bouillon, 1981; Delvin, 1982; Markestad, 1984), and maternal vitamin D levels have been shown to be the major effector of fetal vitamin D levels (Novakovic *et al.*, 2012).

Human studies

The SWS has revealed that maternal vitamin D status during pregnancy is associated with offspring bone indices and body composition. Specifically, low maternal vitamin D levels were associated with increased femur metaphyseal cross-sectional area and increased femur splaying, both of which are indications of rickets (Mahon *et al.*, 2010). This idea is further supported by data showing a positive association between maternal vitamin D levels with femur proximal metaphyseal diameter and femur volume at 34 weeks of gestation (Ioannou *et al.*, 2012). The effect of vitamin D on bone development was also observed specifically in the female neonate, with a positive association between maternal late pregnancy vitamin D and neonatal bone size (Harvey *et al.*, 2008). Maternal vitamin D levels were also associated with body composition of the offspring. Fat mass of the neonate at birth was positively associated with maternal vitamin D levels, while at 6 years of age the relationship reversed, and lower maternal vitamin D levels during pregnancy were associated with increased fat mass of the offspring (Crozier *et al.*, 2012).

In support of data published from the SWS, work on another Southampton cohort showed that low maternal 25(OH)D during pregnancy was correlated with reduced whole-body BMC, BA and areal BMD in the offspring at 9 years (Javaid *et al.*, 2006), demonstrating that effects of maternal vitamin D may persist throughout childhood. In addition, in the Raine prospective birth cohort in Australia maternal vitamin D deficiency at 18 weeks of gestation was associated with reduced peak bone mass in offspring at 20 years of age (Hart *et al.*, 2015). This again supports the idea that vitamin D deficiency during pregnancy may have long lasting consequences for the offspring. However, another study found no association of maternal vitamin D status with measures of BMC in offspring at 9-10 years of age

(Lawlor *et al.*, 2013). Although in this study the percentage of women with vitamin D deficiency was low at only 6%, while 67% of women were vitamin D sufficient. This would have resulted in less power to detect associations with the lower levels of maternal vitamin D. Alongside the effects on infant skeletal development, maternal vitamin D levels have also been implicated in contributing to numerous other outcomes in offspring, including impaired growth, diabetes, asthma, and schizophrenia (Figure 1.16).

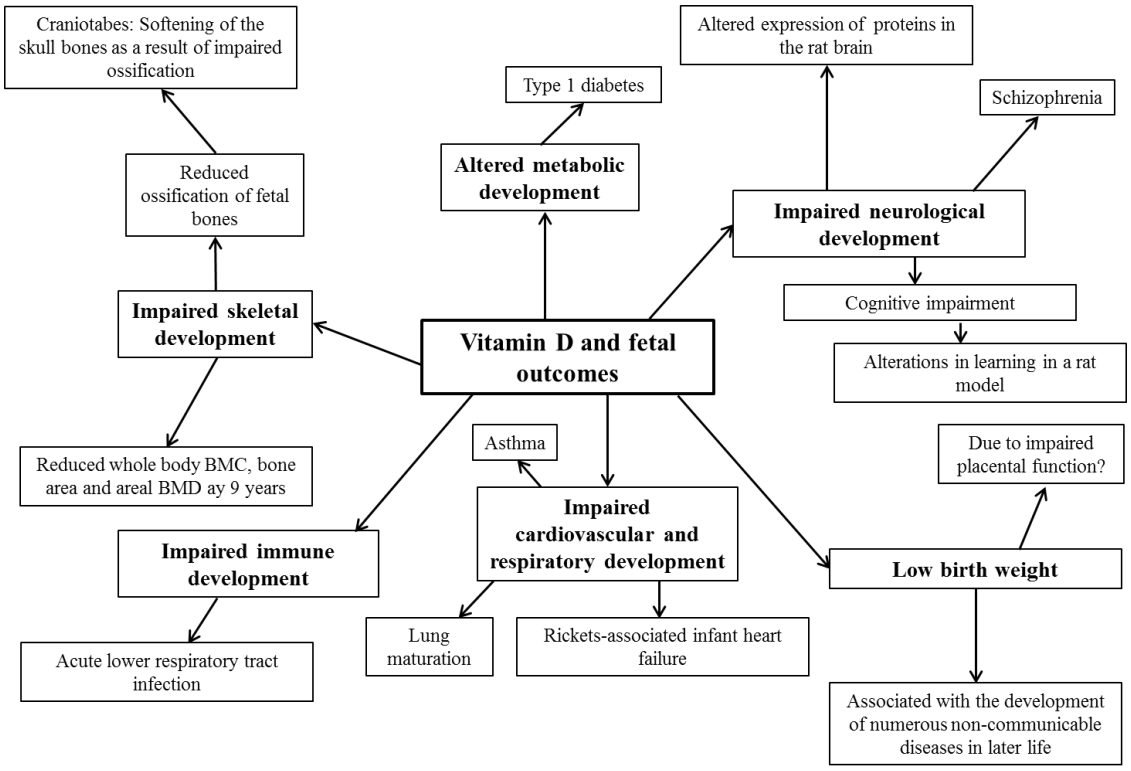

Figure 1.16: Impaired offspring outcomes associated with low maternal and/or fetal vitamin D levels. Impaired skeletal development: (Reif *et al.*, 1988; Javaid *et al.*, 2006; Yorifuji *et al.*, 2008). Altered metabolic development: (Mathieu *et al.*, 2005). Impaired neurological development: (Altschuler, 2001; Eyles, 2003; Becker *et al.*, 2005; Kesby *et al.*, 2006; Almeras *et al.*, 2007; Kinney *et al.*, 2009). Low birth weight: (Brooke *et al.*, 1980; Brooke *et al.*, 1981; Scholl and Chen, 2009). Impaired cardiovascular and respiratory development (Nguyen *et al.*, 2004; Litonjua and Weiss, 2007; Maiya *et al.*, 2008; Brehm *et al.*, 2009). Impaired immune development (Karatekin *et al.*, 2009).

Inconsistent findings have been reported between maternal vitamin D levels and measurements of offspring size and birth weight. Some studies have reported no association between neonatal anthropometry measurements and maternal vitamin D status (Javaid *et al.*, 2006; Gale *et al.*, 2008; Dawodu *et al.*, 2013). However, other studies have reported positive links between maternal vitamin D levels and fetal growth. Bowyer *et al.* (2009) reported lower birth weight in babies of mothers with vitamin D deficiency. Additionally, supplementation of vitamin D to pregnant Asian women (1000 iu) reduced the proportion of IUGR babies relative to a placebo group, which was severely vitamin D deficient. At birth only a trend was observed for increased birth weight with vitamin D supplementation, however, at 3, 6, 9 and 12 months of age infant weight was significantly greater in those whose mothers had received vitamin D supplementation during pregnancy (Brooke *et al.*, 1980;

Brooke *et al.*, 1981). Maternal vitamin D levels during pregnancy have also been associated with measures of offspring size and body composition in later childhood. For example, maternal vitamin D levels were positively associated with offspring head circumference, weight, fat and lean mass at 9 years of age (Gale *et al.*, 2008). Vitamin D may also play a role in neurodevelopment as maternal vitamin D deficiency was associated with reduced cognitive function in offspring at 5 and 10 years of age (Hart *et al.*, 2015).

Animal studies

Findings from human epidemiological studies investigating the effects of maternal vitamin D levels have been replicated in animal models. In rats, offspring of vitamin D deficient mothers displayed impaired endosteal bone formation contributing to trabecular bone loss, possibly due to insufficient bone mineralisation (Marie *et al.*, 1986). VDR mediates the effects of vitamin D (see section 1.5.2), and knockout of this receptor in the mother resulted in reduced birth weight in mouse offspring (Kovacs *et al.*, 2005). In addition, while vitamin D deficiency during pregnancy did not affect offspring birth weight in mice, pups from vitamin D deficient mothers weighed less from day 15 onwards. These offspring also showed impaired glucose tolerance, suggesting that the *in utero* exposure to low vitamin D levels may have programmed metabolic adaptations (Reichetzeder *et al.*, 2014). Also in mice, maternal vitamin D deficiency resulted in increased body and fat mass of offspring at 6 months of age, as well as metabolic disturbances including impaired glucose tolerance, increased insulin secretion and increased triacylglycerol levels (Nascimento *et al.*, 2013). However, it is not clear whether these metabolic disturbances are a direct result of the gestational effects of low vitamin D or are due to the increased fat mass of these offspring. Maternal vitamin D deficiency also affects body composition resulting in smaller muscle fibres in rat offspring (Max *et al.*, 2014). However, as with human epidemiological studies, the effects of maternal vitamin D deficiency are not always consistent in animal studies (Kovacs, 2012). Mice fetuses lacking *VDR* showed no differences in skeletal length, morphology or mineralisation, suggesting that vitamin D-mediated transcription may not be essential for fetal bone development (Kovacs *et al.*, 2005). However, a number of the reported effects of maternal vitamin D deficiency are observed after birth, and in this study it is unclear what the long-term effects would be as mice were not examined past the fetal stage.

Why the inconsistent results?

Associations between maternal vitamin D status and fetal outcomes have been described in human epidemiology studies as well as in animal models of vitamin D deficiency. While these mostly observational human studies provide exciting new possibilities in the vitamin D and pregnancy field, concerns about the over-emphasis placed on these data have been highlighted (Harvey and Cooper, 2012). Furthermore, systematic reviews and meta-analyses have revealed issues including publication bias towards positive results as well as a great deal of heterogeneity in studies (Reid *et al.*, 2013; Harvey

et al., 2014a). Furthermore, issues including varying methods for measuring vitamin D levels, as well as variation in the cut off used to define vitamin D deficiency add a further level of complexity when interpreting these data. The Institute of Medicine guidelines state that ≤ 30 nmol/l 25(OH)D is classed as deficient, 31 – 50 nmol/l 25(OH)D is insufficient and > 50 nmol/l 25(OH)D is sufficient (IOM, 2011). However, these cut off levels are not universally used in publications. For example, Hart *et al.* (2015) define vitamin D deficiency as 25(OH)D concentrations < 50 nmol/l, while Lawlor *et al.* (2013) define deficiency as 25(OH)D < 27.5 nmol/l. These differing cut off points for defining vitamin D deficiency could explain the discrepancies in results between the two studies and highlight the lack of consistency in the field. In addition, the levels defined by the Institute of Medicine are based on levels required for bone health, so it is currently unclear whether these levels are adequate for the additional described effects of vitamin D. However, as many of these additional effects are associations more concrete evidence is required before set points for deficiency to prevent these effects can be determined (IOM, 2011).

The method used to measure 25(OH)D levels adds a further level of complication when interpreting data describing associations with maternal vitamin D and fetal outcomes, as some methods are more robust than others. High performance liquid chromatography (HPLC) is considered the gold standard for 25(OH)D measurements and requires the separate measurement of $25(OH)D_2$ and $25(OH)D_3$ which are then combined to give total 25(OH)D levels. However, this method is slow and requires a high level of expertise to carry out, therefore radioimmunoassays are commonly used for 25(OH)D measurement. While these have a higher sample throughput and require less specialist equipment, radioimmunoassays may be slightly less accurate compared to HPLC. The most widely used commercially available radioimmunoassays measure both $25(OH)D_2$ and $25(OH)D_3$, however, most may underestimate $25(OH)D_2$ levels, presumable due to the slightly different chemical structure of this molecule. This can result in underestimation of total 25(OH)D levels, which may be a particular issue for countries such as America, where supplementation with vitamin D_2 is common (Wootton, 2005; Hollis, 2008).

Meta-analysis showed only weak positive associations between maternal vitamin D status with infant birth weight and serum calcium (Harvey *et al.*, 2014a). While some evidence supports the impact of maternal vitamin D levels on fetal growth and bone mass, the current evidence is not adequate to support recommendations of vitamin D supplementation during pregnancy. A great deal more research is needed; both in terms of basic laboratory science and large scale randomised controlled trials, before conclusions are made regarding the role of vitamin D in pregnancy pathologies and fetal growth. An interesting possibility is that the contrasting effects of vitamin D on fetal development result from differences in placental function. The placenta provides the link between the mother and fetus, therefore it seems plausible that differences in placental function could mediate the effects of vitamin D on fetal development. This idea is supported by the fact that in two studies, while no

association was found between maternal vitamin D levels with measures of neonatal size and body composition, positive associations were observed with cord blood vitamin D levels and fat mass, head circumference, abdominal circumference and femur length of the neonate (Walsh *et al.*, 2013; Godang *et al.*, 2014). Maternal vitamin D levels may be a poor proxy for fetal vitamin D supply. The placenta can actively metabolise vitamin D (see 1.6.3) and the mechanism for vitamin D transport into the placenta is unclear, therefore we need to understand the effects of the placenta in determining the level of vitamin D which reaches the fetus.

- **Maternal vitamin D levels may influence fetal growth and development which could have consequences for the lifelong health of the offspring. However, these effects are not consistently observed which could reflect actions of the placenta mediating the impact of vitamin D on the fetus. Placental transport and metabolism of vitamin D may be important.**

1.6. Vitamin D, pregnancy and the placenta

1.6.1. Vitamin D and pregnancy

Vitamin D is thought by some to have beneficial effects to the mother during pregnancy, including regulation of the immune response to pregnancy, calcium homeostasis and metabolism (Figure 1.17).

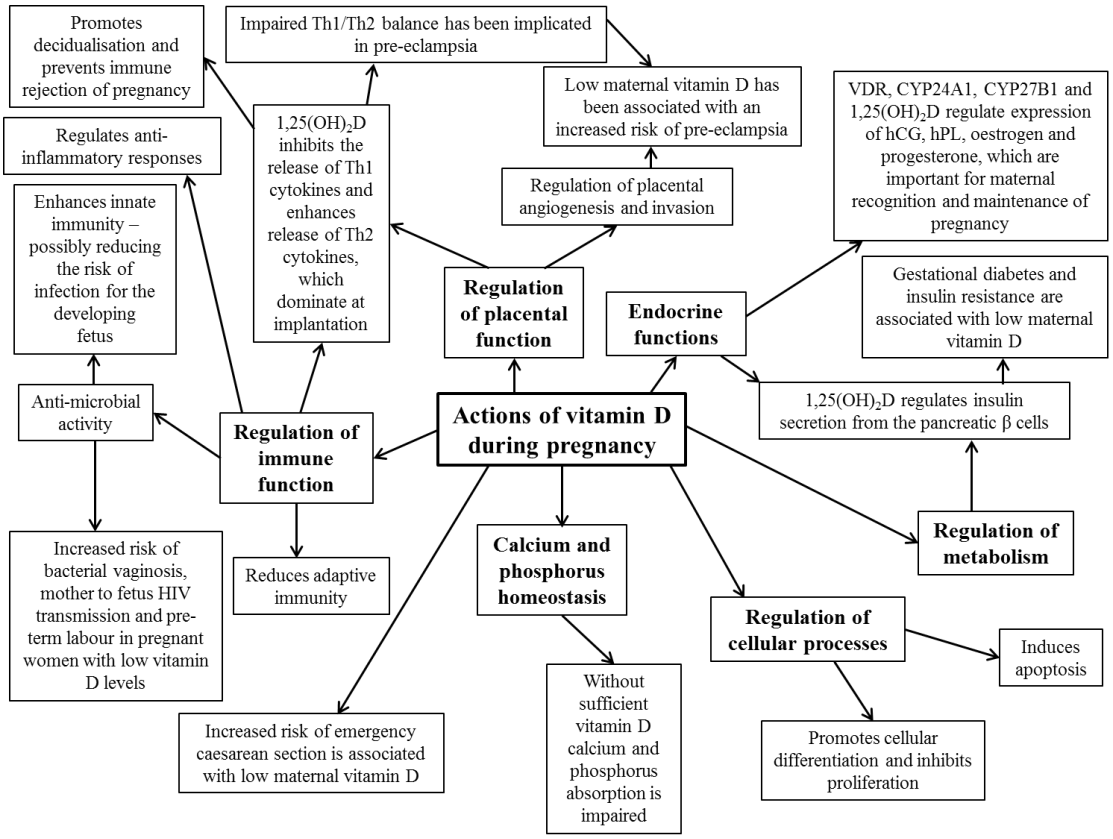

Figure 1.17: The potential wide ranging physiological effects of vitamin D during pregnancy. Regulation of immune function: (Halhali *et al.*, 1991; Saito *et al.*, 1999; Piccinni *et al.*, 2000; Zehnder, 2002; Adams and Hewison, 2008; Bodnar *et al.*, 2009; Mehta *et al.*, 2009; Lewis *et al.*, 2010b; Shin *et al.*, 2010; Dawodu and Nath, 2011; Liu *et al.*, 2011; Liu and Hewison, 2012). Regulation of placental function: (Saito *et al.*, 1999; Piccinni *et al.*, 2000; Zehnder, 2002; Daftary and Taylor, 2006; Bodnar *et al.*, 2007b). Endocrine functions and regulation of metabolism: (Kadowaki and Norman, 1984; Stephanou *et al.*, 1994; Barrera *et al.*, 2007; Barrera *et al.*, 2008; Maghbooli *et al.*, 2008; Zhang *et al.*, 2008; Shin *et al.*, 2010; Liu and Hewison, 2012). Regulation of cellular processes: (Samuel and Sitrin, 2008; Shin *et al.*, 2010). Calcium and phosphorus homeostasis: (Kovacs, 2008; Mulligan *et al.*, 2010; Liu and Hewison, 2012). Emergency caesarean section: (Merewood *et al.*, 2009).

Vitamin D may play an important role in maternal calcium homeostasis during pregnancy. Maternal calcium demands are increased during pregnancy, as maternal calcium is used by the placenta and fetus for mineralization of the fetal skeleton. Placental calcium transport must occur without depriving maternal tissues of their calcium requirements. This increased calcium demand seems to be met largely through increased intestinal absorption, which is thought to be mediated partly by $1,25(OH)_2D$ (Kovacs and Kronenberg, 1997). In a porcine model, maternal $1,25(OH)_2D$ levels correlated with neonatal calcium and phosphorus concentrations at birth (Goff, 1984), suggesting that vitamin D may play an important role in placental transport of calcium and phosphorus.

Vitamin D has also been proposed to have a potential role in placental function, including implantation, angiogenesis, and inflammation. For example, trophoblast cell culture revealed increased hCG production upon the addition of 0.1 to 10 nM $1,25(OH)_2D$ in a dose-dependent manner via cyclic adenosine monophosphate (cAMP) activation (Barrera *et al.*, 2008). Furthermore, the immunoregulatory properties of $1,25(OH)_2D$ are thought to be involved in mediation of immune

responses between maternal and embryonic tissues during implantation, and thus prevent rejection of the embryo (Bodnar *et al.*, 2007b). For example, cell culture studies have shown that culture with $1,25(OH)_2D$ inhibits interleukin-6 activity (Muller *et al.*, 1991).

The importance of vitamin D in placental function is highlighted by the possible relationship between low maternal vitamin D levels and pre-eclampsia. In an observational study, women who later developed pre-eclampsia during pregnancy showed a 15% lower serum 25(OH)D than non-pre-eclamptic pregnant women (Bodnar *et al.*, 2007b). However, in a systematic review only half of the studies identified showed a significant inverse relationship between maternal vitamin D status and pre-eclampsia risk. This review highlights the need for further studies to investigate the links between vitamin D and health problems, including pre-eclampsia, that are apparent only in a subset of observational studies (Harvey *et al.*, 2014a).

1.6.2. Vitamin D homeostasis during pregnancy

During pregnancy both $1,25(OH)_2D$ and DBP levels rise, while there is no change in maternal serum 25(OH)D. Due to the concomitant rise in both $1,25(OH)_2D$ and DBP it is thought that free $1,25(OH)_2D$ levels are unchanged during pregnancy (Van Hoof *et al.*, 2001). Although one study has reported that while the percentage of free $1,25(OH)_2D$ is reduced, due to the increased $1,25(OH)_2D$ the total amount of free $1,25(OH)_2D$ is increased during pregnancy (Bikle *et al.*, 1984). On the other hand, the increase in DBP results in a reduction in free 25(OH)D during pregnancy (Bouillon, 1981). While total 25(OH)D levels are not thought to change in pregnancy it is possible that increased 25(OH)D may be driving increased $1,25(OH)_2D$ production, and therefore would be undetectable if the turnover of active vitamin D formation is increased during pregnancy. 25(OH)D levels are highly correlated with maternal dietary vitamin D intake and UV exposure (Lapillonne, 2010), highlighting the importance of environmental influences in maximising maternal vitamin D levels.

The rise in maternal $1,25(OH)_2D$ during pregnancy is thought mainly to occur as a result of increased maternal renal production as opposed to placental production. The placenta has been shown to express CYP27B1 and to produce $1,25(OH)_2D$ (Delvin and Arabian, 1987; Diaz *et al.*, 2000). It is currently unclear whether placentally produced $1,25(OH)_2D$ acts locally upon the placenta or whether it is transported into the fetal or maternal circulations. The fetus is known to produce its own source of $1,25(OH)_2D$ as CYP27B1 is active within the fetal kidneys (Fenton and Britton, 1980), however it is unknown whether placentally produced $1,25(OH)_2D$ also contributes to fetal $1,25(OH)_2D$ levels. Some evidence suggests that a small portion of placentally produced $1,25(OH)_2D$ contributes to maternal levels. For example, in pregnant nephrectomised rats, injection of 25(OH)D led to the appearance of $1,25(OH)_2D$ in the maternal circulation. This was not observed with non-pregnant nephrectomised rats (Weisman *et al.*, 1978). In addition, a case study of a pregnant woman with chronic renal failure

reported a small increase in her $1,25(OH)_2D$ levels during pregnancy. However, this increase did not raise vitamin D levels to the usual level observed in pregnancy (Turner *et al.*, 1988), which suggests that placentally produced $1,25(OH)_2D$ does not make a major contribution to maternal $1,25(OH)_2D$ levels. This idea is supported by a porcine model in which *CYP27B1*$^{-/-}$ sows showed no increase in $1,25(OH)_2D$ levels when carrying a *CYP27B1*$^{+/-}$ fetus (Lachenmaier-Currle and Harmeyer, 1989). It is currently unclear as to what drives the increased maternal production of $1,25(OH)_2D$ during pregnancy as levels of PTH, which normally acts to stimulate CYP27B1-mediated conversion of 25(OH)D to $1,25(OH)_2D$, do not increase. This suggests that in pregnancy another factor is driving increased formation of active vitamin D. Various candidate molecules for this include, IGF1 (Halhali *et al.*, 1999), PTHrP, prolactin, hPL, calcitonin, osteoprotegerin or oestrogen (Lapillonne, 2010; Mulligan *et al.*, 2010; Shin *et al.*, 2010).

1.6.3. Placental metabolism of and response to vitamin D

The placenta expresses all the components required to metabolise and respond to vitamin D. Of the 25-hydroxylase enzymes, *CYP2J2* and *CYP2R1* have both been detected within human placental tissue (Nishimura *et al.*, 2003; Bieche *et al.*, 2007). However, controversy exists over whether CYP3A4 is also expressed. Some studies report no detection of *CYP3A4* mRNA (Nishimura *et al.*, 2003; Bieche *et al.*, 2007), while others have reported detection in some human placental samples. However, no enzymatic activity of CYP3A4 was detected (Hakkola *et al.*, 1996a; Hakkola *et al.*, 1996b), therefore it seems likely that this enzyme is not functional within human placental tissue. The expression of the other 25-hydroxylase, CYP27A1, has not previously been investigated in placental tissue. The expression and activity of CYP27B1 has been demonstrated (Delvin and Arabian, 1987; Diaz *et al.*, 2000), as has the expression of *CYP24A1,* therefore the placenta is able to both activate and inactivate vitamin D. In addition, in human primary placental trophoblast culture, the classical increase in *CYP24A1* mRNA and decrease in *CYP27B1* mRNA was observed, indicating that the placenta can respond to active vitamin D levels. Furthermore, this response was abrogated by a VDR antagonist, proving that the response is VDR-mediated (Avila *et al.*, 2007b). In support of this, expression of both *VDR* and *RXRa* has been demonstrated in the human placenta (Holdsworth-Carson *et al.*, 2009; Pospechova *et al.*, 2009). The fact that VDR and CYP27B1 are both expressed within the placenta suggests that the placenta can respond to locally produced $1,25(OH)_2D$.

Altered expression of specific components of the vitamin D metabolism and signalling system has been reported in pathological placentas (Figure 1.18). For example, protein expression of RXRα was reduced in placentas from pre-eclamptic pregnancies, while CYP27B1 and VDR protein levels were unaltered (Anderson *et al.*, 2015). Further studies have reported a reduction in both CYP2R1 and VDR protein expression, and an increase in CYP27B1 and CYP24A1 in pre-eclamptic placentas compared

to normal controls (Ma *et al.*, 2012), while *CYP2J2* mRNA expression was up-regulated in placentas from pre-eclamptic women (Herse *et al.*, 2012). While these studies do not report the same effects of pre-eclampsia on expression of vitamin D-related genes, they do consistently show that expression of components of the vitamin D metabolising system are altered in placentas from pre-eclamptic pregnancy. Furthermore, syncytiotrophoblasts cultured from pre-eclamptic placentas showed reduced basal and IGF1-mediated induction of *CYP27B1* mRNA. This reduction in *CYP27B1* mRNA expression had physiological consequences as $1,25(OH)_2D$ production was significantly lower in the pre-eclamptic syncytiotrophoblasts (Diaz *et al.*, 2002). This suggests that the placentas ability to metabolise vitamin D may be lessened in pre-eclampsia. Altered expression of vitamin D-related genes has been reported in placentas from other pathological pregnancies. For example, placentas from mothers who suffered from gestational diabetes showed increased mRNA expression of *CYP24A1*, while mRNA levels of *VDR* and *CYP27B1* were unaltered (Cho *et al.*, 2013). In addition, *VDR* mRNA and protein expression was significantly reduced in placentas from infants with idiopathic FGR compared to AGA infants (Nguyen *et al.*, 2015), while placental VDR protein above the median level was associated with longer fetal femur length (Young *et al.*, 2014). This could indicate that the placentas ability to respond to vitamin D is important for optimal fetal growth (Figure 1.18).

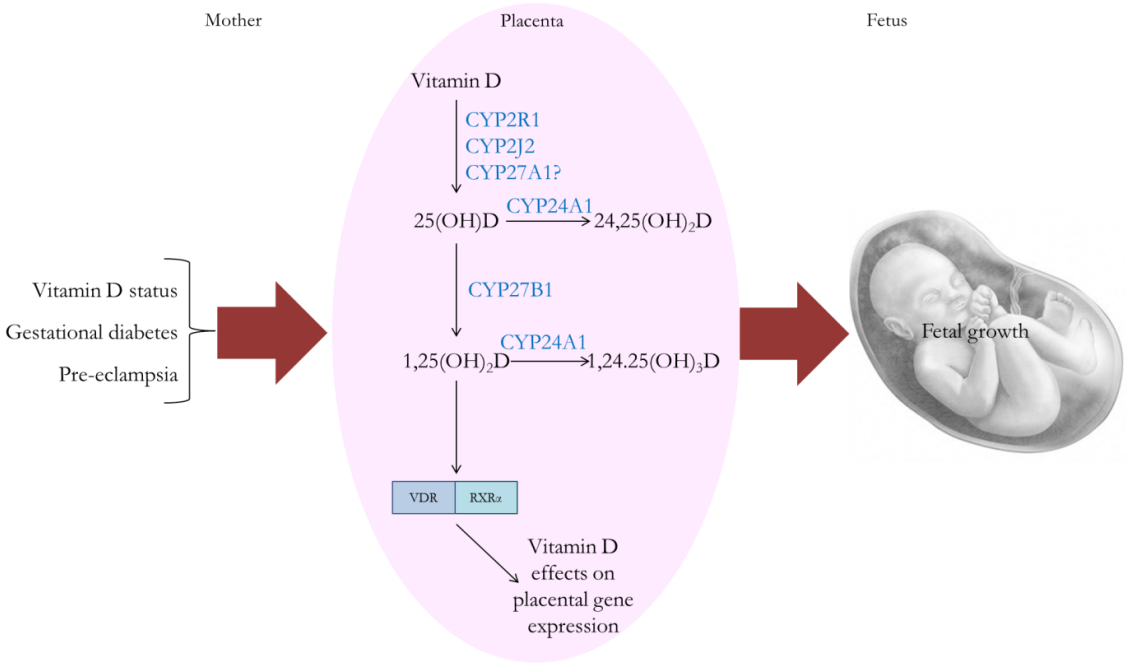

Figure 1.18: Placental metabolism of vitamin D. The placenta can metabolise and respond to vitamin D. This response may be altered by specific pregnancy pathologies. Subsequently, altered metabolism and response to vitamin D by the placenta may impact fetal growth.

It is unclear whether the altered gene and protein expression in pathological placentas is a result of the pathology or whether it contributes to development of disease. Primary placental cell culture has shown that specific external stimuli can result in alterations to vitamin D-related gene expression within the placenta. For example, altered expression of *CYP27B1* and *CYP24A1* mRNA was observed

in primary human trophoblasts cultured with 8-bromo-cAMP, a syncytialising agent (Avila *et al.*, 2007b). In addition, an *in vivo* lipopolysaccharide challenge in pregnant mice resulted in increased *VDR* and *CYP27B1* mRNA (Liu *et al.*, 2011), while treatment of primary human trophoblasts with inflammatory cytokines resulted in increased mRNA expression of *CYP27B1* and *CYP24A1* (Noyola-Martinez *et al.*, 2014). This suggests that the placental response to vitamin D is enhanced in response to an immune challenge.

Maternal vitamin D status may alter expression of vitamin D metabolising enzymes within the placenta. mRNA expression of *VDR*, *RXRa*, *CYP27B1* and *DBP* was up-regulated, whilst *CYP2R1* and *CYP27B1* were down-regulated in placentas from mothers classed as vitamin D deficient (≤ 50nmol/l) compared to those classed as vitamin D sufficient (≥ 75 nmol/l; (Vijayendra Chary *et al.*, 2015). In addition, *CYP27B1* mRNA expression in the placenta at term was positively associated with maternal $1,25(OH)_2D$ in mid-gestation (O'Brien *et al.*, 2014). These studies may represent an effect of vitamin D on expression of vitamin D genes within the placenta, although they may also represent co-regulation of placental mRNA expression and maternal vitamin D levels by an unidentified factor. *In vitro* cell culture studies would show whether vitamin D has a direct impact on expression of these genes.

Vitamin D may modulate placental functions such as syncytialisation and immune response. In cultured human trophoblast cells, $1,25(OH)_2D$ inhibited tumour necrosis factor α-mediated induction of inflammatory cytokines (Diaz *et al.*, 2009), while mice lacking *VDR* showed an altered placental cytokine response following an lipopolysaccharide challenge compared to wildtype mice (Liu *et al.*, 2011). Exposure to $1,25(OH)_2D$ for 6 h resulted in increased *hCGβ* mRNA and increased hCG secretion in human syncytiotrophoblast culture. When the length of exposure to $1,25(OH)_2D$ was increased to 48 h a reduction in *hCGβ* mRNA and hCG secretion was observed. hCG is secreted from the placental syncytiotrophoblast and is therefore used as a marker of placental syncytialisation. The short-term $1,25(OH)_2D$ treatment therefore appeared to enhance syncytialisation, while longer term treatment appeared to reduce syncytialisation (Barrera *et al.*, 2008). Knockdown of *VDR* expression within the BeWo cell line resulted in increased electrical impedance, an indicator of syncytium formation (Nguyen *et al.*, 2015), suggesting that syncytium formation was increased in the absence of vitamin D-mediated effects on transcription as was observed with the longer term $1,25(OH)_2D$ treatment by Barrera *et al.* (2008). It is unclear from this study how vitamin D may affect placental syncytialisation *in vivo*, however the study highlights that differing responses to vitamin D may occur depending on the timing of the treatment. Most studies in which placental cells are cultured with $1,25(OH)_2D$ have a duration of 8 h, therefore the bulk of the published literature represents effects of short-term exposure to vitamin D as opposed to the long-term effects which would likely manifest in the placenta over the course of pregnancy.

- **The placentas ability to respond to and metabolise vitamin D may mediate the effects of vitamin D on the placenta and on the developing fetus. This response could be altered in response to the maternal environment. Differences in the placental response to vitamin D could underlie the fact that associations between maternal vitamin D levels and fetal growth are not consistently observed.**

1.6.4. Placental transport of vitamin D

25(OH)D is thought to cross the placenta due to fact that maternal and cord blood 25(OH)D are highly correlated (Bouillon, 1981; Delvin, 1982; Hollis and Pittard, 1984; Markestad, 1984; Novakovic *et al.*, 2012). $1,25(OH)_2D$ was assumed not to cross the placenta (Shin *et al.*, 2010; Liu and Hewison, 2012), however data on correlations between maternal and cord blood $1,25(OH)_2D$ are contradictory (Fleischman *et al.*, 1980; Delvin, 1982; Hollis and Pittard, 1984; Ishida *et al.*, 1988), therefore it is currently unknown whether $1,25(OH)_2D$ does cross the placenta. The lack of association between maternal and cord blood $1,25(OH)_2D$ could represent maternally derived $1,25(OH)_2D$ being transported into the placenta and acting upon the placenta, therefore $1,25(OH)_2D$ influx into the placenta may still occur. Figure 1.19 summarises what is currently thought about placental vitamin D transport and what is still unknown.

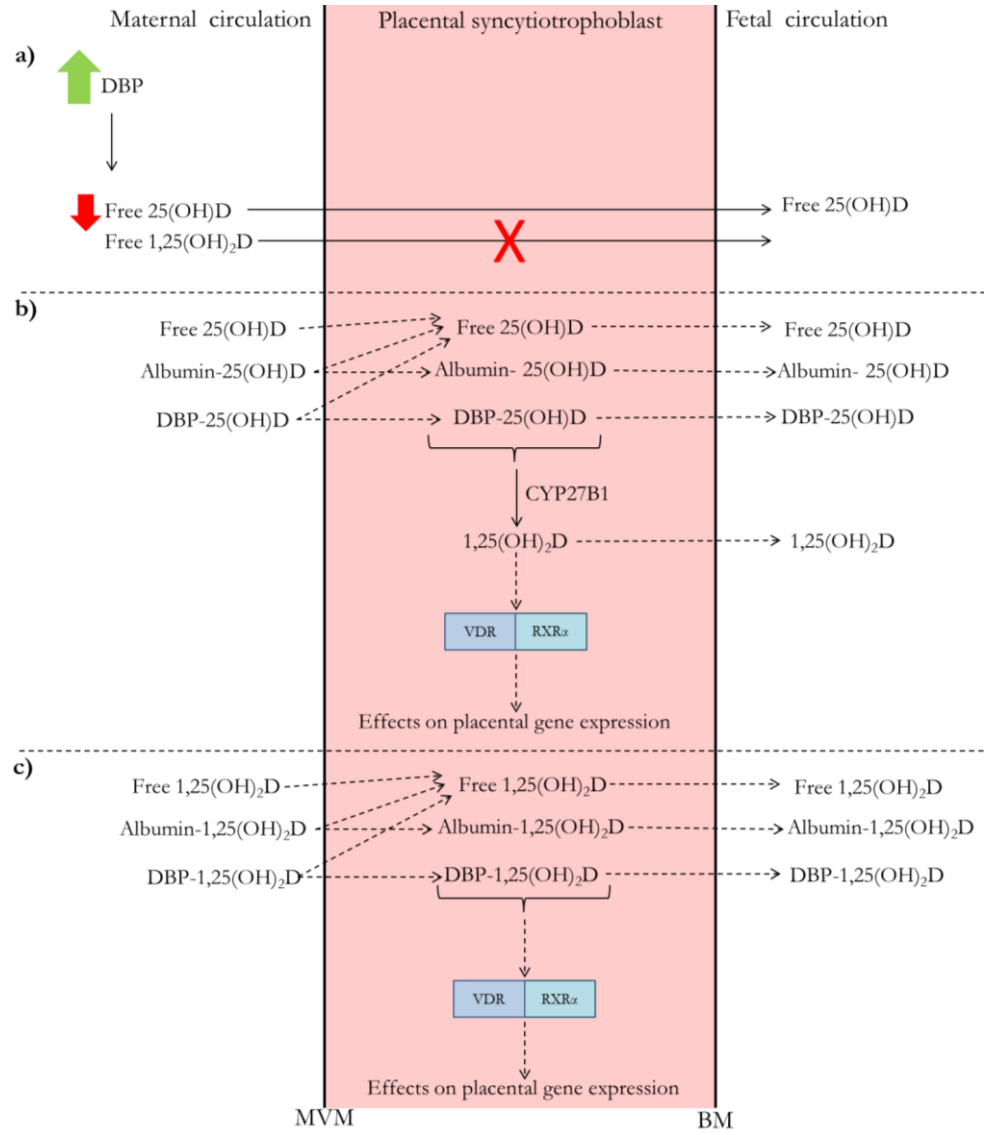

Figure 1.19: Placental transport of vitamin D. a) The current accepted theory of vitamin D transport across the placenta; 25(OH)D crosses the placenta whilst $1,25(OH)_2D$ does not. b) Placental influx and efflux of 25(OH)D. 25(OH)D is known to cross the placenta, however, it is not known whether this transport is mediated by the presence of DBP or albumin. In addition, it is unclear if carrier protein-mediated uptake results in efflux of the 25(OH)D or whether the carrier protein is degraded inside the placenta and free 25(OH)D is delivered to the fetus. While the placenta is known to metabolise 25(OH)D into $1,25(OH)_2D$, it unclear how much of the maternally derived 25(OH)D is metabolised, or whether placentally derived $1,25(OH)_2D$ acts upon the placenta or is transported into the fetal circulation. c) Placental influx and efflux of $1,25(OH)_2D$. It is unknown whether $1,25(OH)_2D$ is transported into the human placenta. Furthermore, if $1,25(OH)_2D$ is transported into the placenta it is unknown whether uptake involves free or carrier protein bound $1,25(OH)_2D$. Once inside the placenta it is unknown whether $1,25(OH)_2D$ acts upon the placenta resulting in changes in mRNA expression or whether it is transported into the fetal circulation. Dotted lines indicate what is currently not known.

The mechanism of transport of 25(OH)D (and $1,25(OH)_2D$) into the placenta is unknown. Due to the reduction in free 25(OH)D levels in pregnancy there would not be a large pool of free 25(OH)D available for transport into the placenta. It is possible that carrier-mediated transport into the placenta is operating, this would give the placenta access to a much larger pool of both 25(OH)D and $1,25(OH)_2D$ as it would be able to access the large carrier protein bound portion of these metabolites too. This could involve megalin- and cubilin-mediated uptake of DBP and albumin bound vitamin D

as occurs in the kidney (Section 1.5.3). Expression of both megalin mRNA and protein and cubilin protein has been demonstrated within the human placenta (Sahali *et al.*, 1992; Lundgren *et al.*, 1997). Furthermore, co-localisation of these receptors has been described in a rat yolk sac cell line (Hammad, 2000), suggesting they may function as a partnership like in the kidney. Both receptors are expressed within the placental syncytiotrophoblast (Burke *et al.*, 2013), and their protein expression increases throughout gestation in human placenta. This suggests a role in placental nutrient transport and that their expression increases with advancing gestation to meet the demands of the developing fetus. Megalin has specifically been localised to the MVM of the placental syncytiotrophoblast (Lambot *et al.*, 2006), supporting the notion that it may play a role in uptake of vitamin D into the placenta.

One previous study has looked at transfer of 25(OH)D and 1,25$(OH)_2$D across the human placenta using the placental perfusion model. This study showed transfer of both of these vitamin D metabolites. However, huge variations were observed in the transfer of 25(OH)D in different placentas. The transfer of 25(OH)D occurred at higher rates than that of 1,25$(OH)_2$D in some placentas, while in others 25(OH)D transfer was less than 1,25$(OH)_2$D. In addition, the study looked at the effect of DBP and reported a reduction in 25(OH)D transfer in the presence of DBP. However, the source of DBP for this study was male human serum, it was therefore not a pure preparation of DBP and the concentration of DBP as well as what other proteins were present within the serum is unknown. This study is also complicated by the fact that the buffer used for all experiments contained albumin, therefore the observed vitamin D transport may have been of vitamin D-albumin complexes (Ron *et al.*, 1984). A more recent study investigated albumin uptake in explants from human term placenta. This study showed rapid internalisation of albumin into placental explants, which was reduced by a decrease in temperature, suggesting that internalisation of albumin into the placenta occurs via a non-diffusion mediated mechanism. Furthermore, uptake of albumin was reduced by specific inhibitors of clathrin-dependent endocytosis, but not by megalin-specific inhibitors (Lambot *et al.*, 2006). This study therefore suggests that transport of albumin into the placenta occurs via a mechanism that is not megalin-mediated. However, the inhibitor of megalin used was not RAP which has been shown to inhibit both megalin- and megalin-cubilin-mediated endocytosis (Nykjaer *et al.*, 2001). Therefore, it is unclear whether the inhibitor used in this study would inhibit internalisation occurring via cubilin binding to the ligand.

Fluorescently labelled albumin has been observed within the placental syncytiotrophoblast but not in the fetal capillaries, suggesting albumin is either recycled back to the maternal circulation or degraded within the syncytiotrophoblast (Lambot *et al.*, 2006). In addition, expression and activity of legumain has been described in the human placenta (Chen *et al.*, 1997; Chen *et al.*, 1998) suggesting that if endocytosis of carrier protein bound vitamin D occurs within the placenta, the placenta has the

capacity to liberate the bound vitamin D so that it could be metabolised or exert signalling functions within the placenta.

While megalin- and cubilin-mediated endocytosis of carrier protein bound vitamin D seems a promising candidate for placental uptake, other potential mechanisms could involve receptor-mediated uptake by another receptor. For example, the neonatal FC receptor (FcRn) has been shown to bind albumin (Andersen *et al.*, 2012), while in the intestine cholesterol transporters have been implicated in vitamin D uptake (Reboul *et al.*, 2011). Uptake of DBP has been described in cells which do not express megalin (Chun *et al.*, 2010) supporting the idea that an alternative method of DBP uptake does occur. The endocytic mechanism for receptor-mediated uptake requires the formation of clathrin coated pits which are then released from the membrane via the action of dynamin (Doherty and McMahon, 2009; Figure 1.20). Alternatively, uptake may occur via another endocytic mechanism. Caveolae-dependent endocytosis involves the formation of a flask-shaped invagination in the membrane which is mediated by caveolin proteins. The invagination is then excised from the membrane by the action of dynamin, in a similar manner to clathrin-dependent endocytosis (Figure 1.20; Mayor and Pagano, 2007; Doherty and McMahon, 2009). It is unlikely that caveolae-dependent endocytosis is mediating placental uptake of vitamin D, as caveolin protein has not been detected within the human placental syncytiotrophoblast (Lyden *et al.*, 2002; Linton *et al.*, 2003). One further possibility is that pinocytosis is involved in uptake of vitamin D in the placenta. Pinocytosis involves membrane ruffles which form as a result of actin driven protrusions of the plasma membrane. The protrusions extend into the external environment of the cell, and engulf part of the external fluid when the protrusion fuses with another protrusion or with itself (Figure 1.20). This results in the formation of the pinosome which is internalised into the cell (Jones, 2007; Kerr and Teasdale, 2009).

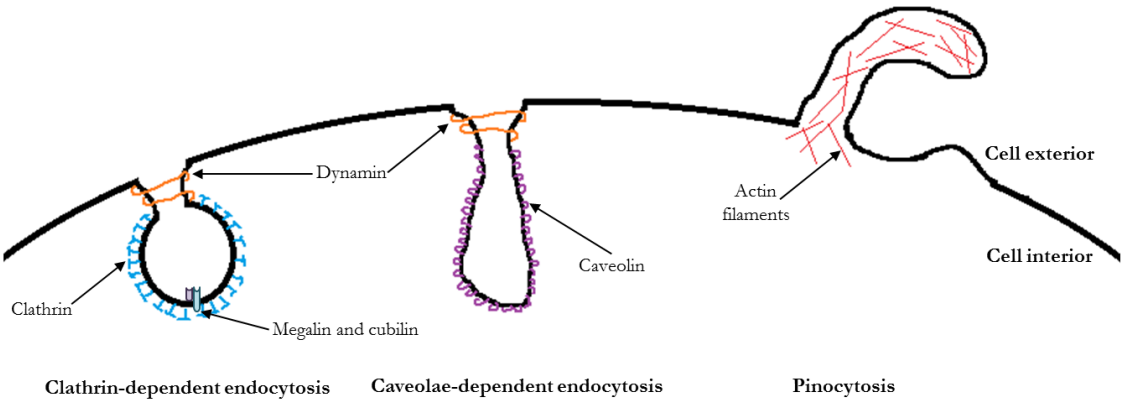

Figure 1.20: Potential mechanisms for the uptake of DBP and albumin bound vitamin D. Modified from Mayor and Pagano (2007); Doherty and McMahon (2009).

Endocytosis was altered in mouse blastocysts in response to a low protein diet in early gestation. Exposure to a low protein diet resulted in increased numbers of lysosomes as well as increased protein

expression of megalin in blastocysts (Sun *et al.*, 2014). This study suggests that endocytosis is altered in response to the *in utero* environment, and could be one mechanism in which programming effects on the fetus are mediated.

- **The placentas ability to transport vitamin D is crucial in terms of fetal development, however, relatively little is known about the types of vitamin D transported and the mechanisms of their transport. In order to gain a better understanding of how vitamin D may affect the placenta we also need to know how and which types of vitamin D are transported into the placenta.**

1.7. Potential effectors/mediators of the actions of vitamin D

The actions of vitamin D are linked to a number of other physiological systems. These systems may potentiate, but also mediate, the effects of vitamin D. Within this thesis the functions of PTHrP, LPL, thioredoxin-interacting protein (TXNIP) and DNA methylation will be investigated alongside vitamin D.

1.7.1. Parathyroid hormone-related peptide

PTHrP levels rise during pregnancy and may play a role in mediating the increase in $1,25(OH)_2D$ that is observed in pregnancy. These actions may be effected through the PTH/PTHrP receptor, PTH1R. Specifically, vitamin D may result in down-regulation of PTHrP, as treatment of a chondrocyte cell line with $1,25(OH)_2D$ resulted in reduced PTHrP protein, and *PTH1R* mRNA. This effect was inhibited by knockdown of *VDR* within the cell line and analysis of the promoter regions of these genes revealed VDREs within both promoter regions. These could be nVDRES indicating a direct effect of vitamin D on expression of PTHrP and PTH1R (Bach *et al.*, 2014). Furthermore, in placentas from *VDR*$^{-/-}$ fetuses *PTHrP* mRNA levels were increased (Kovacs *et al.*, 2005), providing further support for negative regulation of PTHrP by vitamin D. PTHrP may also affect vitamin D-mediated responses, as treatment of a chondrocyte cell line with PTHrP resulted in increased *VDR* mRNA expression, suggesting the PTHrP could increase the sensitivity of the cell to active vitamin D levels (Bach *et al.*, 2014). Furthermore, both vitamin D and PTHrP may regulate placental calcium transport (see section 1.4.7). *PTHrP*$^{-/-}$ mice had altered maternal to fetal calcium gradients, and altered calcium transfer compared to *PTHrP*$^{+/+}$ mice (Kovacs *et al.*, 1996; Bond *et al.*, 2008). Results on PTHrP and calcium transport are inconsistent, with one study suggesting calcium transport to the fetus was increased by PTHrP knockout (Bond *et al.*, 2008), while another showed the opposite effect (Kovacs *et al.*, 1996). These PTHrP-mediated effects on calcium transport may occur alongside vitamin D-

mediated effects. Furthermore, as vitamin D may regulate PTHrP and PTH1R expression, these PTHrP-mediated effects may be modified in response to maternal vitamin D levels.

1.7.2. Lipoprotein lipase

Placental LPL hydrolyses lipoproteins resulting in the release of free fatty acids for transport to the fetus (Dube *et al.*, 2012). Free fatty acids in the circulation are often bound to albumin (van der Vusse, 2009) therefore, an increase in LPL expression could result in increased fatty acids binding to albumin, resulting in lower availability of albumin for vitamin D binding. Furthermore, megalin transports fatty acids as well as vitamin D (Hammad, 2000), therefore increased fatty acid levels in the maternal circulation as a result of LPL activity, could increase megalin-mediated fatty acid uptake and reduce vitamin D uptake. LPL activity was increased in placentas of obese mothers (Dube *et al.*, 2012). If placental LPL does affect bioavailability of vitamin D for placental and fetal transport, this would further exacerbate the reduced bioavailability of vitamin D already observed in obesity as a result of sequestration in adipose tissue. Further links between LPL and vitamin D have been described. Culture of an adipocyte cell line with $1,25(OH)_2D$ resulted in increased LPL mRNA and activity (Vu *et al.*, 1996; Querfeld *et al.*, 1999), while individuals with vitamin D deficiency or insufficiency had lower plasma LPL levels (Huang *et al.*, 2013). In addition placental expression of LPL may be important for fetal growth, as altered expression has been observed in IUGR placentas (Magnusson *et al.*, 2004; Tabano *et al.*, 2006).

1.7.3. Thioredoxin-interacting protein

$1,25(OH)_2D$ has been shown to increase mRNA expression of *TXNIP* (Nishiyama *et al.*, 1999; Hamilton *et al.*, 2014). TXNIP is involved in regulation of the redox system within cells through its interaction with thioredoxin, but also has a variety of other roles within the cell, including mediation of mRNA transcription, and regulation of insulin secretion, glucose homeostasis and fatty acid metabolism (Chung *et al.*, 2006; Chong *et al.*, 2014). For example, *TXINP* deficient mice show dyslipidaemia, hypoglycaemia and hepatic and renal dysfunction when subjected to fasting conditions (Oka *et al.*, 2006), while $TXNIP^{-/-}$ fetuses have reduced glucose and increased insulin levels when mothers were subjected to 24 h of fasting, compared to $TXNIP^{+/+}$ fetuses (Mogami *et al.*, 2010). This suggests that TXNIP may have a role in metabolic sensing of the maternal environment. As TXNIP can be up-regulated by active vitamin D this effect may be mediated by maternal vitamin D levels. TXNIP also has various effects on mRNA transcription including suppression and activation of transcription, as well as interactions with histone deacetylases (Chung *et al.*, 2006; Kim *et al.*, 2007b), although $TXNIP^{-/-}$ mice showed no alterations in placental mRNA expression of *GLUT1* and *GLUT3* (Mogami *et al.*, 2010). However, this is the only study to date which has looked at TXNIP in the placenta. As TXNIP affects a wide range of physiological functions, if TXNIP within the placenta is

responsive to vitamin D, vitamin D could affect a wide range of metabolic processes within the placenta. In addition, the metabolic functions such as glucose homeostasis that are associated with TXNIP could provide a link between vitamin D and gestational diabetes.

- **PTHrP, TXNIP and LPL may mediate the effects of vitamin D through alterations in binding protein availability and VDR expression. In addition, some of the actions of $1,25(OH)_2D$ may be mediated via effects on these genes.**

1.7.4. DNA methylation

Alongside its direct effects on mRNA expression, vitamin D is also thought to act through affecting DNA methylation. DNA methylation is an epigenetic mechanism for regulation of gene expression. In the context of DOHaD, epigenetics could provide a mechanism that allows the effects of an altered *in utero* environment to be maintained permanently throughout development. Epigenetics refers to stable, heritable changes to the DNA structure without alterations to the DNA sequence, and includes DNA methylation, histone modifications, and micro RNAs (miRNAs). These epigenetic modifications influence gene expression, therefore impacting on mRNA, protein levels and cellular function.

The most widely investigated epigenetic mechanism is DNA methylation. DNA methylation of genes tends to be associated with the repression of transcription through preventing the binding of transcription factors and facilitating the recruitment of co-repressor complexes (Wu *et al.*, 2012). Furthermore, DNA methylation can promote more long-term transcriptional silencing through promoting the recruitment of methyl CpG-binding protein 2 (MeCP2), which in turn recruits complexes to initiate histone deacetylation and methylation, therefore creating a closed chromatin state that is unfavourable for transcription (Fuks *et al.*, 2003; Jaenisch and Bird, 2003; Allegrucci *et al.*, 2005; Gicquel *et al.*, 2008). However, DNA methylation is not always associated with transcriptional silencing; for example, DNA methylation may block the binding of repressive elements and induce mRNA transcription (Li, 2002).

In mammals, DNA methylation occurs mainly on the fifth carbon of the cytosine (C) base forming 5-methylcytosine. This occurs almost exclusively at CpG dinucleotides, which are a cytosine guanine (G) nucleotide pair, with the 'p' representing the phosphodiester bond between the two nucleotides. CpGs are unevenly distributed throughout the genome. Areas of the genome with a high proportion of CpGs are termed, CpG islands. In contrast to CpGs located outside of CpG islands, CpGs within these high frequency areas tend to show lower levels of methylation. 50% of CpG islands identified reside within the promoter region of genes, implicating these areas as important in regulation of mRNA expression (Bird, 2007; Gibney and Nolan, 2010).

The process of cytosine methylation involves the transfer of a methyl group (CH_3) from S-adenosylmethionine (SAM) to the 5 carbon of cytosine (Figure 1.21). This reaction is catalysed by DNA methyltransferases (DNMTs; (Gibney and Nolan, 2010).

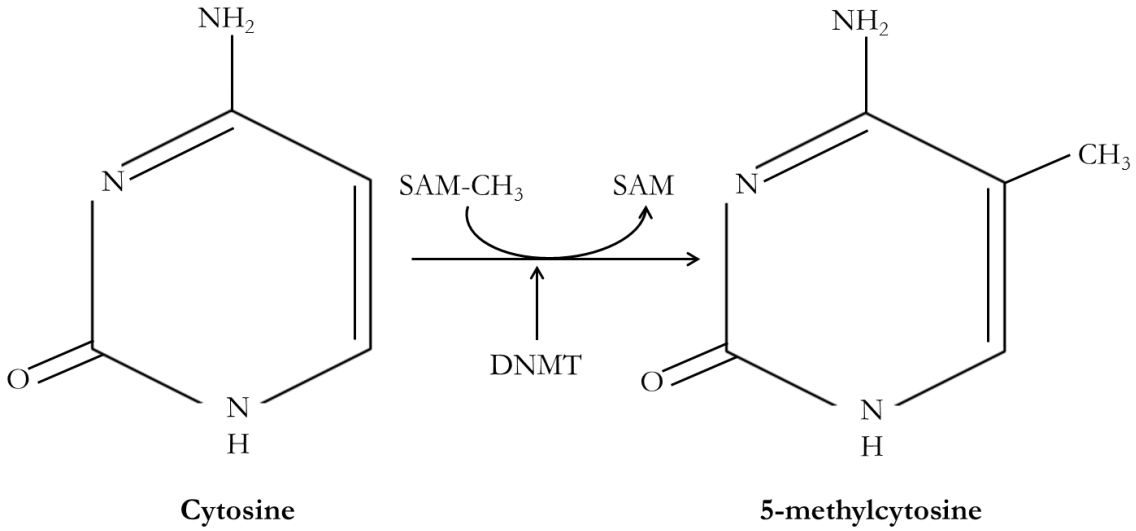

Figure 1.21: Conversion of cytosine to 5-methylcytosine by DNMTs.

Four specific DNMTs are expressed in mammalian cells, DNMT1, DNMT3a, DNMT3b and DNMT3L (Brenner and Fuks, 2006). DNMT1 is known as the maintenance DNMT and is thought to methylate hemi-methylated DNA following mitosis. DNMT3a and 3b are *de novo* DNMTs. DNMT3L lacks catalytic activity but forms a complex with DNMT3a or 3b and can then stimulate their activity (Wu *et al.*, 2012). However, the roles are not precise and DNMT1 is thought to have some *de novo* activity (Okano *et al.*, 1999), while maintenance of DNA methylation has been shown in the absence of DNMT1 expression (Bird, 2002).

Interestingly, separate knockouts of individual DNMTs in mice are all lethal, but show different phenotypes. *DNMT1*$^{-/-}$ mice exhibit genome-wide demethylation. While *DNMT3a*$^{-/-}$ mice show malformation of the gut and defects in spermatogenesis, and *DNMT3b*$^{-/-}$ mice show demethylation at minor satellite DNA and mild neural tube defects. *DNMT3a*$^{-/-}$*DNMT3b*$^{-/-}$ mice show failure to initiate *de novo* methylation after implantation. The timings of lethality with the different knockouts vary; *DNMT1*$^{-/-}$ and *DNMT3a*$^{-/-}$*DNMT3b*$^{-/-}$ show arrest in early gestation. While *DNMT3b*$^{-/-}$ is also embryonic lethal but at a later stage of gestation, *DNMT3a*$^{-/-}$ is not embryonic lethal, but pups die around four weeks of age (Li, 2002). The differing phenotypes and variation in time of death for the different DNMT knockouts highlights that specific DNMTs may exhibit specific functions, and the lack of *de novo* methylation shown in the *DNMT3a*$^{-/-}$*DNMT3b*$^{-/-}$ mouse contradicts findings of *de novo* activity by DNMT1 proposed by Okano *et al.* (1999).

DNA methylation and DOHaD

Maternal nutritional status has been shown to affect DNA methylation and gene expression. Offspring of protein-restricted pregnant rats had significantly lower methylation at specific CpG islands in the hepatic peroxisome proliferator-activated receptor α and glucocorticoid receptor promoter regions. These reductions in DNA methylation correlated with increased mRNA expression of these, and their target, genes (Lillycrop *et al.*, 2005; Lillycrop *et al.*, 2008). Further alterations to the epigenetic status of these offspring were also observed. *DNMT1* and *MeCP2* mRNA expression were reduced, as was the ability of both proteins to bind the glucocorticoid receptor promoter region (Lillycrop *et al.*, 2007). These findings illustrate that maternal nutritional status can alter the epigenetic status of the offspring, which in turn affects the downstream gene and target gene expression, and could result in alterations to metabolic pathways within the offspring.

Human cohorts have illustrated similar findings. Peri-conceptional exposure to the Dutch Hunger Winter was associated with a 5.2% lower methylation in the *IGF2* differentially methylated region in adulthood in comparison to non-exposed siblings. This hypomethylation was not observed in those exposed to the famine in late gestation, thus establishing the importance of the timing of a nutritional insult. Furthermore, the study highlights the persisting nature of epigenetic changes, as the hypomethylation was present six decades after the peri-conceptional nutritional deprivation (Heijmans *et al.*, 2008). Altered DNA methylation may also be associated with pregnancy pathologies, as DNMT1 protein expression and global DNA methylation were reduced in placental villous tissue of women with early pregnancy loss compared to control tissue at the same gestational age. Furthermore, administration of a DNMT1 inhibitor to pregnant mice resulted in a significant reduction in the number of embryos that had implanted by day 11 and the embryos that had implanted showed a significant reduction in global DNA methylation and abnormal development (Yin *et al.*, 2012). This shows that DNMT1 is important for establishing the methylation status of the placenta in early gestation, and suggests that disturbances to DNMT1 expression and function may result in disturbances in fetal development.

DNA methylation and the placenta

Epigenetic regulation of mRNA transcription is important in placental development and function. Furthermore, as epigenetics can be altered by the environment, they may represent a plausible mechanism for introducing changes to placental physiology and fetal growth in response to the maternal condition. In comparison to all human somatic tissues, the placenta shows the lowest 5-methylcytosine content of about 3.1% (Fuke *et al.*, 2004). The placenta also shows a DNA methylation status, which has been compared to that seen in cancer cells, with tumour associated methylation of specific genes. However, unlike cancer cells, there is no evidence of a general silencing of tumour suppressor genes via DNA methylation within placental tissue (Novakovic *et al.*, 2008), suggesting that the epigenetic status within the placenta shows a higher degree of regulation. The methylation status of

the placenta changes over the course of gestation, as heat map analysis showing methylation clustering revealed distinct methylation patterns in first, second and third trimester human placenta samples. There was also an increase in methylation between the second and third trimester placental samples (Novakovic *et al.*, 2011b). The methylation status of specific glucose transporters within the placenta was also shown to vary over gestation, with some showing increased methylation, and others reduced methylation with increasing gestational age (Novakovic *et al.*, 2013). These studies highlight the notion that DNA methylation within the placenta is dynamic and could mean that it is more adaptable and therefore more susceptible to environmental perturbations.

The epigenetic status of the placenta can be influenced by the environment. For example, maternal smoking is associated with altered DNA methylation within cord blood cells (Novakovic *et al.*, 2014), while maternal alcohol intake is associated with reduced placental DNA methylation. Furthermore, the increasing use of assisted reproductive technology over recent years has provided some evidence for early environmental factors influencing placental methylation status, with hypomethylation of DNA in full term placenta from *in vitro* fertilisation (IVF) pregnancies. Furthermore, some studies have linked human IVF to increased incidence of imprinting disorders. However, this finding is not replicated in other studies (Novakovic and Saffery, 2012). Animal models have shown similar influences of the maternal environment on placental methylation status. For example, maternal stress in a rat model resulted in increased *DNMT3a* mRNA levels within the placenta (Jensen Pena *et al.*, 2012), while IVF in sheep reduced placental *DNMT1* mRNA and resulted in increased fetal loss (Ptak *et al.*, 2013).

It seems reasonable that the placenta does respond to environmental stimuli, as of all the tissues from the conceptus the placenta is exposed to the greatest degree of environmental influences (Novakovic *et al.*, 2010). It is therefore plausible that epigenetic modifications in response to environmental stimuli result in alterations to placental mRNA expression, which can impact fetal growth and development in preparation for the perceived environment. In support of this, methylation of specific genes within the placenta has been associated with alterations in fetal growth. Placentas from small for gestational age (SGA) and IUGR infants demonstrated hypomethylation of 14 specific genes and hypermethylation of 8 specific genes (Banister *et al.*, 2011). Moreover, long interspersed nuclear element-1 hypomethylation was seen in low birth weight infants (Non *et al.*, 2012). Furthermore, altered placental DNA methylation has also been observed in pre-eclamptic placentas, with 34 loci showing reduced methylation in placentas from patients with early onset pre-eclampsia (Yuen *et al.*, 2010). However, it is currently unknown whether the altered methylation observed is a driving force behind the pre-eclamptic condition, or a response to the altered maternal environment.

DNMT1, DNMT3a, DNMT3b and DNMT3L are all expressed within the human placenta (Robertson, 1999), and abrogation of DNA methylation through depletion of DNMT3a and DNMT3b resulted in increased promoter activity of cell-adhesion molecules and inhibited trophoblast

migration in the BeWo cell line (Rahnama *et al.*, 2006). Increased expression of placental DNMT1 and DNMT3b has also been described in pre-eclampsia (Zhuang *et al.*, 2014). Deficiency of the oocyte-specific *DNMT1* transcript in mice resulted in abnormal placental morphology, lipid accumulation within the placenta and impaired X chromosome inactivation. Furthermore, this resulted in altered embryonic growth patterns (McGraw *et al.*, 2013; Himes *et al.*, 2015). In a rat model where dams were treated with the demethylating agent, 5-Aza-2'deoxycytidine (AZA) at specific time points during pregnancy, altered placental phenotypes were observed. Treatment with AZA in early gestation resulted in significant reduction in placental weight, while disturbances to the labyrinth layer of the placenta were more severe the earlier in gestation the exposure to AZA occurred (Serman *et al.*, 2007). These studies not only show the importance of DNA methylation for placental development and function, but also highlight that the timing of exposure to a stimulus is crucial in the effect on placental development.

Differences in placental DNA methylation and DNMTs may underlie some of the sex differences seen between male and female placentas. DNMT1 was shown to be expressed at lower levels in male in comparison to female placentas, which may result in loss of methylation of some genes within male placentas. In addition, in response to *in utero* stress DNMT1 expression was increased in female but not male placentas in comparison to sex matched controls. This corresponded with an increased expression of specific genes in male but not female placentas alongside maladaptive stress responses in male mice. This suggests the placental sex differences may underlie the physiological differences between male and female adult mice following *in utero* stress exposure (Mueller and Bale, 2008).

Vitamin D and DNA methylation

Vitamin D may act via altering DNA methylation of specific genes. Vitamin D status has been associated with methylation status of specific genes in some human cohorts. For example, vitamin D intake was negatively associated with DNA methylation of Dickkopf-related protein 1 and Wnt 5a in colorectal cancer patients in Newfoundland. This association was not replicated in a cohort in Ontario, where UV exposure is abundant, and presumably dietary vitamin D does not provide the major source of vitamin D (Rawson *et al.*, 2012). Furthermore, plasma vitamin D levels were negatively associated with methylation status of cation-transporting P-type ATPase 1, and showed positive associations with long interspersed nuclear element-1 methylation (Tapp *et al.*, 2013). These studies support the notion that vitamin D can modulate DNA methylation, however, the mechanism by which this occurs is currently unknown (Fetahu *et al.*, 2014). It is possible that vitamin D could induce DNA methylation through altering mRNA expression of DNMTs.

As well as the potential effect of vitamin D on DNA methylation, the methylation status of vitamin D-related genes may affect cellular responsiveness to vitamin D. In T cells, treatment with AZA reversed human immunodeficiency virus induced down-regulation of *VDR* (Chandel *et al.*, 2013), suggesting

that *VDR* mRNA expression is controlled by methylation. Additional studies have also reported that AZA treatment of a number of cell lines resulted in increased *VDR* mRNA. *VDR* mRNA expression was increased further in response to treatment with AZA and 1,25$(OH)_2$D combined (Marik *et al.*, 2010; Essa *et al.*, 2012), suggesting that vitamin D and DNA methylation may act antagonistically. However, in other cell lines *VDR* mRNA expression was not altered by AZA treatment and the *VDR* promoter region was shown to be unmethylated (Habano *et al.*, 2011). This could indicate cell type-specific differences in methylation of *VDR*. *VDR* methylation has been reported to be low in human placental tissue (Novakovic *et al.*, 2009), which could mean *VDR* is not regulated by methylation within the placenta.

Expression of vitamin D metabolising enzymes may also be regulated by DNA methylation status. Removal of DNA methylation by AZA treatment increased *CYP3A4* mRNA expression in 4 out of 6 cell lines tested (Habano *et al.*, 2011). *CYP24A1* may also be regulated by DNA methylation in a cell type-specific manner, as treatment of cell lines with AZA resulted in up-regulation of *CYP24A1* mRNA and activity in half of the cell lines tested. The methylation status of the *CYP24A1* promoter has been investigated in detail in human placental tissue and was shown to be much higher than that in non-placental tissue, 56.53% compared to 1.56% respectively. This increase in methylation status within the placenta abrogated 1,25$(OH)_2$D-mediated induction of *CYP24A1* mRNA expression (Novakovic *et al.*, 2009). Methylation of vitamin D metabolism enzymes may be particularly important in mediating the response to vitamin D as DNA methylation of *CYP24A1* and *CYP2R1* was reduced in participants whose serum 25(OH)D levels increased following 12 months of vitamin D supplementation, compared to those whose serum 25(OH)D levels were unchanged. In addition, vitamin D itself may play a role in mediation of *CYP24A1* methylation status, as in all the participants *CYP24A1* methylation level was reduced with supplementation (Zhou *et al.*, 2014b).

DNA methylation of vitamin D-related genes may also be altered in pregnancy pathologies. For example, in placentas from pre-eclamptic pregnancies DNA methylation of *CYP27B1*, *VDR* and *RXRa* was increased. For RXRα this increase in DNA methylation resulted in reduced protein expression (Anderson *et al.*, 2015). Furthermore, *CYP24A1* methylation was lower in placentas from pre-eclamptic pregnancies (Novakovic *et al.*, 2009). In addition, DNA methylation of vitamin D genes *in utero* has been linked with fetal programming effects. The methylation status of *RXRa* within umbilical cord samples was associated with childhood body composition. Specifically, increased methylation of *RXRa* correlated with increased child fat mass at both 6 and 9 years of age (Godfrey, 2011). This was shown in two separate human cohorts, one of which was the SWS (6 year old fat mass), demonstrating the clinical relevance of epigenetic marks as possible biomarkers of later phenotype. Furthermore, *RXRa* promoter methylation at 5 CpGs was negatively associated with size-corrected and percentage BMC of infants at 4 years of age in the SWS cohort. Methylation at 1 of these CpGs was also associated with maternal free 25(OH)D levels, suggesting the effect may have

been partly mediated via maternal vitamin D status (Harvey *et al.*, 2014c). These findings suggest that methylation status of vitamin D-related genes may have functional relevance in producing phenotypic variation.

- **DNA methylation may be an important modifier of placental function. 1,25(OH)$_2$D is thought to exert some of its actions via DNA methylation. In addition, the methylation status of vitamin D genes within the placenta may mediate the placentas ability to metabolise and respond to vitamin D.**

1.8. Placental cell culture models

One of the aims of this thesis is to investigate the effect of vitamin D on the mRNA expression of nutrient transporters within the human placenta. Human epidemiological studies are useful as these can provide information on associations between factors such as maternal vitamin D levels with expression of genes within the placenta. However, epidemiological studies tend to have a number of confounding variables, for example maternal fat mass, which may influence data. In addition, while human epidemiological studies can provide interesting associations, additional *ex vivo* and *in vitro* studies are required to show a direct link between the two factors in question. Placentation in animals varies widely from that in humans (Carter, 2007) therefore it is sometimes unclear whether findings in animal models are applicable to human placenta. As vitamin D may have tissue and species-specific effects, this thesis will also aim to establish a suitable cell culture model in which to examine the direct effects of vitamin D on placental mRNA expression.

1.8.1. Current placental cell culture models

The ideal model in which to study human placental function is primary human placental tissue as villous explants, villous fragment or primary trophoblast culture. However, primary trophoblasts no longer proliferate in culture (Orendi *et al.*, 2011), therefore limiting the amount of data which can be obtained from each sample. A disadvantage of the villous explant model is that the original syncytiotrophoblast dies off and is then regrown (Siman *et al.*, 2001). It is therefore unclear exactly what cell type this new syncytiotrophoblast is and whether it responds in the same manner as the original syncytiotrophoblast. Further disadvantages of primary trophoblast culture are that the procedure is lengthy and expensive, which could result in reduced sample numbers. Placental villous fragment culture involves the dissection of villous samples from the placenta, which are then cultured with experimental buffers and has previously been used to study uptake of radiolabelled amino acids in human placenta (Lewis *et al.*, 2010a). The use of fresh human placental tissue poses the major disadvantage of being reliant on the availability of fresh human placental tissue from normal

pregnancies. A lack of sample availability could result in a major reduction in sample numbers. Due to these issues, it was decided to pursue a cell culture model to study human placental nutrient transport.

There are over 40 placental cell lines. These can be classified into three groups based on their origin; those which arose spontaneously from cultured cytotrophoblasts, those which were immortalised by transfection with viral genes, and those which originate from choriocarcinoma. The methods of immortalisation can result in differences in the cells, for example cell lines created by viral transfection have alterations to the genome, which may alter mRNA and protein expression (Gogusev et al., 1988). In the last 15 years there has been a realisation about the lack of formal guidelines for characterisation of placental cell lines (King *et al.*, 2000; Shiverick *et al.*, 2001; Sullivan, 2004). For example, one of the most widely used features to characterise a cell line as placental is hCG expression. However, this has also been shown to be a common feature in cancer cell lines. Furthermore, *in vivo* placental hCG secretion is largely restricted to the syncytiotrophoblast cells. hPL secretion is also used to characterise placental cell lines, and as with hCG has been shown to occur in some cancer cells (Heyderman *et al.*, 1985).

Placental structure, mRNA and protein expression varies throughout gestation. For example, CYP27B1 expression is reduced in late-gestation (Ma *et al.*, 2012), therefore cell lines created from first trimester tissue may vary greatly from those generated from term tissue. The placenta is also composed of a variety of cell types, including villous cytotrophoblast, stroma, extravillous cytotrophoblast, syncytiotrophoblast and endothelial cells. This heterogeneity in cell type also makes the characterisation of placental cell lines more difficult, as some features are common to specific cell types within the placenta. For example, the secretion of hCG by the syncytiotrophoblast. As this study is focused on nutrient transport, a cell culture model for the syncytiotrophoblast is required, as this is the major transport layer of the placenta. Two potential models for the syncytiotrophoblast are; the BeWo choriocarcinoma, and the human embryonic kidney 293 (HEK293) cell lines.

1.8.2. BeWo choriocarcinoma cell line

The BeWo cell line was established in culture in 1966 and is the most extensively used cell model for human placenta. The cell line was established from choriocarcinoma which had metastasised to the cerebrum. The cerebral metastasis was transplanted into a hamster cheek pouch and maintained though serial transfers for 8 years. The BeWo cell line was then derived when this cell population was explanted from the hamster cheek pouch and co-cultured with human decidual tissue (Hertz, 1959; Pattillo and Gey, 1968; Pattillo *et al.*, 1971). The cell line was classified as trophoblastic based on the secretion of hCG (Pattillo and Gey, 1968; Pattillo *et al.*, 1971), but this may not be a reliable indicator of the placental origins of a cancerous cell line. It is also unclear how comparable a cancerous placental

cell is to a normal healthy placental cell or which placental cell population the BeWo cell line represents.

Despite, these potential disadvantages, BeWo cells are commonly used to model placental transport. For example, placental transport of substances including glucose (Vardhana and Illsley, 2002), leptin (Wyrwoll *et al.*, 2005), unconjugated bilirubin (Pascolo *et al.*, 2001), iron (Heaton *et al.*, 2008), iodide (Manley *et al.*, 2005) and fatty acids (Liu *et al.*, 1997) have all been modelled using the BeWo cell line. Furthermore, the BeWo cell line has previously been used to investigate regulation of amino acid transport (Fang *et al.*, 2006). However, to the best of our knowledge, the influence of vitamin D on calcium and amino acid transporters has not been investigated in this cell line. Other pathways have also been investigated in the BeWo cell line, and it was found that a number of components of the renin-angiotensin system present in placenta are not expressed in BeWo cells (Marques *et al.*, 2011; Wang *et al.*, 2012a). The same may be true for components of the vitamin D signalling pathway, as low *VDR* expression in BeWo cells compared to placenta has been reported. This low expression was increased by agents which interfere with the epigenetic status of gene expression; AZA and sodium butyrate, which inhibit DNA methylation and histone acetylation, respectively, suggesting that the epigenetic status of these cells may differ to normal placenta (Pospechova *et al.*, 2009).

BeWo cells can syncytialise and form a multinucleated cell layer, similar to the placental syncytiotrophoblast. Syncytialisation of BeWo cells is achieved by increasing cAMP; through addition of cAMP itself, its analogue 8-bromo-cAMP or through forskolin, which acts via adenylyl cyclase to increase intracellular cAMP (Wice *et al.*, 1990). Syncytialisation of BeWo cells is generally assessed by an increased hCG production and reduced E-cadherin expression (Al-Nasiry *et al.*, 2006).

1.8.3. Human embryonic kidney 293 cell line

The HEK293 cell line, like the placental syncytiotrophoblast, is an epithelial transport layer. The cell line was established from primary cultures of human embryonic kidney cells that were immortalised through transfection with sheared adenovirus type 5 DNA (Graham and Smiley, 1977; Harrison *et al.*, 1977). HEK293 cells are a common cell model used in transport studies, and have previously been used to study amino acid transport. For example they have been used to investigate the regulation of L-arginine transport by nitric oxide (Cui *et al.*, 2005), and to demonstrate a link between proton-assisted amino acid transporters 1 and 4 and the mammalian target of rapamycin complex 1 pathway (Heublein *et al.*, 2010). However, like the BeWo cell line, the expression of amino acid and calcium transporters in response to vitamin D does not appear to have been previously investigated in this cell line. HEK293 cells are also commonly transfected with transporter genes to study transport (Yamamoto *et al.*, 2004; Khunweeraphong *et al.*, 2012). This raises the possibility of using transfection to introduce transporter genes if our genes of interest are not naturally expressed.

To the best of our knowledge the expression of vitamin D-related genes in HEK293 cells has not been compared to placental expression. However, expression of *VDR*, *CYP27B1* and *CYP24A1* has been demonstrated in these cells. Furthermore, a reduction in *CYP27B1* and an increase in *CYP24A1* expression have been shown in response to culture with $1,25(OH)_2D_3$ (Turunen *et al.*, 2007). This suggests that HEK293 cells respond in a classical manner to vitamin D and may therefore be a good model to study the regulation of transporter expression by vitamin D.

- **A placental cell culture model is required to investigate placental uptake of vitamin D, as well as the effects of vitamin D on placental mRNA expression *in vitro*. BeWo or HEK293 cells may provide adequate cell culture models for the human placenta.**

1.9. Summary

Studies have revealed associations between maternal vitamin D levels and fetal growth. In addition, placental transport of calcium and amino acids is also linked to fetal growth. Fetal growth is important as reduced fetal growth is associated with an increased risk of non-communicable disease such as diabetes and osteoporosis in later life. Vitamin D acts at the transcriptional level by altering mRNA expression of its target genes. It is therefore possible that maternal vitamin D levels modulate fetal growth via an effect on placental nutrient transporter expression. In addition, the placenta is likely to play an active role in the response to vitamin D, through expression of genes involved in vitamin D metabolism and signalling. Expression of these genes within the placenta may mediate the effects of vitamin D. Furthermore, placental uptake of vitamin D will also play an important role in the effects of vitamin D on the placenta. It is currently unknown whether $1,25(OH)_2D$ is transported into the human placenta. While 25(OH)D is thought to be transported into the placenta, the mechanism for this uptake is still unclear.

1.10. Aims and hypothesis

1.10.1. Aims

The aims of this investigation are:

1. To investigate associations between maternal vitamin D levels and placental nutrient transport expression in a human cohort.
2. To develop a cell culture model in which to study the effects and transport of vitamin D in the human placenta.
3. To investigate the uptake of 25(OH)D and $1,25(OH)_2D$ into the human placenta.

4. To explore the effect of vitamin D on mRNA expression of placental nutrient transporters in an appropriate placental model.
5. To examine the effect of maternal vitamin D supplementation during pregnancy on mRNA expression of nutrient transporters in the human placenta.

1.10.2. Hypothesis

This investigation hypothesises that both forms of vitamin D will be transported into the human placenta, possibly via a carrier-mediated mechanism and that vitamin D effects mRNA expression of specific amino acid and calcium transporters within the human placenta.

Chapter 2:
General Methods

2.1. Introduction

In order to investigate the mechanisms of vitamin D entry and the actions of vitamin D within the placenta a variety of approaches were used including, human epidemiology studies, cell culture, placental fragment culture, mRNA expression measures, methylation measurements and immunofluorescence microscopy studies.

2.2. Human cohorts

Two human cohorts were used to investigate relationships between maternal vitamin D and placental mRNA expression; the Southampton Women's Survey (SWS) and the Maternal Vitamin D Osteoporosis Study (MAVIDOS). These studies were conducted according to the guidelines in the Declaration of Helsinki, and the Southampton and South West Hampshire Research Ethics Committee approved all procedures. Written informed consent was obtained from all participating women and by parents or guardians with parental responsibility on behalf of children.

2.2.1. Southampton Women's Survey

Maternal measurements

Non-pregnant Southampton women aged 20-34 years (n = 12,570) were recruited to the SWS (Inskip *et al.*, 2006). These women were interviewed about their diet, body composition, physical activity, social circumstances and lifestyle. Women who became pregnant were followed throughout pregnancy (n = 3159). Maternal anthropometry measurements were taken by trained research nurses at study entry, and at early (11 weeks) and late (34 weeks) gestation in those who became pregnant. Measurements taken included BMI, sum of skinfold thickness, fat mass, calf circumference, ratio of subscapular/triceps skinfold thicknesses, mid-upper arm circumference, arm muscle area and height. Skinfold thickness from the triceps, biceps, subscapular and supra-iliac was measured on the non-dominant side using Harpendon skinfold callipers. Measurements were taken in triplicate to the nearest 0.1 mm. Mid-upper arm and calf circumference was measured using a tape measure (Harrison *et al.*, 1988). The logarithm of skinfold thickness was used to estimate fat mass using the method described by Durnin and Womersley (1974). Arm muscle area was calculated by (Heymsfield *et al.*, 1982):

$$\frac{(\text{mid-upper arm circumference} - \Pi \times \text{triceps skinfold thickness})^2}{4\,\Pi} - 6.5$$

Maternal lifestyle factors were also recorded before and during pregnancy. Diet was assessed using a validated food frequency questionnaire (Robinson *et al.*, 2004; Crozier *et al.*, 2008). Principal Component Analysis was used to summarise the dietary data, resulting in two principal components

that indicated the level of concordance with healthy eating recommendations (the prudent diet score) and the level of energy in the diet (the high energy score). These scores were standardised to provide z-scores indicating the prudent and high energy diet components (Crozier *et al.*, 2006). Women were asked to estimate the hours spent standing or walking per week and the frequency of strenuous exercise undertaken per week. A dichotomous variable was derived indicating whether mothers had stated they had taken strenuous exercise over the previous three months. Maternal smoking before and during pregnancy was assessed by questionnaire and a dichotomous variable was derived for each time point. Also recorded were the mother's own reported birth weight, social class, educational level and parity.

Maternal venous blood samples were taken at study recruitment, and at 11 and 34 weeks of gestation. Samples were centrifuged, and the serum separated and stored at –80°C. Maternal vitamin D was measured in 11 and 34 week blood samples. 25(OH)D and DBP concentrations at 34 weeks were measured by radioimmunoassay (Diasorin Stillwater, Minnesota, USA) at Kings College London, UK (coefficient of variability < 10%). The 25(OH)D assay measures both $25(OH)D_2$ and $25(OH)D_3$. The detection range for this 25(OH)D assay is 3.8-250 nmol/l. The assays met the requirements of the UK National Vitamin D External Quality Assurance Scheme, and intra- and inter-assay coefficients of variance were < 10%. 25(OH)D at 11 weeks of gestation was measured by HPLC chromatography and tandem mass spectrometry. Serum samples were processed by protein denaturation with zinc sulphate and methanol. An internal standard was also used, and $25(OH)D_2$ and $25(OH)D_3$ were extracted from both the samples and the internal standard into hexane. This was then dried and reconstituted in the mobile phase. Components within samples were subsequently separated by liquid chromatography and detected via tandem mass spectrometry (Waters, Milford, MA, USA). Free 25(OH)D at 34 weeks of gestation was calculated by the ratio of total 25(OH)D to DBP levels.

Fetal, neonatal and childhood measurements

Fetal anthropometry measurements were taken by ultrasound at 11, 19 and 34 weeks of gestation. Following delivery, neonatal anthropometry measurements and placental samples (see section 2.2.3) were taken. Children were followed up at 4 (n = 1076) and 6 (n = 1477) years of age (Inskip *et al.*, 2006). Neonatal and childhood measurements recorded were birth weight, head circumference, abdominal circumference, crown-heel length, subscapular skinfold thickness and mid-upper arm circumference. A subset of infants underwent dual X-ray absorptiometry (DXA) measurements within 2 weeks after birth to analyse body composition, including lean and fat mass and bone indices; BA, BMC and BMD (Harvey *et al.*, 2010a; Harvey *et al.*, 2010b). At the time of DXA scan the child's length, using a Leicester height measurer, and weight, using calibrated digital scales (Seca Ltd., Birmingham, UK) were measured. Whole body neonatal DXA was carried out using a Lunar DPX-L instrument (GE corporation, Madison, Wisconsin, USA) with specific paediatric software (paediatric small scan

mode, v 4.7c). A Hologic Discovery Instrument (Hologic Inc., Bedford, Massachusetts, USA) in paediatric scan mode was used for DXA from 4 years onwards. These measures were carried out by the SWS Study Group.

2.2.2. Maternal Vitamin D Osteoporosis Study

MAVIDOS is a randomised, double-blind placebo controlled trial of vitamin D supplementation during pregnancy (Harvey *et al.*, 2012a; Figure 2.1).

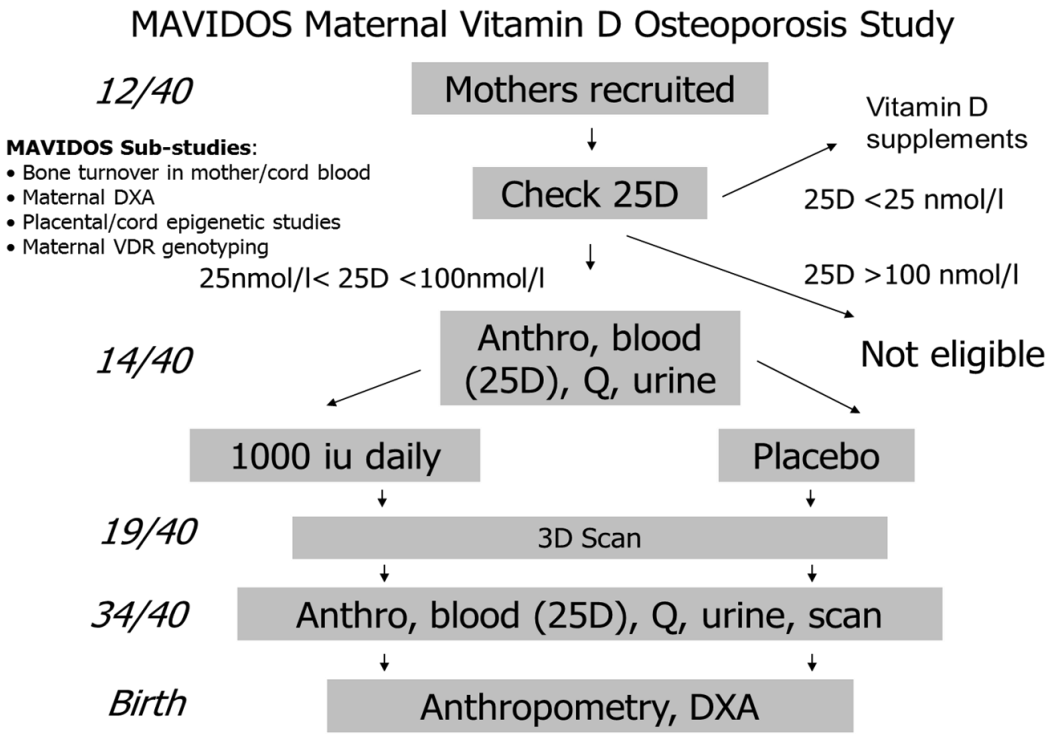

Figure 2.1: MAVIDOS trial outline (Harvey *et al.*, 2012a).

Women were recruited to the study at 3 trial centres (Southampton, Sheffield and Oxford). This investigation is restricted to samples collected in Southampton. Circulating 25(OH)D was measured in maternal serum using a Diasorin Liaison radioimmunoassay on an automated platform in a central laboratory (MRC, Human Nutrition Research Centre, Cambridge, UK). Women with levels between 25-100 nmol/l were recruited to the study. These women were randomised to an oral vitamin D supplement of 1000 iu cholecalciferol daily or matched placebo from 14 weeks of gestation until delivery of the baby. At 14 weeks of gestation a blood sample was taken for measurement of 25(OH)D, calcium and albumin. This was repeated at 34 weeks of gestation. Serum calcium measurements in maternal blood samples were based on the reaction between calcium ions and Arsenzo III (2,2'-[1,8-Dihydroxy-3,6-disulphonaphthylene-2,7-bisazo]-bisbenzenear-sonic acid). The reaction results in formation of an intense purple coloured complex. Absorbance at 660/700 nm was then measured on an AU Beckman Coulter analyser (Beckman Coulter Inc., Brea, California, USA)

with absorbance being directly proportional to the calcium concentration in the sample. Serum albumin in maternal blood samples was also measured on an AU Beckman Coulter analyser. The albumin was bound to Bromocresol Purple to form a coloured complex. The absorbance of the complex at 600 nm was then measured on an AU Beckman Coulter Analyser with absorbance directly proportional to the albumin concentration within the sample.

Following delivery, placental (see section 2.2.3) weight was recorded and placental tissue samples were collected. Neonatal anthropometry measurements (weight, crown-heel length, subscapular and triceps skinfold thicknesses, and head, mid-upper arm, chest and abdominal circumferences) were taken and neonatal DXA was carried out using a Hologic Discovery Instrument to measure whole body BA, BMC, and BMD as well as lean and fat mass (Harvey *et al.*, 2012a).

2.2.3. Placenta collection

Placenta samples from two human cohorts (SWS and MAVIDOS) were collected within 30 min of delivery to preserve RNA quality. 300 placenta samples from the SWS were collected by Dr R. Lewis. 74 placenta samples from MAVIDOS were collected by C. Simner. Following collection, the placental membranes were trimmed, the cord was removed and the placenta was weighed. A random stratified sampling grid printed onto clear acetate (Figure 2.2) was placed over the placenta and used to select 10 areas of the placenta to sample. The random sampling grid used displayed 16 sites as the placenta did not usually cover the entire grid. If the placenta was covered by more than 10 sites, sites were selected to ensure that all 4 quadrants of the placenta were sampled. Cubes of ~0.5cm^2 were cut from the placenta and the maternal decidual tissue removed. The samples were snap frozen in liquid nitrogen and stored at -80°C.

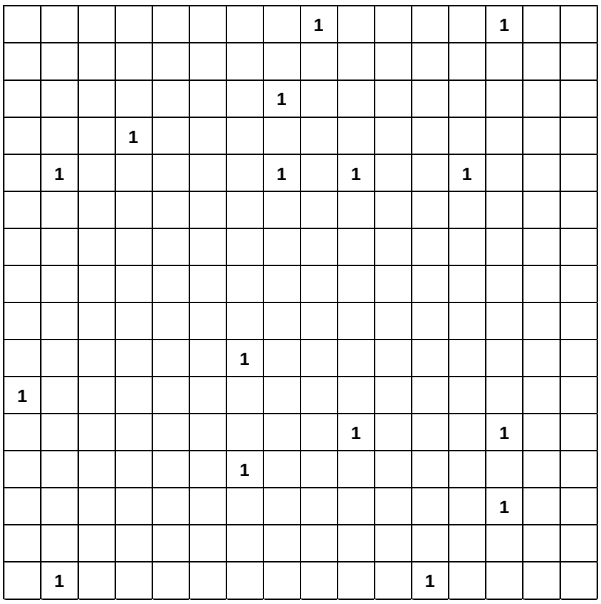

Figure 2.2: An example of a random stratified sampling grid used for placenta sampling.

All 10 placental samples were ground together into a powder and mixed before RNA or DNA extraction. Samples were ground on dry ice in a frozen tissue press which had been chilled in liquid nitrogen to ensure samples did not thaw during the process. The powdered placental tissue was then transferred to pre-chilled cryovials and stored at -80°C.

2.3. Cell culture

Cell culture studies, with HEK293 and BeWo cells, were used to investigate a suitable model system to study placental transport. Subsequently, they were used to study the effects of vitamin D and removal of DNA methylation on nutrient transporter mRNA expression. Transfection of BeWo cells with green fluorescent protein (GFP)-tagged *TAT1* was also investigated as a method to study the effects of vitamin D on placental nutrient transporters.

2.3.1. Materials and working solutions for cell culture

Table 2.1: Materials used for cell culture work.

Material	Supplier
$1,25(OH)_2D$	Cayman Chemical, Michigan, USA
25(OH)D	Cayman Chemical
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Missouri, USA
5-Aza-2'deoxycytidine (AZA)	Sigma-Aldrich
Ampicillin	Fisher Scientific, Loughborough, UK
BeWo cells	HPA Culture Collections, Salisbury, UK
Circular coverslips 19 mm diameter	Appleton Woods, Birmingham, UK
DH5α™ competent cells	Invitrogen, Paisley, UK
Dimethyl sulfoxide (DMSO) hybrid-max sterile-filtered	Sigma-Aldrich
Dulbecco's modified Eagle's medium (DMEM)	Lonza, Switzerland
Dulbecco's phosphate buffered saline (DPBS)	Lonza
Fetal bovine serum (FBS)	Lonza
Forskolin	Fisher Scientific
FuGENE®HD	Promega, Wisconsin, USA
Gelatin (from bovine skin)	Sigma-Aldrich
GeneJET™ Plasmid Maxiprep Kit	Fermentas, Germany
Glycerol (sterile)	Sigma-Aldrich
Glycine	BDH Laboratory Supplies
Ham's F12	Lonza
HEK293 cells	Dr M. Darley, University of Southampton, UK
hCG enzyme-linked immunosorbent assay (ELISA)	DRG diagnostics, Germany
Isopropanol	Fisher Scientific
L-Glutamine 200 mmol/l	Lonza
Lipofectamine® 2000	Invitrogen
Luria Bertani (LB) agar, Miller	Fisher Scientific
LB broth, Miller	Fisher Scientific
Mouse monoclonal anti-desmoplakin I+II	Abcam
Nanofectin	PAA, Somerset, UK
Neutral buffered formaldehyde 10% V/V	Surgipath, Cambridgeshire, UK
Penicillin 10,000 iu/ml; Streptomycin 10,000 iu/ml	Lonza
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich
Polyclonal rabbit anti-mouse IgG fluorescein isiothiocyanate (FITC)	Dako, Denmark
TAT1 ORF pCMV6-AC-GFP vector	OriGene, Maryland, USA
Trypsin ethylenediaminetetraacetic acid (EDTA) 0.25%	Lonza

Working solutions

DMEM/Ham's cell culture media

1:1 DMEM (250 ml) and Ham's F12 (250 ml), 10% FBS (50 ml), 500 iu/ml penicillin 500 iu/ml streptomycin (5 ml), and 1 mmol/l L-glutamine. Pre-warmed to 37°C in water bath before use.

Freeze medium

70% DMEM/Ham's cell culture medium, 20% FBS and 10% DMSO.

LB agar

40 g LB agar powder dissolved in 1 L H_2O gives 15 g agar, 10 g tryptone, 10 g NaCl and 5 g yeast extract per L. Autoclaved before use. 1 μg/ml ampicillin added after autoclaving.

30 ml LB agar was poured into 90 mm petri dishes and allowed to cool. Plates were cooled and stored at 4ºC until use.

LB broth

25 g LB broth powder dissolved in 1 L dH_2O gives 10 g tryptone, 10 g NaCl and 5 g yeast extract per L. Autoclaved before use. 1 μg/ml ampicillin added after autoclaving.

PBS

1 PBS tablet dissolved in 200 ml dH_2O gives 10 mmol/l phosphate buffer, 2.7 mmol/l KCl and 137 mmol/l NaCl, pH 7.4 at 25°C.

2.3.2. Cell culture

Retrieving cells from liquid nitrogen

Cells were stored in freeze medium in liquid nitrogen. Cells were thawed for 1-2 min at 37°C and mixed with 10 ml of DMEM/Ham's cell culture media. Cells were centrifuged at 1200 rpm for 10 min and the supernatant removed. The pellet was resuspended in 20 ml of DMEM/Ham's medium and transferred to a T75 flask.

Splitting BeWo and HEK293 cells

Cells were maintained at 37°C in 5% CO_2, media changed every 48 h and once ~80% confluent they were split. Medium was removed and the monolayer washed with 10 ml pre-warmed DPBS. 3 ml of 0.25% trypsin EDTA was then added to the flask and incubated for 3 min at 37°C. Cell lifting was checked under a LH50A microscope (Olympus, Japan) and trypsinisation was stopped with 7 ml DMEM/Ham's medium. Cells were then transferred to a falcon tube and centrifuged at 1200 rpm for 10 min, the supernatant was discarded and cells were resuspended in DMEM/Ham's medium. When

seeding into new flasks, HEK293 and BeWo cells were both seeded 1:3. Cell counting was used when cells were seeded into plates (see below).

Cell counting

Cell counting was used when cells were seeded at specific densities into 32 mm well plates. Cells were counted using a Neubauer improved bright-line haemocytometer (Marienfeld, Germany); 10 µl per chamber. Each 1 mm corner square was counted using the Leitz Wetzlar SM-Lux microscope (Leica, Germany). Cells touching the top and left lines were counted and those touching the bottom and right lines were not counted (Figure 2.3).

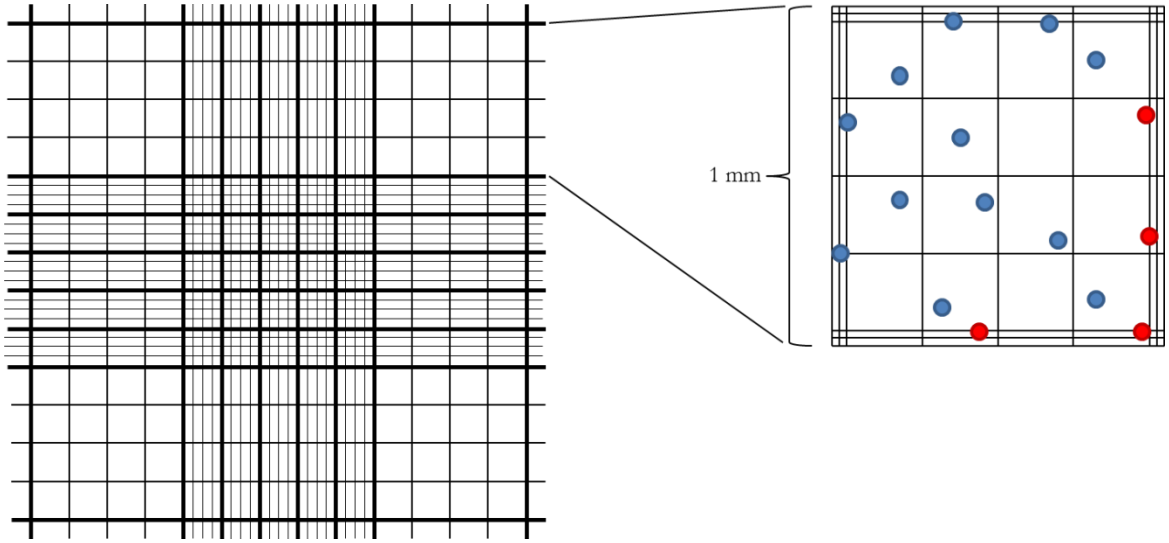

Figure 2.3: Neubauer improved bright-line haemocytometer. The left hand image shows the entire grid of the neubauer chamber, and the right hand image shows an enlargement of one of the 1 mm corner squares. Cells are indicated as round circles. The circles in blue were included in the cell counting and those in red were not.

Total cell number was counted by the calculation:

$$\frac{\text{Chamber 1 total}}{4} = n1 \qquad \frac{\text{Chamber 2 total}}{4} = n2$$

$$\frac{n1 + n2}{2} = \text{number of cells/ml}$$

Gelatin-coating

For transfection and cell staining experiments, cell culture plates and coverslips were gelatin-coated to increase cell adherence. 2 ml of 0.1% gelatin in DMEM/Ham's cell culture media pre-warmed to 37°C was added per 32 mm well (with or without coverslips in the wells). After 30 min the excess was removed and cells plated out. For experiments requiring protein extraction, plates were not gelatin-coated as this was shown to affect the protein assay (Appendix 1).

Treatment with vitamin D and AZA

BeWo and HEK293 cells were treated with AZA to investigate the effect of removal of DNA methylation on mRNA expression. HEK293 cells were also treated with 25(OH)D and 1,25$(OH)_2$D to investigate the effect of vitamin D on mRNA expression. 24 h after plating cell culture media was removed and replaced with media containing AZA or vitamin D. After 48 h, cell culture media was removed, and RNAzol® was added to the well. Cells were scrapped into RNAzol®, transferred to an eppendorf and stored at -80ºC until RNA extraction.

Syncytialisation of BeWo cells

Syncytialisation of BeWo cells was attempted with forskolin and assessed by hCG secretion and desmoplakin staining. Full methods are displayed in Appendix 2.

Freezing BeWo and HEK293 cells

BeWo and HEK293 cells were frozen into liquid nitrogen storage. At ~80% confluency, medium was removed and the cell monolayer washed in pre-warmed DPBS. Cells were trypsinised (as described above). After centrifugation, the pellet was resuspended in freeze medium (1 ml per cyrovial). Cryovials were placed in a Nalgene® Mr Frosty® freezing container (Fisher Scientific) filled with isopropanol in a -80°C freezer overnight and then transferred to liquid nitrogen storage the following day. The Nalgene® Mr Frosty® freezing container achieves the recommended cooling rate of -1°C per min.

Primary cytotrophoblast cell culture

Cytotrophoblast cells were extracted from full term human placenta and cultured by Dr J. Cleal. RNA was extracted as described in Section 2.5.2

2.3.3. Transfection

GFP-tagged *TAT1* was transfected into BeWo cells. Glycerol stocks of GFP-*TAT1* were grown on agar plates and DNA purified using Plasmid Maxiprep Kit before transfection into BeWo cells. Initial preparation of GFP-*TAT1* by transformation into DH5α™ competent cells, purification and formation of glycerol stocks was performed by Dr E. Lofthouse.

Transformation

Vector containing GFP-*TAT1* (*TAT1* ORF pCMV6-AC-GFP vector; 10 ng; Figure 2.4) was added to 100 µl DH5α cells and incubated on ice for 30 min. The cell and vector mixture was heat shocked for 45 s at 42°C and then incubated on ice for 2 min. 0.9 ml LB Broth was added at room temperature (RT) and the mixture shaken for 1 h at 225 rpm. 100-200 µl of cell mixture was spread onto LB agar

plates and incubated overnight at 37°C. The following day a single colony was picked at random, added to 250 ml LB Broth and incubated overnight in a 37°C shaker.

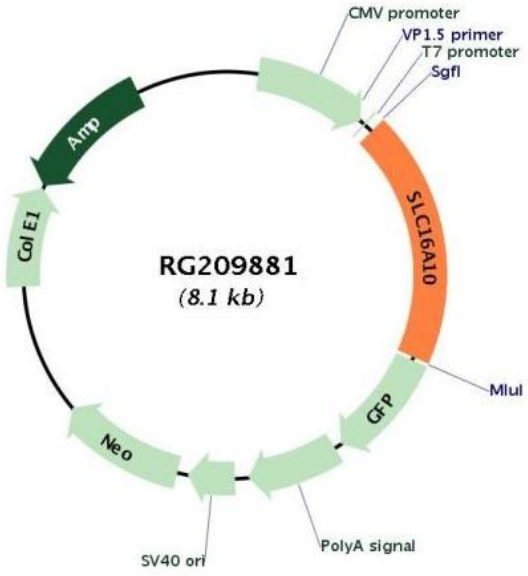

Figure 2.4: *TAT1* ORF pCMV6-AC-GFP vector.

Maxiprep

GFP-*TAT1* DNA was purified via GeneJET™ Plasmid Maxiprep Kit, according to manufacturer's instructions. Briefly, the cells in LB Broth were pelleted via centrifugation for 10 min at 5000 x g. The pellet was resuspended in 6 ml of Resuspension Solution, with ribonuclease (RNase) A Solution. 6 ml of Lysis Solution was then added, mixed gently and incubated for 3 min at RT to liberate the plasmid DNA. Then, 6 ml of Neutralization Solution and 0.8 ml of Endotoxin Binding Reagent were added and mixed to re-anneal plasmid DNA. The solution was incubated for 5 min at RT and the cell debris, chromosomal DNA and sodium dodecyl sulphate (SDS) precipitate were pelleted via centrifugation at 4600 x g for 30 min. The supernatant was mixed with 1 volume of 96% ethanol and 20 ml of this was added to a purification column in a collection tube, centrifuged at 2000 x g for 3 min and the flow through discarded. This was repeated for any remaining sample. The high salt content of the plasmid DNA facilitates binding of the DNA to the spin column. The sample was then cleaned by; one wash with 8 ml of Wash Solution I, and two washes with 8 ml of Wash Solution II. Between washes, samples were centrifuged at 3000 x g for 2 min and for 5 min after the final wash to remove wash solution. 1 ml of Elution buffer was added to the purification column and incubated for 2 min at RT, DNA was eluted via centrifugation at 3000 x g for 5 min. DNA yield was determined via NanoDrop™ (Thermo Scientific; see section 2.5.3) and the DNA was stored at -20°C.

Glycerol Stock

The first time the vector was grown up in culture glycerol stocks were made from the Maxiprep Kit product. 0.5 ml of purified DNA was added to 0.5 ml of 80% sterile glycerol in dH_2O (autoclaved before use). Glycerol stocks were stored at -80°C.

To obtain adequate amounts of vector for transfection, glycerol stocks were streaked onto an agar plate. The following day a single colony was picked and left to grow overnight in LB broth. The DNA was then purified using a Plasmid Maxiprep Kit (as described previously) and stored at -20°C.

Transfection of cells

Cells were plated at ~2.5 x 10^5 per 32 mm well with gelatin-coated coverslips (see section 2.3.2). 24 h after plating, cell culture media was removed, cells were washed once in pre-warmed DPBS and fresh media was supplied. The transfection reagent/DNA mixture was added (see below) for 12 h. Transfection reagent was removed, cells were washed with pre-warmed DPBS and fresh media was added. Cells were fixed and stained (see section 2.3.4) at 24 and 48 h post-transfection. For each transfection, a DNA negative control was prepared, where cells were transfected with transfection reagent but no DNA. A variety of transfection reagents were trialled; FuGENE®, Lipofectamine® 2000 and Nanofectin. Fixation and nuclei staining were used to determine transfection efficiency (see section 2.3.4).

Transfection Reagents:

FuGENE® HD: Plasmid DNA (3.33 μg) in ddH_2O to a final volume of 155 μl was mixed with 9.8 μl of FuGENE® HD. Following 10 min incubation at RT, 150 μl was added to each well.

Lipofectamine® 2000: Before the addition of transfection reagent, cell culture media was replaced with serum-free cell culture media. Plasmid DNA (4 μg) in serum-free media to a final volume of 250 μl and 10 μl of Lipofectamine® 2000 in 240 μl of serum-free media were incubated separately at RT for 5 min. Solutions were combined, incubated at RT for 20 min and 500 μl was added per well.

Nanofectin: Before the addition of transfection reagent, cell culture media was replaced with serum-free media. Plasmid DNA (2 μg) in serum-free media to a final volume of 100 μl was added to 10 μl Nanofectin in 90 μl serum-free media. Following incubation for 20 min at RT, 200 μl was added per well.

2.3.4. DAPI staining of transfected cells

Cells were plated onto coverslips and transfected as described in section 2.3.3. 24 or 48 h post-transfection cells were washed for 5 min in PBS (0.5 ml/32 mm well). Cells were then fixed in 10 % formaldehyde for 5 min. Following fixation, cells were washed twice with PBS, wells were then incubated with DAPI (1 µl 1mg/ml DAPI in 1 ml PBS) for 15 min in the dark. Cells were washed twice in PBS, and the coverslips mounted onto microscope slides with Mowiol (From Dr Dave Johnston, Biomedical Imaging Unit, University of Southampton). Slides were stored at 4°C and visualised either with an Axiovert 200 fluorescent microscope (Zeiss, Oberkochen, Germany) or a SP5 fluorescent confocal microscope (Leica). Transfection efficiency was determined by the following equation:

% transfection efficiency = (Number of transfected cells/Number of DAPI stained nuclei) x 100

2.4. Placental villous fragment culture

Placental villous fragment culture was used to investigate uptake of vitamin D into the human placenta as well as the regulation of nutrient transporter expression in the human placenta in response to vitamin D. Inhibitors of specific endocytic processes were used to determine the mechanism of vitamin D entry into the placenta. Fluorescence microscopy was also used in placental villous fragments to investigate the uptake of albumin, a vitamin D carrier protein. These studies were conducted according to the guidelines in the Declaration of Helsinki, and the Southampton and South West Hampshire Research Ethics Committee approved all procedures. Written informed consent was obtained from all participating women.

2.4.1. Materials and working solutions for placental villous fragment culture

Table 2.2: Materials used for placental villous fragment work.

Material	Supplier
$1,25(OH)_2D$	Cayman Chemical
25(OH)D	Cayman Chemical
2,2'thiodiethanol (TDE)	Sigma-Aldrich
Amiloride hydrochloride hydrate	Sigma-Aldrich
Biotinylated *Datura Stramonium* Lectin (DSL)	Vector Laboratories Inc., California, USA
Bovine serum albumin (BSA)	Fisher Scientific
Calcium chloride dihydrate ($CaCl_22(H_2O)$)	Fisher Scientific
Chlorpromazine hydrochloride	Sigma-Aldrich
D-(+)-glucose	Sigma-Aldrich
DAPI	Sigma-Aldrich
DBP (Gc-globulin from human plasma)	Sigma-Aldrich
DMSO hybrid-max sterile-filtered	Sigma-Aldrich
Dynasore	Dr I. O'Kelly, University of Southampton
Ethanol	Fisher Scientific
FITC labelled albumin	Sigma-Aldrich
FITC labelled *Aleuria Aurantia* Lectin (AAL)	Vector Laboratories Inc.
FITC labelled dextran	Sigma-Aldrich
Gelatin (from bovine skin)	Sigma-Aldrich
Gentamicin solution 50 mg/ml	Sigma-Aldrich
HEPES	Acros Organics, Belgium
L-amino acids	Sigma-Aldrich
Magnesium chloride hexahydrate ($MgCl_26(H_2O)$)	Acros Organics
PBS tablets	Sigma-Aldrich
Potassium chloride (KCl)	Fisher Scientific
Rhodamine labelled *Pisum Sativum* Agglutinin (PSA)	Vector Laboratories Inc.
Sodium chloride (NaCl)	Fisher Scientific
Sodium hydroxide (NaOH)	Fisons Scientific Equipment, Loughborough, UK
Sodium hydroxide (NaOH) solution	Fisher Scientific
Streptavidin 680LT	LI-COR, Lincoln, Nebraska, USA
Triton X 100	Sigma-Aldrich

Working solutions

50 X amino acid mix

13.38 mmol/l Ala, 2.2 mmol/l Arg, 0.5 mmol/l Asp, 4.3 mmol/l Asn, 1.4 mmol/l Cys, 4.75 mmol/l Glu, 18.98 mmol/l Gln, 6.6 mmol/l Gly, 3.9 mmol/l His, 2.15 mmol/l Iso, 3.93 mmol/l Leu, 5.9

mmol/l Lys, 0.98 mmol/l Met, 2.03 mmol/l Phe, 6.3 mmol/l Pro, 4.65 mmol/l Ser, 7.6 mmol/l Thr, 2.05 mmol/l Trp, 1.75 mmol/l Tyr and 6.68 mmol/l Val.

PBS

1 PBS tablet dissolved in 200 ml dH_2O gives 10 mmol/l phosphate buffer, 2.7 mmol/l KCl and 137 mmol/l NaCl, pH 7.4 at 25°C.

Tyrodes

135 mmol/l NaCl, 10 mmol/l HEPES, 5.6 mmol/l D-(+)-glucose, 5 mmol/l KCl, 1.8 mmol/l $CaCl_22(H_2O)$, 1 mmol/l $MgCl_26(H_2O)$ and dH_2O to 1 L. pH to 7.4 with NaOH solution.

2.4.2. Placental villous fragment culture

Full term human placentas were collected within 30 min of delivery. Villous fragments of ~10 mg were cut from the maternal facing side of the placenta and any maternal decidual tissue was removed. Fragments were stored in Tyrodes buffer at RT while the remaining fragments were dissected. Placental villous fragments were exposed to 25(OH)D alone or with albumin, or $1,25(OH)_2D$ alone, with DBP or albumin to investigate the effect of carrier proteins on vitamin D uptake into the placenta. The mechanism of vitamin D uptake was investigated through culturing placental villous fragments with each type of vitamin D alongside carrier proteins combined with exposure to inhibitors of specific endocytic processes. Villous fragments that were to be exposed to endocytic blockers for the duration of the experiment were incubated in a pre-incubation buffer (Table 2.3) containing the specific blocker for 30 min at 37ºC. Villous fragments that were not to be exposed to endocytic blockers for the experiment were incubated in Tyrodes at 37ºC for the pre-incubation period. Then fragments were transferred to experimental buffers at 37°C for the duration of the experiment. Experiments for microscopy were incubated for a maximum of 1 hr, while experiments for RNA had a duration of 8 hr. At the end of microscopy experiments, the buffer was removed and the fragment placed in 4 % paraformaldehyde (PFA) in PBS (From Carol Roberts, University of Southampton). At the end of experiments for RNA, the buffer was removed and the fragments snap frozen on dry ice and stored at -80°C.

Table 2.3: Buffers used for placental villous fragment studies.

Buffer	Components
Pre-incubation buffers	
Amiloride hydrochloride hydrate Tyrodes	5 mmol/l amiloride hydrochloride hydrate (in DMSO) in Tyrodes
Chlorpromazine hydrochloride Tyrodes	1.4 mmol/l chlorpromazine hydrochloride (in dH_2O) in Tyrodes
Dynasore Tyrodes	80 μmol/l dynasore (in DMSO) in Tyrodes
Gentamicin Tyrodes	100 μmol/l gentamicin solution (in dH_2O) in Tyrodes
Experimental buffers	
25(OH)D Tyrodes	20 μmol/l 25(OH)D (in ethanol) in Tyrodes +/- 0.7 mmol/l BSA +/- 5 mmol/l amiloride hydrochloride hydrate
1,25$(OH)_2$D Tyrodes	50 nmol/l 1,25$(OH)_2$D (in ethanol) in Tyrodes +/- 0.7 mmol/l BSA +/- 5 μmol/l DBP +/- 1 X amino acid mix (20 μl 50 X mix/1 ml) +/- gelatin (to match BSA levels) +/- 5 mmol/l amiloride hydrochloride hydrate +/- 1.4 mmol/l chlorpromazine hydrochloride +/- 80 μmol/l dynasore +/- 100 μmol/l gentamicin
FITC-albumin Tyrodes	150 nmol/l FITC-albumin (10 μg/ml) in Tyrodes +/- 5 mmol/l amiloride hydrochloride hydrate +/- 80 μmol/l dynasore
FITC-dextran Tyrodes	1.43 μmol/l FITC-dextran (100 μg/ml) in Tyrodes
Vehicle control buffers	Ethanol (to match 25(OH)D and 1,25$(OH)_2$D levels) and 0.7 mmol/l BSA in Tyrodes +/- DMSO (to match amiloride hydrochloride hydrate levels) +/- 5 mmol/l amiloride hydrochloride hydrate +/- 1.4 mmol/l chlorpromazine hydrochloride +/- 80 μmol/l dynasore +/- 100 μmol/l gentamicin

2.4.3. Lectin staining of placental villous fragments

Placental villous fragments were fixed in 4% PFA on the day of the experiment. The following day samples were transferred to 0.1% sodium azide (5 ml 1% sodium azide in 45 ml PBS; From Dr Rohan Lewis, University of Southampton) and stored at 4°C until staining. Samples were washed three times in PBS for 10 min. Samples were then permeabilised for 2 hr in 1% Triton X 100 (500 μl Triton X 100 in 50 ml PBS). This was followed by three more 10 min washes in PBS. Samples were then incubated in lectin stains in PBS for 1 hr at RT and left overnight at 4°C.

The following day, samples were washed three times in PBS on a shaker at RT. Samples were then incubated with Streptavidin 680LT (1:500 in PBS) for 2 hrs at RT on a shaker, so that the biotinylated lectin could be visualised. Villous fragments were washed three times in PBS and incubated with DAPI (2 μl 1mg/ml DAPI in 1 ml PBS) for 15 min. This was followed by three washes in PBS, before clearing in TDE. Samples were cleared through a series of TDE concentrations; 10%, 25%, 50%, 97%, 97% and 97% (in PBS) and stored at 4°C in 97% TDE. Placental villous fragments were visualised on the SP5 fluorescent confocal microscope. Images were obtained through a series of z-sections through the placental fragment. 6 sections were taken for each image at 10 μm intervals beginning at the tip of the villi, and a minimum of 5 images were taken for each placental villous fragment. A minimum of 2 images were taken for each FITC-dextran placental fragment. For more detailed images, a greater number of z-sections were taken at smaller intervals to create z-stacks.

2.5. RNA

RNA was used to determine and compare expression levels of genes of interest from cell culture and human placental samples. RNA was extracted and converted to complementary DNA (cDNA). cDNA was used for reverse transcription polymerase chain reaction (rt-PCR) to determine whether a gene of interest was present, and quantitative rt-PCR (qrt-PCR) was used to determine expression levels of genes of interest. qrt-PCR was used to investigate the effects of vitamin D and removal of methylation of expression of specific placental genes.

2.5.1. Materials and working solutions for RNA work

Table 2.4: Materials for RNA work.

Material	Supplier
Acid-phenol:chloroform	Ambion, Paisley, UK
Agarose	Sigma-Aldrich
Chloroform	BDH Laboratory Supplies, Poole, UK
Deionised formamide	BDH Laboratory Supplies
Deoxyribonuclease (DNase) I kit	Sigma-Aldrich
DNA ladders: 100 base pairs (bp) and 1 kb	Promega
Ethanol	Fisher Scientific
External RNA Controls Consortium RNA Spike-In Mix 1	Ambion
GelRed™	Biotium, San Francisco, USA
geNorm HKG selection kit	Primer Design, Southampton, UK
Isopropanol	Fisher Scientific
Lightcycler 480 Probes Master Mix	Roche, West Sussex, UK
Loading dye	Ambion
miRNeasy mini kit	Qiagen, West Sussex, UK
mirVana™ miRNA isolation kit	Ambion
Moloney Murine Leukaemia Virus (M-MLV) Reverse Transcriptase	Promega
Polymerase chain reaction (PCR) master mix 2 X	Promega
PCR nucleotide mix	Promega
Precision nanoScript™2 reverse transcription (rt) kit	Primer Design
Primers (rt-PCR and Roche qrt-PCR)	Eurogentec, Liege, Belgium
Primer/probe mix (Perfect Probe qrt-PCR)	Primer Design
Random hexamer primers	Promega
Recombinant RNAsin RNase inhibitor	Promega
RNA 6000 Nano/Pico kit	Agilent, California, USA
RNase-free DNase Set (for use with miRNeasy mini kit)	Qiagen
RNAzol®	Sigma-Aldrich
TRI Reagent®	Sigma-Aldrich
Tris borate EDTA (TBE) buffer 10 X	Fisher Scientific
TruSeq™ Stranded mRNA sample preparation kit	Illumina, San Diego, California, USA
Universal probe library (UPL) probes	Roche
Wizard® SV gel and PCR clean-up system	Promega

Working solutions

1 X TBE

100 ml 10 X TBE and 900 ml dH_2O.

2.5.2. RNA extraction

A number of RNA extraction methods were trialled; TRI Reagent®, RNAzol®, mirVana™ miRNA isolation kit, miRNeasy and the use of DNase alongside these methods. Unless stated otherwise RNAzol® was used for RNA extraction from HEK293 and BeWo cell culture and the mirVana™ miRNA isolation kit or miRNeasy kit was used for extraction of mRNA from human placental tissue.

TRI Reagent®

TRI Reagent® was used to extract RNA from syncytiotrophoblast cells. 1 ml of TRI Reagent® was added per 32 mm well and cells were scraped and transferred to a pre-cooled eppendorf. Samples were homogenised and incubated on ice for 5 min. Chloroform (0.2 ml/ml TRI Reagent®) was added to allow phase separation. Samples were shaken vigorously for 30 s and incubated on ice for 5 min. Following centrifugation (15 min at 12,000 x g at 4°C) the aqueous phase was transferred to a fresh eppendorf, mixed with isopropanol (0.5 ml/ml TRI Reagent®) and left overnight at -20°C. The following day, the sample was centrifuged (10 min at 12,000 x g at 4°C) to pellet the RNA. The RNA pellet was resuspended in 75% ethanol (1 ml/ml TRI Reagent®) and centrifuged (7500 x g for 5 min at 4°C) to clean the pellet. The supernatant was removed and the pellet air dried for 45 min before resuspending in 30 µl ddH_2O and dissolving at 60°C.

RNAzol®

1 ml of RNAzol® was added per 32 mm well and cells were scraped and transferred to a pre-cooled eppendorf. DNA, protein and polysaccharide were precipitated from the sample by the addition of RNase-free water (0.4 ml/ml RNAzol®). The sample was shaken vigorously for 15 s and then incubated at RT for 15 min. The sample was then centrifuged at 12,000 x g for 15 min and the upper supernatant, containing RNA, was transferred to a fresh tube and an equal volume of 70% isopropanol added to precipitate RNA. After incubating at RT for 10 min the mixture was centrifuged at 12,000 x g for 10 min to pellet the RNA. RNA pellets were washed three times with 75% ethanol (0.5 ml/ml supernatant used for precipitation). Samples were centrifuged at 8000 x g for 3 min to pellet any remaining RNA and the alcohol was removed. The RNA pellet was then resuspended in 100 µl of RNase-free water.

mirVana™ miRNA isolation kit

For extraction of total RNA from tissue samples, 50 mg of powdered tissue was added to a pre-chilled homogenization tube with 1 ml of Lysis/Binding buffer. Samples were homogenised and centrifuged at 10,000 x g for 5 min and the supernatant transferred to a fresh tube. 100 µl of miRNA Homogenate Additive was added and samples were vortexed and incubated on ice for 10 min. Following incubation, 1 ml of acid-phenol:chloroform was added and samples were vortexed for 1 min. Samples were centrifuged for 5 min at 10,000 x g and the aqueous phase transferred to a new tube. 1.25 volumes of

100% ethanol were added to the sample and 700 µl of this was added to a filter cartridge column in a collection tube at a time. Between each addition of sample, the collection tubes with filter cartridge were centrifuged for 20 s at 10,000 x g until the entire sample was added to the filter-tube assembly. The RNA bound to the filter was then washed through a series of three wash steps; 700 µl miRNA Wash Solution 1, and two washes with 500 µl miRNA Wash Solution 2/3. Between each wash samples were centrifuged for 20 s at 10,000 x g. After the final wash, samples were centrifuged at 10,000 x g for 1 min to remove any residual ethanol. After placing the filter in a fresh collection tube, 100 µl of RNase-free water, at 95°C, was added. The sample was left to stand for 30 s and then centrifuged at 10,000 x g for 30 s to elute the RNA.

miRNeasy

30 mg of powdered placental tissue was homogenized in 700 µl of Lysis Reagent. The homogenate was incubated at RT for 5 min, then 140 µl of chloroform was added and the sample shaken vigorously for 15 s. The homogenate was then incubated at RT for 3 min before being centrifuged at 12,000 x g at 4°C for 15 min. Following centrifugation, 350 µl of the upper aqueous phase was transferred to a new tube and mixed with 525 µl of 100% ethanol. 700 µl of the sample was then transferred to an RNeasy spin column in a collection tube. The sample was spun at 8000 x g for 15 s at RT and the flow through discarded. This was repeated for the remainder of the sample. 350 µl of Buffer RWT was then added to the sample, centrifuged at 8000 x g for 15 s at RT and the flow through discarded. 80 µl of DNase I incubation mix (10 µl DNase and 70 µl Buffer RDD per sample) was added directly to the membrane of the RNeasy spin column and left at RT for 15 min. Following the DNase incubation, 350 µl of RWT Buffer was added to the sample and centrifuged at 8000 x g for 15 s at RT and the flow through discarded. This was followed by two washes with 500 µl of Buffer RPE. For the first wash the sample was spun at 8000 x g for 15 s at RT, for the second wash the time was increased to 2 min to remove any remaining ethanol. The RNeasy spin column was then moved to a fresh tube and 50 µl of RNase-free water added. RNA was eluted by centrifugation at 8000 x g for 1 min at RT.

DNase treatment

Following RNA extraction with RNAzol®, BeWo samples were treated with DNase I to degrade any remaining genomic DNA. 2 µl of DNase and Reaction Buffer were added to 2 µg of RNA and the reaction volume adjusted to 20 µl by the addition of ddH_2O. The mixture was incubated for 20 min at RT, and the reaction stopped by the addition of 2 µl of Stop Solution and incubation at 70°C for 10 min.

2.5.3. Determination of RNA yield and quality

RNA yield and quality were determined using a NanoDrop™ Spectrophotometer, a Bioanalyser and gel electrophoresis.

NanoDrop™

The NanoDrop™ determines RNA quantity through measuring absorbance at 260 nm. Purity of the sample is assessed by the 260/280 ratio; the ratio of absorbance at 260 and 280 nm. A ratio of ~2.0 is accepted for pure RNA. If the ratio is considerably lower, the sample may be contaminated by protein, phenol or other substances which absorb at 280 nm. A further ratio, the 260/230 ratio, is used as a secondary measure of sample purity. A ratio of ~2.0 to 2.2 is generally accepted as pure nucleic acid. To measure samples using the NanoDrop™, the NanoDrop™ was first blanked with a water measurement. Then 1.5 µl of the sample was loaded and absorbance measured at 260 nm.

Gel electrophoresis

RNA quality was assessed through gel electrophoresis. Gel electrophoresis separates molecules based on size and charge. Due to the presence of phosphate groups, RNA is a negatively charged molecule. When an electric current is passed through the samples in an agarose gel, the negatively charged RNA moves towards the positive electrode. The agarose within the gel forms a mesh-like structure which retards the movement of larger molecules, therefore smaller molecules move further towards the positive electrode. 1% agarose gels were used for RNA quality.

Agarose gels (1%; 1 g in 100 ml 1 X TBE) were prepared with 0.01% GelRed™ (10 µl) to allow visualisation of nucleic acid bands under UV light. Once set, the gel was placed in an electrophoresis tank filled with 1 X TBE buffer. RNA samples were prepared (5 µl ddH_2O, 3 µl neat RNA, 2 µl deionised formamide and 2 µl loading dye), loaded into the gel and run at 100 V for 30 min. Nucleic acid bands were viewed under UV light. Good quality RNA was represented by 2 bands indicating 28S and 18S ribosomal RNA (rRNA). A representative image of an RNA gel is shown in Figure 2.5.

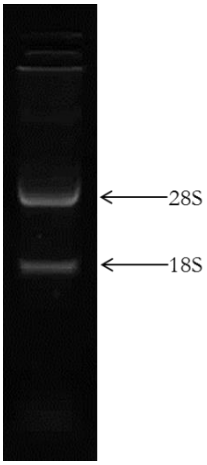

Figure 2.5: Representative image of an RNA gel. Bands represent 28S and 18S rRNA.

Bioanalyser

For RNA from the human cohorts RNA quality was assessed with an RNA 2100 Bioanalyser (Agilent). The Bioanalyser works on the same principle as gel electrophoresis but uses microfluidics. Samples are

loaded onto a chip which contains 12 sample wells and a ladder well. The wells are interconnected by micro channels which become filled with a gel-dye mix during preparation. Electrodes within the Bioanalyser fit into the wells of the chip and the charged RNA moves upon application of a current, allowing separation of the RNA molecules by size. Fluorescent dye intercalates with the RNA strands and this signal is detected by laser-induced fluorescence. Samples are compared to the known sizes of the ladder by the software to determine RNA concentration and rRNA peaks of each sample.

To prepare the gel, 550 µl of RNA 6000 gel matrix was added into a spin filter and centrifuged at 1500 x g for 10 min. 65 µl of filtered gel was then added to a fresh tube along with 1 µl of RNA 6000 dye concentration. This gel-dye mix was then centrifuged at 13,000 x g for 10 min. The RNA chip was placed in the chip priming station and 9 µl of gel-dye mix was added into the first gel well. This was set by a plunger which was released after 30 s. This process was repeated for the remaining 11 gel wells. The wells were then loaded with 5 µl of RNA Marker, and 1 µl of ladder was added into the ladder well. 1 µl of RNA sample was added to each well on the chip, and the chip was spun on an IKA vortexer™ (Agilent) for 1 min at 2400 rpm. The chip was then run on the Bioanalyser for 20 min. Data from the Bioanalyser includes a graph representing rRNA peaks which is also translated into a representative gel image (Figure 2.6), RNA concentration and RNA integrity number (RIN). The RIN is a measure of RNA quality and a RIN of above 6.0 indicates good quality RNA.

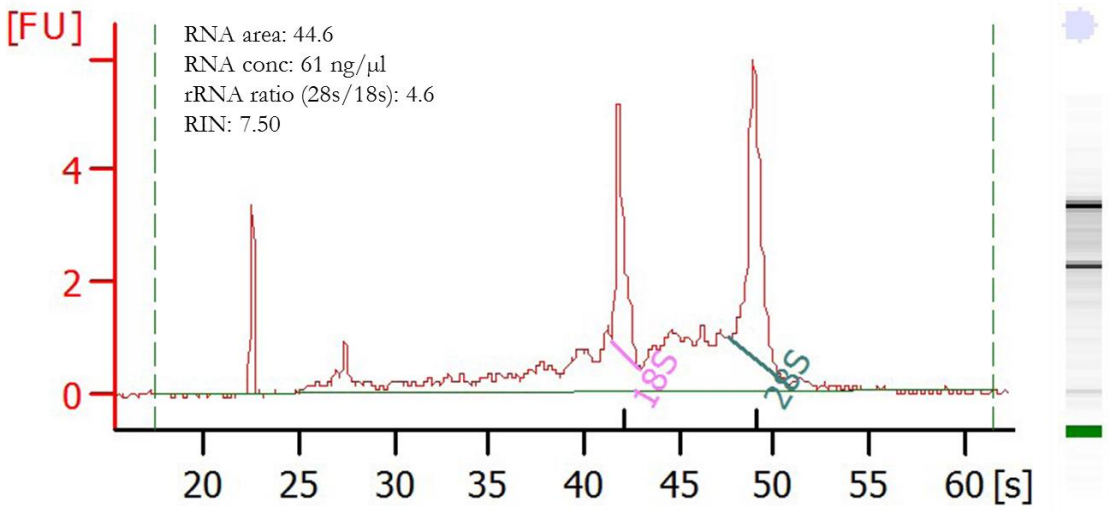

Figure 2.6: A representative image of data from bioanalysis of an RNA sample. The graph shows peaks for 18S and 28S rRNA. When a sample is degraded the baseline is higher and the two peaks are less distinct. The flourescence data from the peaks of the graph is converted into a representative gel image as shown on the right. The analysis also gives RNA concentration and a RIN value which indicates RNA quality.

2.5.4. Reverse transcription

mRNA was reverse transcribed into cDNA for use in mRNA expression studies. Two rt protocols were used; one using the Promega protocol with M-MLV reverse transcriptase and the other with Precision nanoScript™2 rt kit.

Promega M-MLV reverse transcriptase rt

200-500 ng RNA was added to 0.5 μg random hexamer primers and ddH_2O to a final volume of 15 μl and heated for 5 min at 70°C on a Veriti 96 well thermal cycler (Applied Biosystems, California, USA). 25 units (u) Recombinant RNAsin RNase inhibitor, 200 u M-MLV rt, 5 X M-MLV rt reaction buffer, 0.5 mmol/l each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP) and 2.25 μl ddH_2O were added to each RNA reaction mixture. This was then heated at 37°C for 60 min, and 75°C for 10 min in the thermal cycler.

Precision nanoScript™2 rt

200 ng RNA was added to 1 μl random nonamer primers and the volume made up to 10 μl with ddH_2O. The samples were heated to 65°C for 5 min in a thermal cycler and cooled immediately on ice. 5 μl nanoScript2 4 X buffer, 3 μl ddH_2O, 1 μl 10 mmol/l deoxynucleotide triphosphate (dNTP) mix and 1 μl nanoScript2 reverse transcriptase enzyme were added to each RNA reaction mixture. This was then heated to 25°C for 5 min, 42°C for 20 min and the reaction stopped by heating to 75°C for 10 min.

For both methods, all samples were produced in one batch to reduce variation. No enzyme controls (NECs) were made during rt to demonstrate the absence of genomic DNA. For NECs, the cDNA synthesis protocol was identical apart from the addition of an extra 1 μl of ddH_2O in place of rt enzyme. Coefficient of variation (CV) controls were also transcribed. This was 1 mRNA sample created from a pooled RNA stock, which was separated into 6 separate samples, all of which were reverse transcribed at the same time, to test for experimental variation. cDNA was diluted 1:15 in ddH_2O and stored at -20°C.

2.5.5. rt-polymerase chain reaction

rt-PCR was used to determine the presence or absence of specific genes within the placenta or a given cell line. Intron-spanning primers were designed using Primer3 software (National Human Genome Research Institute, USA, http://primer3.ut.ee/) with sequence information obtained from NCBI Nucleotide (National Centre for Biotechnology Information, National Institutes of Health, Maryland, USA, http://www.ncbi.nlm.nih.gov/nuccore). Where possible, primers were designed to cover all transcript variants. The average length of each primer was 20 bp. PCR reactions containing 3.2 ng cDNA, 1 μmol/l forward and reverse primers, 12.5 μl of 2 X PCR Master Mix and ddH_2O to a final volume of 25 μl were run on a 96 well thermal cycler. Cycling conditions were 94°C for 3 min; 40 cycles of 94°C for 30 s for denaturation of DNA molecules into single strands, primer annealing temperature for 30 s and 72°C for 30 s for elongation of PCR products; and 72°C for 7 min to ensure full elongation of any remaining single stranded DNA. The annealing temperature was altered for

specific primer pairs and temperature gradients were run on primer sets that did not show amplification of a product. Controls were run with each PCR run; NECs to prove amplification of cDNA and not genomic DNA and no template controls (NTCs) where water was added to the reaction in place of DNA to prove that amplification was not due to contamination of reagents. Where genes were not detected within placental tissue primer sets were tested on liver or kidney cDNA (From Dr E. Lofthouse, University of Southampton) as a positive control.

2.5.6. Gel electrophoresis

Gel electrophoresis was used to separate PCR products and determine their size relative to a DNA ladder. 1% agarose gels were prepared as described in section 2.5.3. For small product sizes 3-4% agarose gels were used. After allowing the gel to set, DNA ladder (6 µl DNA ladder, 6 µl ddH_2O and 4 µl loading dye) was loaded into the first well of the gel; depending on the expected size of the product, one of two DNA ladders; 100 bp or 1 kb were used (Figure 2.7). DNA samples were prepared (15 µl DNA sample and 3 µl loading dye) and loaded into the remaining wells of the gel. Gels were run at 100 V for 1 h and visualised under UV light.

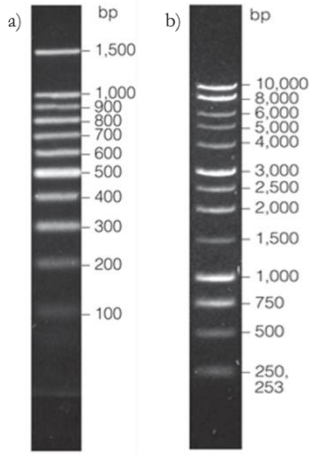

Figure 2.7: DNA ladders. a) 100 bp ladder. b) 1 kb ladder. Images modified from Promega.

2.5.7. Purification of PCR products for sequencing

PCR products were sent for DNA sequencing to confirm the correct product had been amplified. PCR products were purified for sequencing using Wizard® SV Gel and PCR Clean-Up System. Where bands were excised from the gel under UV light, the gel slice was weighed and 10 µl of Membrane Binding Solution was added per 10 mg of gel slice. This was heated at 65°C until the gel slice was fully dissolved. Where product was purified from the remaining PCR solution, this was added to an equal volume of Membrane Binding Solution. The solutions were then transferred to a minicolumn inserted into a collection tube and incubated at RT for 1 min to allow binding of DNA to the minicolumn.

Column assemblies were spun at 16,000 x g for 1 min and the flow through discarded. 700 µl of Membrane Wash Solution was added to each column, followed by centrifuging at 16,000 x g for 1 min. This was followed by a second wash with 500 µl of Membrane Wash Solution and 5 min of centrifugation. After emptying the collection tube, the column assembly was recentrifuged for 1 min with the microcentrifuge lid off to allow evaporation of residual ethanol. The minicolumn was then transferred to a clean microcentrifuge tube, 50 µl of ddH_2O was added and after incubation at RT for 1 min DNA was eluted into the microcentrifuge tube through centrifugation at 16,000 x g for 1 min. DNA concentration was assessed through the NanoDrop™ (section 2.5.3). Samples were then stored at –20°C. Samples were then sent to GATC Biotech (Konstanz, Germany) for DNA sequencing.

2.5.8. Quantitative rt-PCR

qrt-PCR was used to compare the mRNA expression levels of specific genes between samples in relation to appropriate HKGs. qrt-PCR was carried out using Perfect Probe or UPL designed assays and run on the Lightcycler 480 PCR machine (Roche). For each qrt-PCR assay, samples were run in triplicate and the following controls were used; NECs, CV controls and NTCs. A set of 7 standards each containing a known RNA concentration were used to calculate the concentration of cDNA for each gene.

Housekeeping genes

HKGs are used for the normalisation of target gene expression data in qrt-PCR. This normalisation aims to correct for differences between samples that are not a result of the experimental variable being tested. Variables that are controlled for by normalising data to HKGs include differences in the amount of tissue or RNA, different enzyme efficiencies during the rt reaction and differences in overall transcriptional activity in the specific tissues or cells. HKGs are constitutively expressed genes that are involved in normal cellular processes. For example, one common HKG, *UBC*, is involved in protein degradation. For accurate normalisation a combination of HKGs should be used (Vandesompele *et al.*, 2002). Studies have revealed differences in expression of HKGs with specific treatments and between cell types (Thellin *et al.*, 1999; Schmittgen and Zakrajsek, 2000; Suzuki *et al.*, 2000; Warrington *et al.*, 2000; Radonić *et al.*, 2004; Cleal *et al.*, 2010), therefore it is important to establish which HKGs are appropriate for normalisation within a specific experiment

The geNorm HKG selection kit was used to establish the appropriate number and the most stable HKGs to use for qrt-PCR data normalisation. The HKGs tested are listed in Table 2.5. When evaluating HKG expression and stability a minimum of 5 samples per experimental condition were tested. geNorm was used to investigate HKG differences between placental, BeWo and HEK293 cells as well as to examine the appropriate HKGs for MAVIDOS placental samples.

Table 2.5: HKGs tested for stability in cDNA samples.

Gene	Gene name	Accession number (GenBank)
ACTB	*β-ACTIN*	NM_001101.3
ATP5B	*ATP synthase*	NM_001686
CYC1	*Cytochrome C-1*	NM_001916.4
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	NM_002046
RPL13A	*Ribosomal protein L13a*	NM_012423.3, NM_001270491.1
SDHA	*Succinate dehydrogenase complex, subunit A*	NM_004168
TOP1	*Topoisomerase (DNA) 1*	NM_003286.2
UBC	*Ubiquitin C*	NM_021009
YWHAZ	*Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta*	NM_003406.3, NM_145690.2, NM_001135699.1, NM_001135700.1, NM_001135701.1, NM_001135702.1

For geNorm, samples from each condition to be tested were run with each HKG in the qrt-PCR reaction described below. Following qrt-PCR the crossing point (cp) values (see below) of the HKGs were analysed using the qrt-PCR analysis software qbase+ (Biogazelle, Belgium). The analysis uses the gene expression stability measure (M) devised by Vandesompele *et al.* (2002). The M value is based on the principle that for two ideal control genes the expression ratio of the two genes should be identical in all samples. Increasing variation in this expression ratio results in a reduced M value, i.e. reduced expression stability. To determine the M value for each HKG the pairwise expression ratio with all other HKGs is calculated, this value is log transformed and the standard deviation (SD) determined. The average of pairwise variation of the HKG in question with all other control genes is determined to give the M value. The lower the M value the more stable the HKG is for that given experiment. The qbase+ software calculates the M value for all the HKGs tested and produces a graph ranking the HKGs in order of stability. An example graph of M values resulting from geNorm analysis is displayed in Figure 2.8.

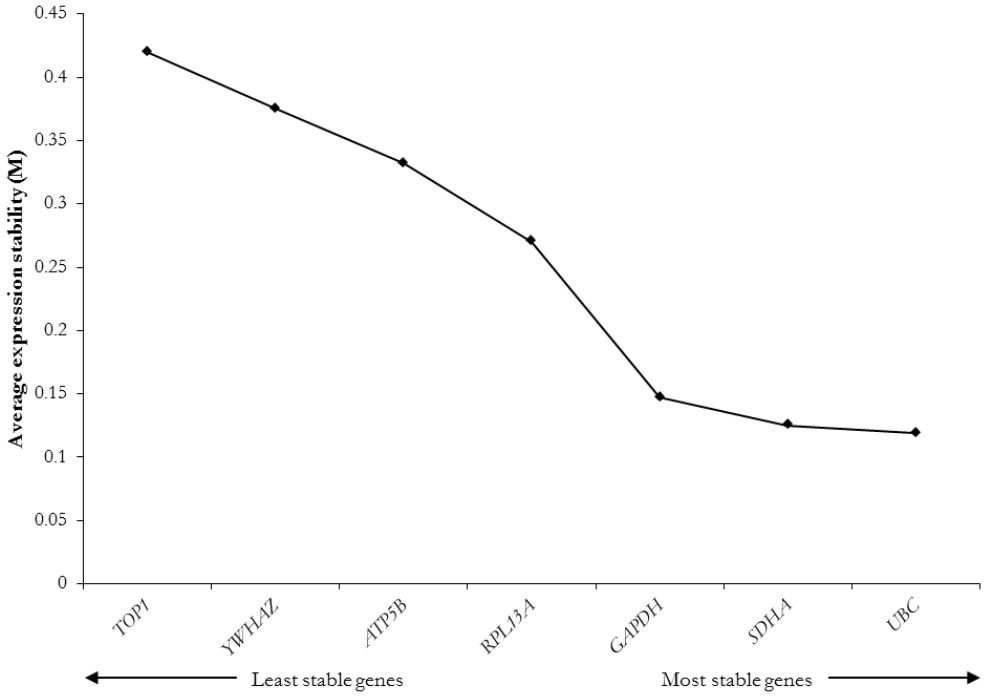

Figure 2.8: An example graph showing M values for HKGs from geNorm analysis. HKGs with the lowest M values are the most stably expressed and therefore better for normalisation of data.

A value called the V score is also determined; this indicates how the normalization factor changes when another gene is included in the calculation. Starting with the most stably expressed genes, as determined by the M values, the sequential addition of each HKG is assessed for the resulting level of variation in the normalisation factor. A V score of 0.15 or below indicates that the additional gene has no significant contribution to the newly calculated normalization factor and is therefore not needed (Figure 2.9).

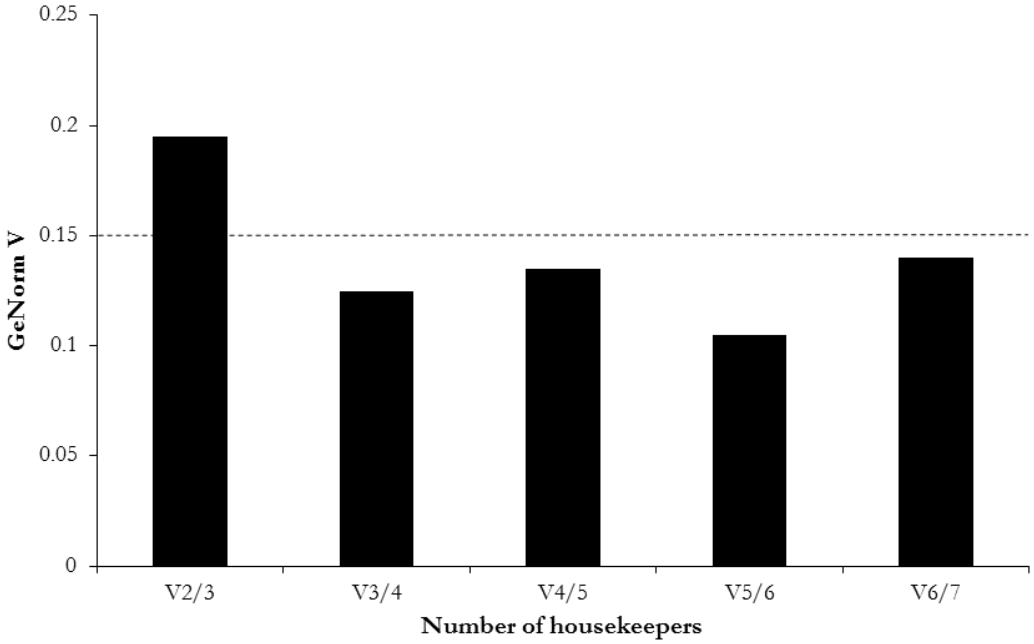

Figure 2.9: An example graph showing V values for HKGs from geNorm analysis. V values are shown with the sequential addition of HKGs. A cut-off value of 0.15 is set, as indicated by the dashed line, after which the inclusion of an extra HKG is not required. For this particular example the V score for 3 or 4 HKGs is the first that reaches a score below 0.15, therefore 3 or 4 HKGs should be used.

Once appropriate HKGs were determined, target gene expression was then normalised to the geometric mean of the HKGs. The geometric mean is used as it controls better for possible outliers than the arithmetic mean.

qrt-PCR reactions

Target gene expression was measured with Perfect Probe or UPL qrt-PCR assays. qrt-PCR reaction components for target gene and HKG assays are displayed in Table 2.6 and cycling conditions are displayed in Table 2.7. Intron spanning oligonucleotide probes and primers were designed using the Roche ProbeFinder Version 2.50. Probes were supplied from the UPL, and primers were synthesised by Eurogentec.

Table 2.6: Concentration of PCR components used for both Roche UPL and Perfect Probe qrt-PCR assays.

PCR component	UPL	Perfect Probe
Lightcycler 480 Probes Master Mix	5 µl	5 µl
Primer/probe mix	N/A	300 nmol/l
Forward primer	33 nmol/l	N/A
Reverse primer	33 nmol/l	N/A
Probe	133 nmol/l	N/A
DNA	4 ng	4 ng
ddH_2O	N/A	To final volume
Final volume	10 µl	10 µl

Table 2.7: Cycling conditions used for Roche UPL, Perfect Probe and Perfect Probe HKG qrt-PCR assays.

Cycling conditions	UPL	Perfect Probe	Perfect Probe HKGs
Polymerase activation:	95°C for 10 min	95°C for 10 min	95°C for 10 min
Product amplification:	45 cycles of:	50 cycles of:	50 cycles of:
	95°C for 10 s	95°C for 10 s	95°C for 15 s
	60°C for 30 s	60°C for 60 s*	50°C for 30 s*
	72°C for 1 s*		72°C for 15 s
Cooling:	50°C for 30 s	50°C for 30 s	50°C for 30 s

* indicates data collection step.

Calculation of gene expression levels

Cp values were calculated using the Second Derivative Max Method (Roche). This method takes the cp value at the point on the amplification curve where acceleration of the fluorescence signal is at its maximum, i.e. where the fluorescence reaches the maximum of the second derivative of the amplification curve. Using this method the cp value should always be located within the middle of the log linear phase of the amplification curve. Cp values were plotted against the known log cDNA concentration for each standard (see Table 2.8 and Figure 2.10 for example) and the cp values for each sample converted to a cDNA concentration through the equation:

$$x = 10^{((y - c)/ m)}$$

Where m refers to the gradient of the line, c refers to the y intercept and y refers to the Cp value of the sample.

Table 2.8: An example of standard curve dilutions and concentrations.

Standard	Dilution		Concentration (ng/5ul)
S1	30 µl stock		2000
S2	15 µl S1	15 µl ddH_2O	1000
S3	15 µl S2	15 µl ddH_2O	500
S4	15 µl S3	15 µl ddH_2O	250
S5	15 µl S4	15 µl ddH_2O	125
S6	15 µl S5	15 µl ddH_2O	62.5
S7	15 µl S6	15 µl ddH_2O	31.25

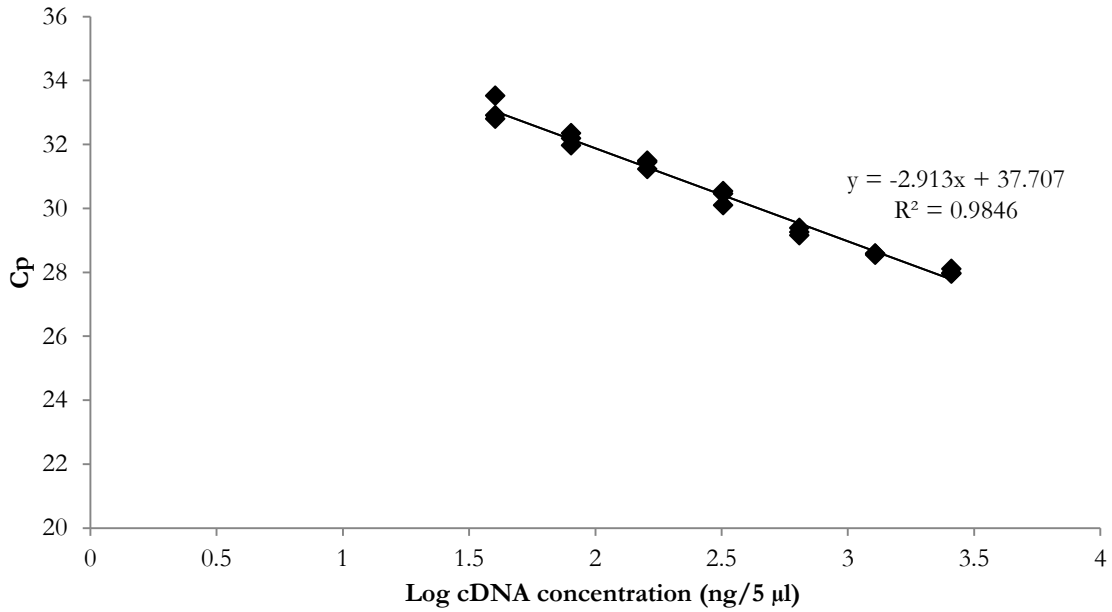

Figure 2.10: An example qrt-PCR standard curve.

After calculation of DNA concentration, samples and CV controls were divided by the geometric mean of the HKGs. Then the CV of the 6 CV controls was calculated by:

$$CV = (SD / mean) \times 100$$

In a few cases where the standard curve failed, qrt-PCR data was analysed by $2^{-\Delta\Delta cp}$. This calculates change in gene expression in relation to a control sample and is calculated as follows:

1. ΔCp = Cp of target gene – geometric mean of HKG cps
2. ΔΔCp = ΔCp of sample – ΔCp of specific control sample
3. $2^{-\Delta\Delta Cp}$ = 2^(- ΔΔCp)

2.5.9. RNA sequencing

Total RNA from placental villous fragment experiments was sent for RNA sequencing to further investigate the effects of 25(OH)D on placental mRNA expression. Samples incubated with 25(OH)D, 25(OH)D with albumin, and vehicle with albumin control were analysed. 450 ng of total RNA was prepared at a dilution of 30 ng/µl and paired-end RNA sequencing was carried out by Expression Analysis Inc (Durham, North Carolina, USA). First cDNA libraries for paired-end sequencing were built from RNA samples using the TruSeq™ Stranded mRNA sample preparation kit. Briefly, 1 µl of a 1:1000 dilution of External RNA Controls Consortium RNA Spike-in mix 1 was added to each sample. Polyadenylated RNA was then purified using magnetic beads bound to oligo-dT. Purified mRNA was then fragmented with heat in the presence of divalent cations. rt with random hexamer priming was used to convert mRNA fragments into single-stranded cDNA. Double-stranded cDNA was generated

from this with deoxyuridine triphosphate (dUTP) in place of dTTP in the second strand master mix. A single 'A' base was added to the cDNA. Forked adapters that include index sequences were ligated to the end of each fragment to enable attachment to the flow cell surface. PCR was then performed on the cDNA to amplify the ligated maternal. The polymerase used for PCR stalls when encountering a dUTP in the DNA template. As only the second DNA strand contains a dUTP, this results in the first strand being the only viable template thereby preserving the strand information. Final cDNA libraries were quantified, normalized and pooled. Pooled cDNA libraries were then bound to the surface of a flow cell. Individual clusters were created from each bound template by clonally amplifying each bound template molecule up to 1000 fold. The DNA sequence was determined using sequencing-by-synthesis technology. Sequencing-by-synthesis involves the addition of labelled nucleotide bases; guanine, cytosine, adenosine and thymine. Four fluorescently labelled nucleotides were flowed over the surface of the flow cell and incorporated into each nucleic acid chain. Each nucleotide label terminates polymerisation, therefore ensuring that in each cycle only one single base is added to each nascent DNA chain. During each cycle the fluorescence was measured for each cluster. The level of fluorescence omitted following laser excitation is then used to determine the identity of the incorporated base. The dye was then enzymatically removed to allow incorporation of the next nucleotide and the cycle was repeated. Sequencing was carried out to a depth of 19 million reads. The raw sequencing information was then aligned against the human transcriptome to align the reads to the genome and identify the genes within each sample.

2.6. DNA methylation analysis

DNA methylation levels of CpGs near to and within a VDRE within the promoter region of *TAT1* were investigated, as these may affect the response to vitamin D of this gene. For analysis of DNA methylation at specific CpGs; DNA was extracted and bisulfite converted, the selected region amplified through PCR and the methylation analysed by pyrosequencing. Bisulfite conversion of DNA converts unmethylated cytosines to uracil while methylated cytosines remain as cytosines. During subsequent PCR the uracil is then converted to a thymine (Figure 2.11). During pyrosequencing the methylation level is determined by measuring the levels of cytosine and thymine at selected CpGs.

Figure 2.11: Changes to the DNA sequence during bisulfite conversion and PCR. During bisulfite conversion unmethylated cytosines are converted to uracil, which is converted to thymine during PCR (shown in red). Methylated cytosines are protected and remain as cytosines throughout the conversion and PCR processes (shown in green).

2.6.1. Materials and working solutions for methylation analysis

Table 2.9: Materials for methylation analysis.

Material	Supplier
Agarose	Sigma-Aldrich
CpGenome™ Universal methylated DNA	Millipore, Billerica, Massachusetts, USA
DNA ladders: 100 bp and 1 kb	Promega
EpiTect® unmethylated control DNA	Qiagen
Ethanol	Fisher Scientific
EZ-96 DNA methylation gold kit™	Zymo Research, California, USA
GelRed™	Biotium
KAPA2G Robust HotStart ReadyMix	Kapa Biosystems, Massachusetts, USA
Loading dye	Ambion
Primers (for pyrosequencing)	Biomers, Germany
Pyromark® 10 X wash buffer	Qiagen
Pyromark® annealing buffer	Qiagen
Pyromark® assay design	Qiagen
Pyromark® binding buffer	Qiagen
Pyromark® gold Q96 CDT reagents-dNTPs, enzyme and substrate	Qiagen
Pyromark MD pyrosequencer	Biotage, Uppsala, Sweden
Pyromark® Q96 capillary tip	Qiagen
Pyromark® Q96 HS plate	Qiagen
Pyromark® Q96 reagent tip	Qiagen
QuickPick™ SML gDNA kit	BN, Finland
Sodium hydroxide (NaOH)	Fisons Scientific Equipment
Streptavidin sepharose beads	Fisher Scientific
TBE buffer 10 X	Fisher Scientific

Working solutions

Denaturation solution

136 mmol/l NaOH (8 g) in 1 L ddH_2O.

1 X TBE

100 ml 10 X TBE and 900 ml dH_2O.

Wash Buffer 1 X

100 ml 10 X Pyromark® Wash Buffer and 900 ml ddH_2O.

2.6.2. DNA extraction

DNA was extracted from 10 mg of ground placenta using the QuickPick™ SML gDNA kit. Protein was degraded and the sample lysed through incubation for 1 h at 56°C with 20 µl of Proteinase K and 200 µl of Lysis Buffer. RNA in the sample was degraded by incubation with 3 µl RNase at RT for 10 min. DNA was bound to 16 µl of magnetic particles by mixing with 500 µl Binding Buffer. The PickPen® was used to transfer the magnetic particles with the bound DNA through two successive tubes of 750 µl Wash Buffer 1, and one tube of 750 µl Wash Buffer 2. The magnetic particles were transferred into a final tube containing 200 µl of Elution Buffer. After continuous mixing at RT for 10 min, the DNA dissociated from the particles and the magnetic particles were removed with the PickPen®. The eluate containing the genomic DNA was centrifuged at 12,000 x g for 30 min and the supernatant removed. Yield and quality of the DNA were assessed and the DNA was stored at –20°C.

2.6.3. Determination of DNA yield and quality

DNA yield and quality were determined through the use of a NanoDrop™ and gel electrophoresis.

NanoDrop™

The NanoDrop™ determines DNA quantity and quality through measuring absorbance at 260 nm. The NanoDrop™ was used as described in section 2.5.3. For pure DNA a 260/280 ratio of ~1.8 indicates pure DNA, and a 260/230 ratio of ~2.0 to 2.2 indicates pure nucleic acid.

Gel electrophoresis

DNA quality was also assessed through gel electrophoresis using 0.8% agarose gels (100 ml 1 X TBE and 0.8 g agarose) with 0.01% (10 µl GelRed™). DNA samples were prepared (7 µl ddH_2O, 5 µl neat DNA sample and 3 µl loading dye) and loaded into wells. Gels were run at 100 V for 30 min. A representative image of a DNA gel is shown in Figure 2.12.

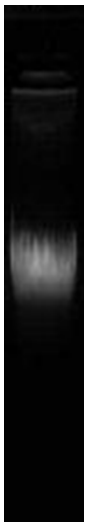

Figure 2.12: Representative image of a DNA gel.

2.6.4. Bisulfite conversion

Bisulfite conversion of DNA was carried out using EZ-96 DNA Methylation Gold Kit™ as per manufacturer's instructions. Briefly, 1 μg of DNA was made to a total volume of 20 μl with ddH_2O in a Conversion Plate and 130 μl Conversion Reagent was added. DNA of known methylation level (0% and 100%) was also bisulfite converted for use as a control during pyrosequencing. The Conversion Plate was heated on a thermal cycler at 98°C for 10 min, to denature the DNA into a single stranded structure, and 64°C for 2.5 h, for the conversion of unmethylated cytosine to uracil sulfonate (Figure 2.13).

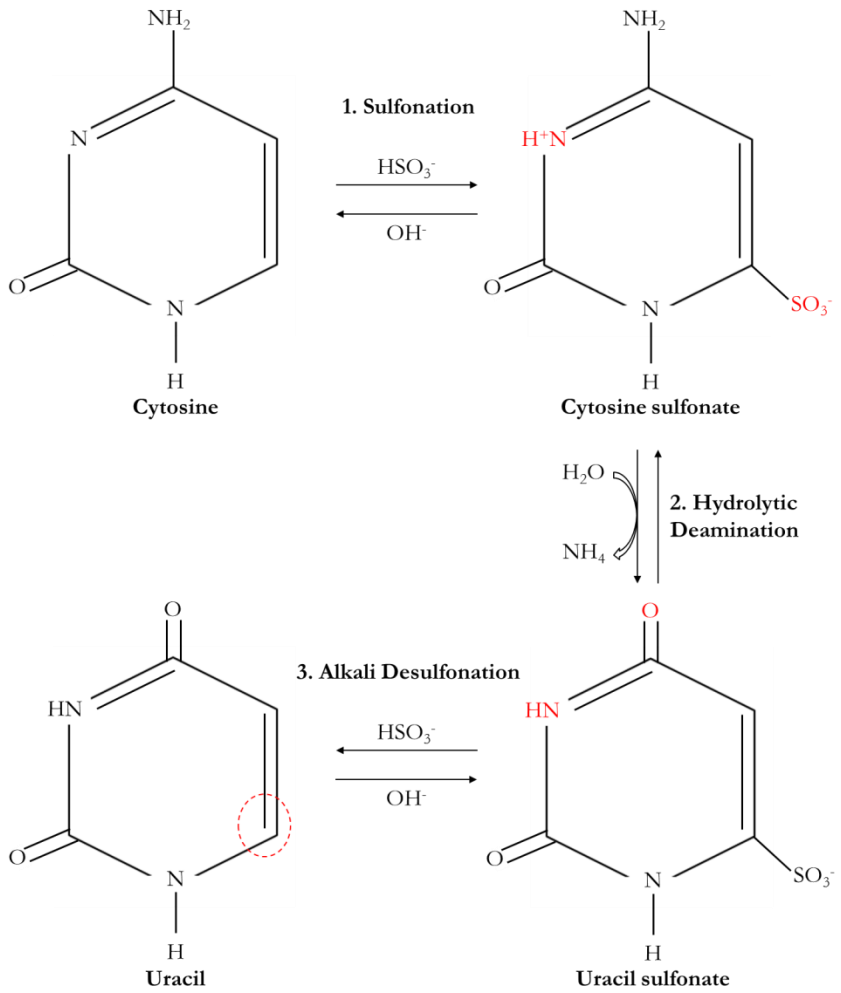

Figure 2.13: Conversion of unmethylated cytosine to uracil during bisulfite conversion. Cytosine to uracil sulfonate conversion occurs during thermal cycling. Uracil is desulfonated whilst in the Silicon-A Binding Plate.

A Silicon-A Binding Plate was prepared by mounting on a Collection Plate and adding 400 µl M-Binding Buffer. Samples were then transferred to the Silicon-A Binding Plate and centrifuged for 3000 x g for 5 min. Samples were washed with M-Wash Buffer and centrifuged for 3000 x g for 5 min. M-Desulphonation Buffer was then added to the samples and left for 20 min at RT to convert uracil sulfonate to uracil (Figure 2.13). Following uracil desulfonation, samples were washed through two successive washes with 400 µl M-Wash Buffer. Each wash was followed by centrifugation at 3000 x g, for 5 and 10 min after the first and second washes, respectively. Bisulfite converted DNA was then eluted into an Elution Plate. 30 µl of ddH_2O was added to DNA in the Silicon-A Binding plate, mounted on the Elution Plate, and after leaving to stand for 5 min the assembly was centrifuged for 3000 x g for 3 min. DNA was stored at -20°C.

2.6.5. PCR

Promoter regions of genes were selected using MatInspector (Genomatix, Munich, Germany) to identify potential transcription factor binding sites. Pyromark® Assay Design was used to design

primers specifically avoiding single nucleotide polymorphisms (SNPs), CpG islands and long runs of thymines. Primers were synthesised by Biomers. The region of interest was amplified using PCR reactions containing 12.5 µl KAPA2G Robust HotStart ReadyMix, 200 nmol/l forward and reverse primers, 66 ng bisulfite converted DNA and ddH_2O to a final volume of 25 µl. One primer in each primer set was biotinylated. 0% and 100% methylated control DNA were amplified with each PCR and NTCs were run alongside samples to check for DNA contamination. Samples were run on a 96 well thermal cycler and cycling conditions were; 95°C for 3 min to activate the polymerase; 45 cycles of 94°C for 15 s, annealing temperature for 15 s, and 72°C for 15 s; and a cooling step of 72°C for 1 min. PCR product (5 µl neat PCR product and 2 µl loading dye) was run on a 2% agarose gel (2 g in 100 ml TBE, 0.01% GelRed™) to check for amplification and negative NTCs.

2.6.6. Pyrosequencing

10 µl of biotinylated PCR product was immobilised onto 2 µl streptavidin sepharose beads in a 96 well plate with 38 µl Pyromark® Binding Buffer, and ddH_2O added to a final volume of 80 µl per well. 0% and 100% methylated control DNA and NTCs from PCR were run on each plate. The mixture was then shaken for 5 min. A Pyromark® Q96 HS Plate was prepared with 417 nmol/l sequencing primer and 11.5 µl Pyromark® Annealing Buffer per well. The 96 well plate was placed in the PCR plate bay of the Pyromark® Q96 Vacuum Prep Worktable (Qiagen) and the PCR product held onto the prongs of the Vacuum Prep tool by the vacuum pump. The PCR product was then washed through 70% ethanol, 136 mmol/l Denaturation Solution and 1 X Wash Buffer for 5 s each before being released into the Pyromark® Q96 HS Plate (Figure 2.14).

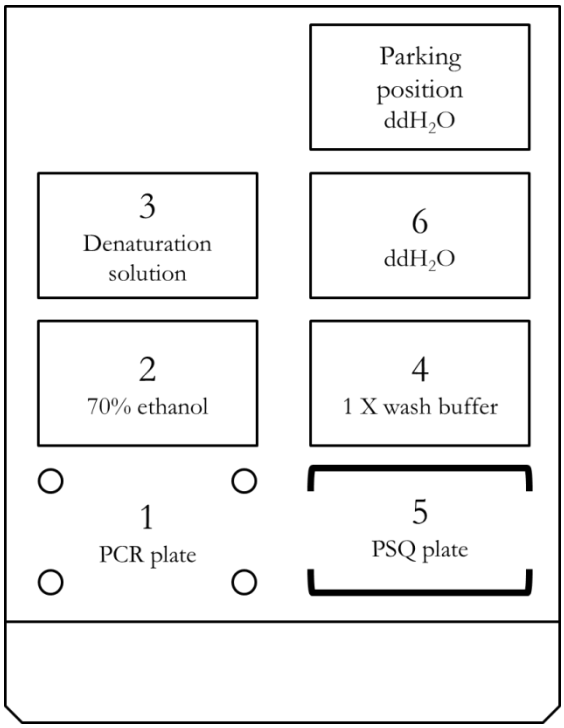

Figure 2.14: Vacuum Prep Worktable. Numbers indicate work flow. The vacuum is turned off before placing the vacuum prep tool into the PSQ plate.

The PSQ plate was then heated at 80°C for 2 min for primer annealing. Pyrosequencing was performed using dNTPs and Pyromark® Gold Q96 enzyme and substrate mixtures on a PyroMark MD pyrosequencer (Biotage, Uppsala, Sweden). In brief, this involves hybridisation of a sequencing primer to a single stranded PCR amplified DNA template. This is incubated with the enzymes; DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates; adenosine 5' phosphate (APS) and luciferin. dNTPs are added one at a time. If complementary, the dNTP is incorporated into the template strand through the action of DNA polymerase. This is accompanied by the release of pyrophosphate (PPi). PPi is converted to ATP through the action of ATP sulfurylase in the presence of APS. The ATP drives the conversion of luciferin to light through luciferase. The quantity of light produced is relative to the number of nucleotides incorporated (Figure 2.15). Apyrase degrades ATP and unincorporated nucleotides, switching off the light in preparation for the addition of the next dNTP. The percentage methylation level at specific CpGs is determined by the proportion of cytosines and thymines sequenced. To check that the bisulfite conversion had completed, a control was used in the pyrosequencing assay that checks that any cytosine not followed by a guanine had been 100% converted to a thymine.

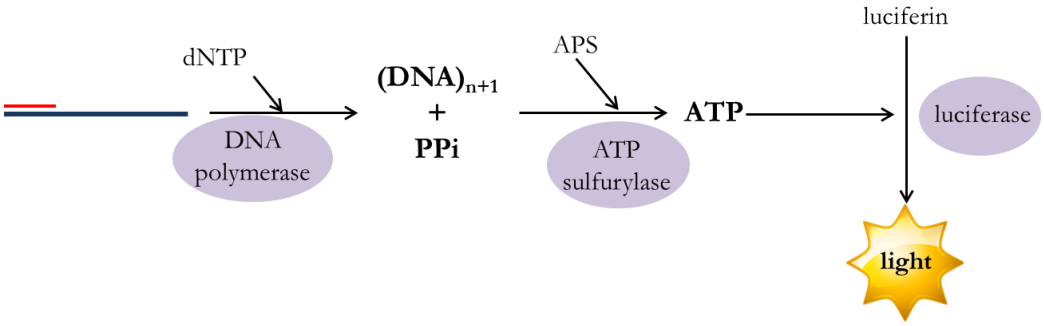

Figure 2.15: The chemistry of pyrosequencing. The PCR amplified DNA is represented by the blue line, and the sequencing primer by the red line. Enzymes are represented in purple ovals.

2.7. Protein

Protein expression of facilitated amino acid transporters was measured in HEK293, BeWo and placenta cells. Protein expression of these transporters was used as a measure of how suitable the cell models were to study the effects of vitamin D on placental amino acid transporter expression. Protein was extracted from cells and protein concentration was determined. The expression of specific proteins was then assessed using western blotting with specific antibodies for the proteins of interest.

2.7.1. Working solutions for protein work

Table 2.10: Materials for protein work.

Material	Supplier
Anti-TAT1 antibody	Sigma-Aldrich
Anti-LAT4 antibody purification	Thermo Scientific, Massachusetts, USA
Bicinchoninic acid (BCA) protein assay	Pierce, Thermo Scientific
Dithiothreitol (DTT)	Abcam, Cambridge, UK
Enhanced chemiluminescence (ECL) western blotting substrate	Promega
ECL Prime™ blocking agent	GE healthcare, Buckinghamshire, UK
Goat-anti rabbit horseradish peroxidase (HRP) conjugated secondary antibody	Abcam
Laemmli Sample Buffer 2 X	Bio-Rad, California, USA
Methanol	Fisher Scientific
Mini-PROTEAN® TGX™ Gels 4-20%	Bio-Rad
Monoclonal anti-β-actin-HRP	Sigma-Aldrich
PBS tablets	Sigma-Aldrich
Precision Plus Protein™ dual colour standard	Bio-Rad
Polyvinylidine fluoride (PVDF) membrane	Bio-Rad
Protease inhibitor cocktail	Sigma-Aldrich
SDS	BDH Laboratory Supplies
Sodium chloride (NaCl)	Fisher Scientific
Sodium deoxycholate	Sigma-Aldrich
SuperSignal® West Femto ECL	Thermo Scientific
Tris	Sigma-Aldrich
Tris/glycine buffer 10 X	Bio-Rad
Tris/glycine/SDS buffer 10 X	Bio-Rad
Triton X 100	Sigma-Aldrich
Tween 20	Fisher Scientific

Working solutions

PBS

1 PBS tablet dissolved in 200 ml dH_2O gives 10 mmol/l phosphate buffer, 2.7 mmol/l KCl and 137 mmol/l NaCl, pH 7.4 at 25°C.

0.1% PBS-Tween (PBS-T)

200 µl Tween 20 in 200 ml PBS.

Radioimmunoprecipitation assay (RIPA) buffer

150 mmol/l NaCl, 50 mmol/l Tris, 1% Triton X 100, 0.5% NaDeoxycholate, 0.1% SDS, 1:25 Protease Inhibitor Cocktail and PBS.

Run buffer

100 ml 10 X Tris/glycine/SDS buffer and 900 ml dH_2O gives 192 mmol/l glycine, 25 mmol/l Tris and 0.1% (w/v) SDS, pH 8.3.

Transfer buffer

100 ml 10 X Tris/glycine buffer, 200 ml methanol and 700 ml ddH_2O gives 192 mmol/l glycine and 25 mmol/l Tris, pH 8.3.

2.7.2. Protein extraction

Protein was extracted using RIPA buffer. For protein extraction from cells, cells were trypsinised and pelleted by centrifugation at 1200 rpm for 10 min at 4°C. To obtain enough protein, cells were pelleted from one T75 flask or by combining two 32 mm wells. Cell pellets were resuspended in 300 µl of RIPA buffer and incubated on ice for 5 min. Samples were sonicated three times for 1 min in a sonicating water bath and were incubated on ice for 5 min between each sonication. Samples were then incubated on ice for 15 min. Nuclear material was spun down at 2500 x g for 15 min at 4°C. Supernatant was removed and stored at -80°C.

2.7.3. Protein assay

Protein concentration was determined using the BCA protein assay. This method uses the Biuret reaction combined with the use of BCA to cause a colour change proportional to protein concentration. The Biuret reaction involves the reduction of copper; Cu^{2+} to Cu^{+} by protein within an alkaline environment. One Cu^{+} ion chelates with two molecules of BCA resulting in a purple reaction product (Smith *et al.*, 1985).

10 µl of each sample (1:10) and standard (Table 2.11) was added in duplicate to a 96 well microplate, followed by the addition of 200 µl of BCA working reagent (50:1 of Reagent A and Reagent B).

Table 2.11: Standards for BCA protein assay.

Standards	Volume of H_2O (µl)	Volume of RIPA buffer (µl)	Volume (µl) and source of BSA	BSA concentration (µg/ml)
A	0	10	290 of stock	1934.7
B	115	10	375 of stock	1500
C	315	10	325 of stock	1000
D	165	10	175 of B	750
E	315	10	325 of C	500
F	315	10	325 of E	250
G	315	10	325 of F	125
H	390	10	100 of G	25
I (blank)	390	10	0	0

The plate was mixed and incubated at 37°C for 30 min. After cooling to RT, absorbance was measured at 550 nm on a Multiskan EX plate reader. Protein concentrations were calculated by subtracting the absorbance of the blank sample from all other standard and sample absorbance measurements. A standard curve of blank-corrected absorbance measurements for the standards was plotted against their BSA concentrations (Figure 2.16). This standard curve was used to determine the protein concentrations of the unknown samples using the equation:

$$x = ((y - c)/ m)$$

Where m refers to the gradient of the line, c refers to the y intercept and y refers to the absorbance.

Protein concentrations obtained from the standard curve were multiplied by 10 to adjust for the 1:10 dilution used in the assay.

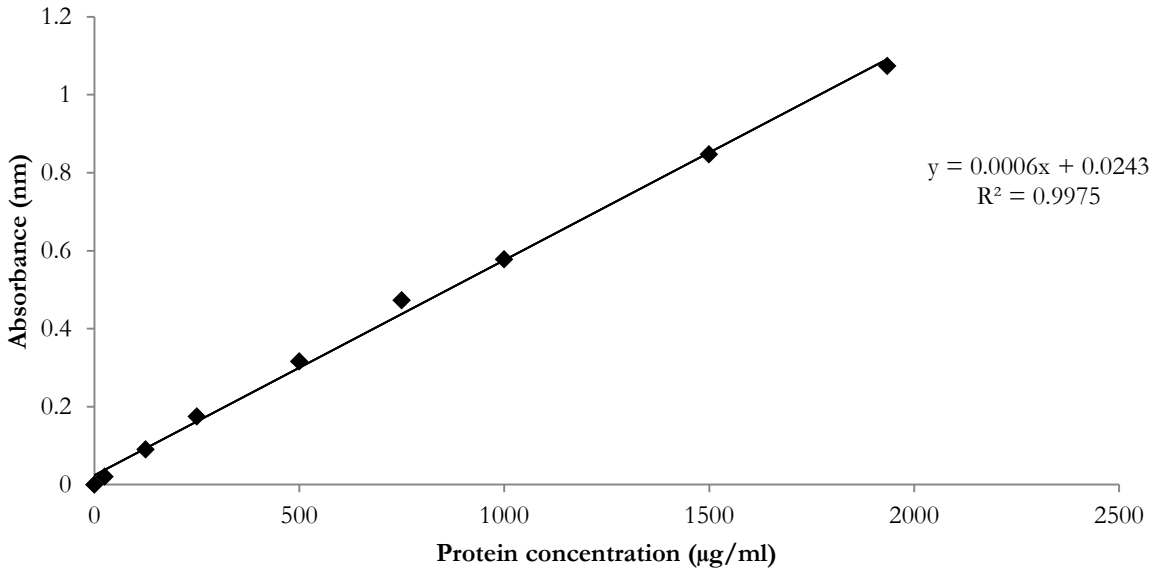

Figure 2.16: An example protein assay standard curve.

2.7.4. Western blotting

Western blotting was used to determine expression of specific proteins. Proteins were separated by size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS linearizes the protein giving a uniform negative charge, allowing the separation of protein by electrophoresis based on size alone. Separated proteins were then transferred to a PVDF membrane, incubated with primary and secondary antibodies and visualised to determine the presence or absence of a specific protein.

40 μg of each protein sample was incubated on ice with DTT (400 mmol/l final concentration after addition of buffer) for 1 h. An equal volume of 2 X Laemmli Sample Buffer was added to each sample and samples were heated to 70°C for 10 min to reduce the protein and disrupt secondary and tertiary structures. Protein samples and ladder (Precision Plus Protein™ Dual Colour Standard; Figure 2.17) were separated by SDS-PAGE in 4–20% Mini-PROTEAN® TGX™ gels in run buffer at 200 V for 45 min.

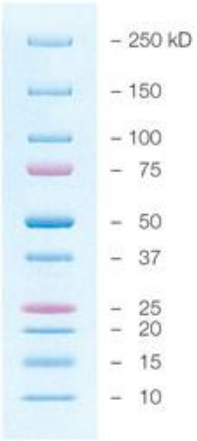

Figure 2.17: Precision Plus Protein™ Dual Colour Standard used for western blotting.

Separated protein samples and ladder were then transferred to PVDF membrane in transfer buffer at 100 V for 1 h. Membranes were blocked for 1 h at RT in 3% ECL Prime™ blocking solution in 0.1% PBS-T. Membranes were incubated with primary antibody overnight at 4°C. The primary antibody for LAT4 had been raised in rabbit and affinity purified and was used at a dilution 1:500. The primary antibody for TAT1 was from Sigma and was used a dilution of 1:150. The following day, membranes were washed with three 10 min washes in PBS-T followed by incubation with an HRP conjugated goat anti-rabbit secondary antibody at RT for 1 or 2 h. The secondary antibody was used at a dilution of 1:50,000. Membranes were washed in three 10 min washes of PBS-T and protein bands were visualised using VersaDoc 4000 MP (Bio-Rad) after a 5 min incubation with SuperSignal® West Femto ECL (1:1 of Stable peroxide solution and Luminol/Enhancer solution). A negative control incubated with just secondary antibody was used for each membrane to demonstrate primary antibody specificity.

Equal loading was demonstrated by incubating membranes with monoclonal anti-β-actin-HRP. Each membrane was washed three times in PBS-T prior to and after incubation with anti-β-actin (1:10,000). Bands were visualised using VersaDoc after 1 min incubation with ECL western blotting substrate (1:1 of peroxide solution and Luminol Enhancer solution). Protein expression was quantified using ImageJ (National Institutes of Health, USA). Each protein band on the blot was selected and measured generating a profile plot. The area of each plot was calculated in ImageJ and expressed as a percentage of the total size of all the profile plots. Relative density of each protein band was then calculated by dividing the percent value for each band by that of a selected control from the same blot. Protein bands from protein of interest blots and β-actin blots were analysed in the same way. To correct for any unequal loading the relative density of the protein of interest was divided by the relative density of β-actin for that sample to give relative density adjusted for β-actin.

2.8. Data analysis

Data are presented as mean + standard error of the mean (SEM) unless otherwise stated. Data were analysed using IBM® SPSS® Statistics Version 20 (IBM®, Armonk, New York, USA). Graphs were created in GraphPad Prism 6 (GraphPad Software Inc., California, USA) or Microsoft Excel 2010. All data were tested for normal distribution before further statistical analysis. Normal distribution was assessed by analysis of frequency histograms as well as the skewness statistic. A skewness value between -1.0 to +1.0 was considered normally distributed. Non-normally distributed data were log transformed or square rooted and then re-tested for normal distribution. Data that were still not normally distributed were analysed using non-parametric methods. All normally distributed data were analysed using parametric statistical tests. For all statistical tests a p value of < 0.05 was considered statistically significant and $p < 0.1$ was considered a trend.

2.8.1. Analysis of data from human cohorts

SWS

Data were tested for effects of sex and gestational age, and adjusted for these variables before further analysis. Pearson's partial correlation was used to determine whether there were associations between mRNA expression or methylation levels and specific maternal and fetal measurements. Data from cohorts are presented as Pearson's correlation r values unless otherwise stated.

MAVIDOS

The effect of maternal vitamin D supplementation on placental mRNA expression was analysed by regression with treatment group as the predictor. As the treatment group is a dichotomous variable (placebo or vitamin D treated) the β value shows the amount of change in placental mRNA expression

with vitamin D treatment. Sex and gestational age were added into the linear regression model to adjust for these variables. Data are presented as β values or mean mRNA expression with 95% confidence intervals (CIs). Pearson's correlation was used to investigate associations between placental mRNA expression with maternal serum and neonatal anthropometry and DXA measures. mRNA expression levels were adjusted for sex and gestational age prior to correlation analysis; linear regression was used to determine the coefficient by which each gene expression value varied with sex and gestational age. Gene expression values were then multiplied by this coefficient to give an adjusted gene expression value. Correlation data are presented as r values.

2.8.2. Analysis of cell culture data

When comparing mRNA and protein expression in HEK293, BeWo and placental cells normally distributed data were analysed using one-way analysis of variance (ANOVA). The Levene homogeneity of variance statistic was used to assess variance within the data set. For data with equal variance, indicated by a non-significant Levene Statistic, Tukey's multiple comparison post-hoc test was used, and for data with unequal variance the Games-Howell post-hoc test was used. For non-normally distributed data, mRNA and protein expression levels were compared using a Kruskal-Wallis test.

Data from AZA treatment of HEK293 and BeWo cells only required the comparison of two groups; control treated and AZA treated, therefore normally distributed data were analysed using a t-test. P values were selected according to results of the Levene test. Non-normally distributed data were analysed using a Mann Whitney U test.

Effects of vitamin D treatment were tested for homogeneity of variance with the Levene test. Data were compared to control treated cells by one-way ANOVA with Dunnett t post-hoc test when the Levene statistic indicated equal variances within the data set. When the Levene statistic was significant, indicating that data did not have equal variance, a Dunnett T3 post-hoc test was used.

2.8.3. Analysis of placental villous fragment data

Fluorescence microscopy data were obtained as described in section 2.4.3. Using ImageJ the amount of biotin-DSL and FITC-albumin or dextran in each image z-section were quantified. For FITC measurements, a threshold was set so that background fluorescence was eliminated. The FITC value was then divided by the DSL value to create a measure of FITC-protein uptake in proportion to the amount of membrane for each placental villous fragment image slice. The FITC:DSL ratios from each slice were then averaged for each image, and average values for each placental fragment were calculated. All image data were normally distributed following transformation.

qrt-PCR data were normalised to HKG expression, and are presented as mean fold change relative to control. All data were normally distributed following transformation.

The Levene statistic was used to test equal variance of data, and p values were selected accordingly. Two-way ANOVA was used to investigate the effect of time and temperature on FITC-albumin uptake, and effect of time and vitamin D treatment on *CYP24A1* mRNA expression. T-tests were used to compare FITC-albumin with FITC-dextran uptake, and to compare FITC-albumin uptake with and without endocytic blockers. One-way ANOVA was used to determine the effect of vitamin D with and without albumin or DBP on *CYP24A1* mRNA expression, as well as to investigate the effect of endocytic blockers on vitamin D-mediated changes to *CYP24A1* mRNA expression. Where the Levene statistic was non-significant Tukey's post hoc test was used, where the Levene statistic was significant, indicating unequal variance in the data, the Games-Howell post hoc test was used. For data that was still not normally distributed following transformation, a Kruskal Wallis test was used to compare groups.

RNA sequencing data were analysed by three two-way comparisons using t-tests to compare the three groups. Data are presented as fold change relative to control. A fold change of 2 was set for the threshold above which a gene was classed as up-regulated, while a threshold of 0.5 was set as the fold change for down-regulation of gene expression.

Chapter 3:

Placental mRNA expression, maternal anthropometry and fetal outcome

3.1. Introduction

3.1.1. Vitamin D and fetal growth

Poor fetal growth is associated with increased risk of perinatal mortality and chronic disease in later life, including osteoporosis, obesity, cardiovascular and metabolic disease (Barker, 1998; Gluckman, 2008). Fetal growth is reliant on an adequate supply of vitamin D for cell growth and bone development (Bowyer *et al.*, 2009; Hart *et al.*, 2015). Vitamin D insufficiency which is common in women of childbearing age (Javaid *et al.*, 2006) or poor placental transport of vitamin D during pregnancy may affect fetal and neonatal development. Low maternal 25(OH)D levels are associated with reduced fetal growth (Bowyer *et al.*, 2009; Hart *et al.*, 2015) and reduced birth weight (Harvey *et al.*, 2014a) which is prevented by vitamin D supplementation in pregnant Asian women (Brooke *et al.*, 1980; Brooke *et al.*, 1981). In the SWS cohort maternal 25(OH)D concentrations correlated with neonatal bone mass (Harvey *et al.*, 2008), while lower maternal 25(OH)D was associated with morphological changes in the fetal femur (Mahon *et al.*, 2010). The effects of maternal vitamin D supply may persist into childhood. Low maternal 25(OH)D correlated with reduced whole-body BMC, BA and BMD in offspring at 9 years (Javaid *et al.*, 2006) and increased fat mass at 6 years of age (Crozier *et al.*, 2012). Vitamin D can only be obtained from the maternal diet or dermal synthesis from sunlight and must be transported by the placenta in order to reach the fetus and influence growth. Alterations in maternal vitamin D levels may equate exactly to levels supplied to the fetal circulation and directly influence fetal growth. Alternatively, maternal vitamin D may induce changes within the placenta, resulting in altered mRNA expression which could act upon vitamin D transport and metabolism within the placenta but also upon transport of other nutrients key for optimal fetal growth (Figure 3.1).

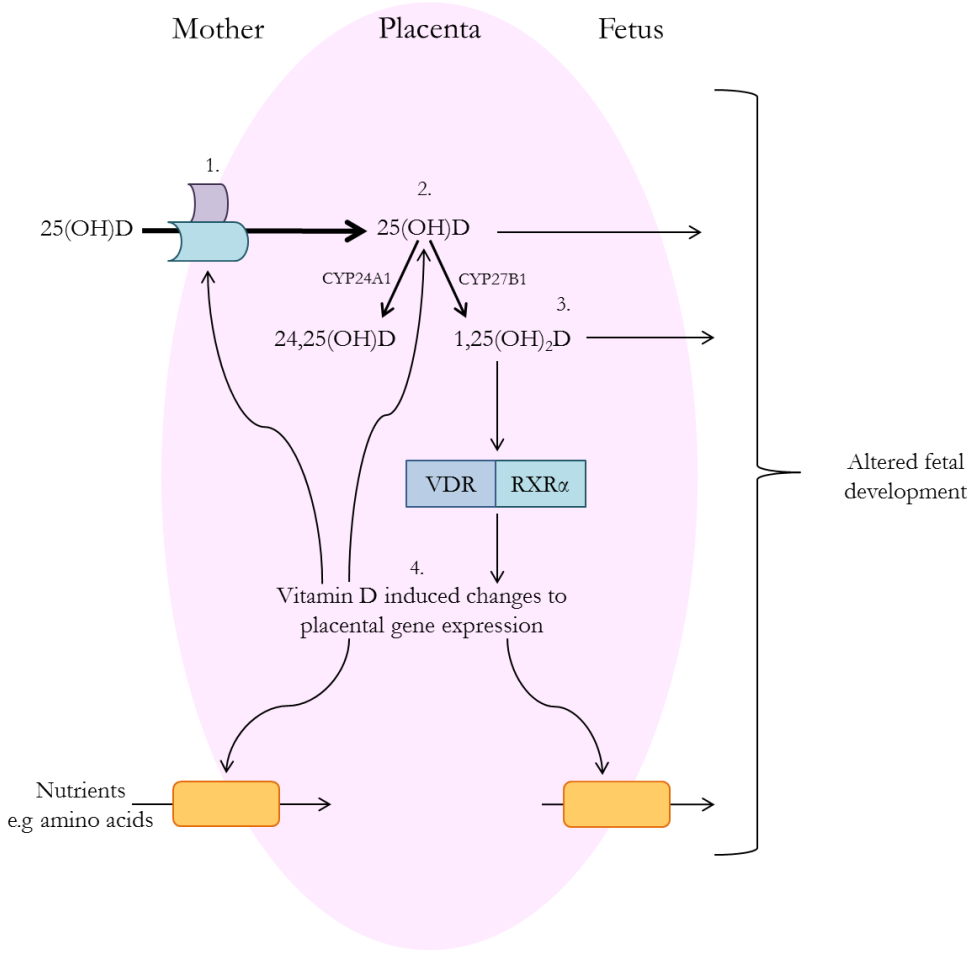

Figure 3.1: The ways in which maternal vitamin D may impact placental function. 1. Placental expression of transporters may mediate uptake of vitamin D into the placenta, therefore affecting the levels available for transport to the fetus. 2. Once transported into the placenta, 25(OH)D may be directly transported into the fetal circulation. Alternatively, it may be metabolised by enzymes within the placenta. CYP24A1 catalyses the formation of the inactive breakdown product 24,25(OH)D from 25(OH)D. CYP27B1, catalyses the formation of the active $1,25(OH)_2D$ from 25(OH)D. 3. $1,25(OH)_2D$ may then be transported into the fetal circulation or may act on placental function via interaction with the nuclear receptors VDR and RXRα. 4. Effects of vitamin D on placental mRNA expression could alter a range of functions affecting fetal growth.

Maternal vitamin D could alter placental function in a number of ways (Figure 3.1). In response to active vitamin D, mRNA expression of *CYP24A1* increases and *CYP27B1* decreases (Turunen *et al.*, 2007), therefore the level of vitamin D reaching the placenta could alter the placental metabolism of vitamin D, which may affect the levels of vitamin D available for transfer to the fetus. In addition, expression of megalin may be affected by vitamin D levels (Chlon *et al.*, 2008), which would impact transport into the placenta. Vitamin D may also alter expression of placental transporters required for uptake or efflux of other nutrients essential for fetal growth, such as amino acids. Previously placental mRNA expression of the calcium transporter, *PMCA3*, (known to be regulated by vitamin D) related to fetal growth in the SWS cohort (Martin *et al.*, 2007). In the same cohort placental amino acid transporters (*TAT1*, *LAT3* and *LAT4*) related to several measures of fetal growth (Cleal *et al.*, 2011). The presence of VDREs in the regulatory regions of these transporter genes, suggests that expression

of these transporters may be modulated by vitamin D. We now need to establish whether these and other placental amino acid transporters are related to maternal vitamin D levels.

3.1.2. Influences on circulating vitamin D

There is a high prevalence of vitamin D deficiency in many populations (Holick *et al.*, 2011). Vitamin D is obtained from the sun and dietary or supplementary sources therefore lifestyle is likely to have a large impact on individual vitamin D status. Maternal lifestyle factors which may impact circulating 25(OH)D levels include levels of physical activity, obesity and smoking status (Bodnar *et al.*, 2007a; Brock *et al.*, 2010; Zhao *et al.*, 2012; Bjorn Jensen *et al.*, 2013; Vimaleswaran *et al.*, 2013; Gill *et al.*, 2014). Physical activity may be an indicator of a healthier lifestyle with increased sun exposure or a direct relationship between exercise and vitamin D status may exist, as following adjustment for sun exposure the association is still present (Brock *et al.*, 2007). In addition, obesity is thought to be causative of vitamin D deficiency as a result of sequestration of vitamin D in the body fat (Wortsman *et al.*, 2000; Vimaleswaran *et al.*, 2013). As these factors may be confounding in identifying relationships between maternal vitamin D levels with placental function, vitamin D data were corrected for walking speed, as a measure of physical activity, sum of skinfold thickness, as a measure of fat stores, and smoking during pregnancy. Although BMI was also available for this data set, previous analysis has demonstrated that the use of BMI as a measure of maternal adiposity did not alter results compared to the use of sum of skinfold thickness (Harvey *et al.*, 2014b). Furthermore, parity may have an effect on placental function and fetal growth (Harvey *et al.*, 2008; Harvey *et al.*, 2010a; Matsuda *et al.*, 2015), therefore this variable was also adjusted for.

3.1.3. Placental handling of vitamin D

The expression of vitamin D-related genes in the human placenta has not previously been investigated in relation to both maternal anthropometry and fetal outcomes in a normal healthy cohort. Expression of vitamin D-related genes and proteins are altered in some pathological placentas, including FGR and pre-eclampsia (Ma *et al.*, 2012; Cho *et al.*, 2013; Anderson *et al.*, 2015). For example VDR mRNA and protein levels were reduced in placentas of infants with FGR (Nguyen *et al.*, 2015). Moreover, higher VDR protein expression was associated with longer fetal femur length, and expression associated with maternal to fetal calcium transfer (Young *et al.*, 2014), supporting the idea that vitamin D may affect both placental function and fetal growth.

A number of different genes involved in the vitamin D pathway will be investigated, including those involved in vitamin D metabolism and signalling, as well as genes that may have a role in vitamin D transport (Figure 3.2). *CYP24A1* mRNA expression is induced upon activation of VDR by $1,25(OH)_2D$ (Turunen *et al.*, 2007), and could provide a biomarker for active vitamin D levels within

the placenta. Therefore, expression of this gene will also be investigated for associations with amino acid transporter genes.

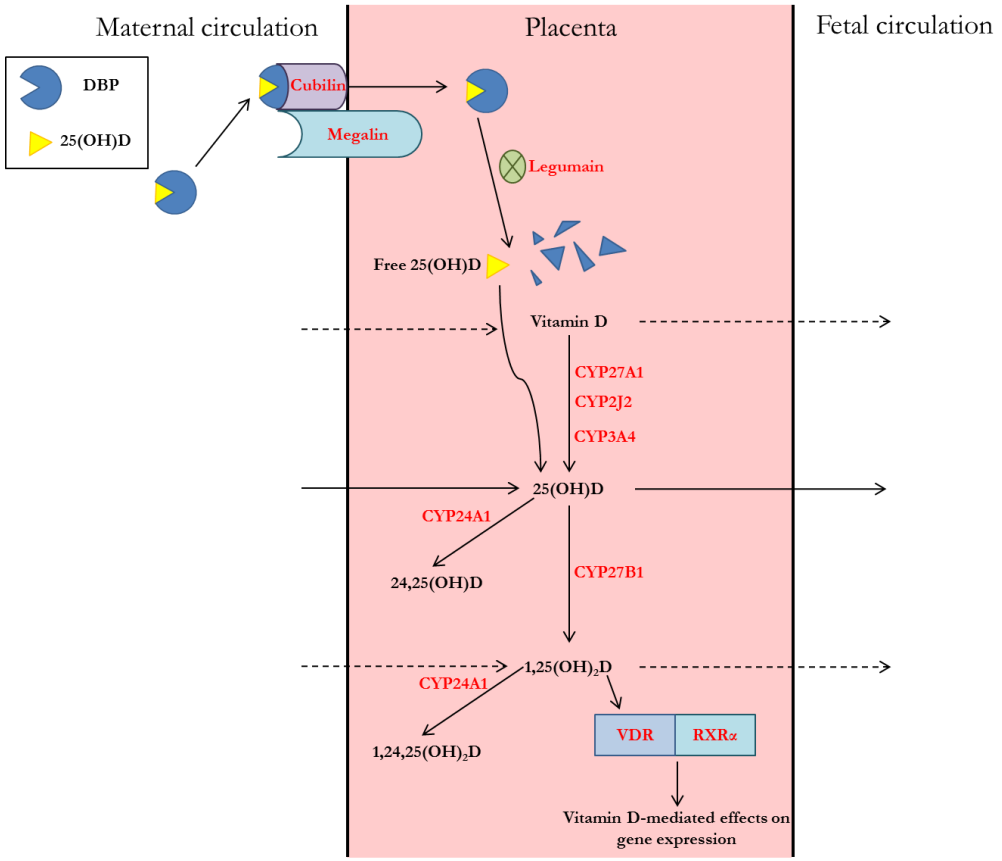

Figure 3.2: Role of vitamin D-related genes in the placenta. Cubilin and megalin may bind and transport vitamin D bound to DBP or albumin (Nykjaer *et al.*, 1999; Nykjaer *et al.*, 2001). Once inside the placenta, legumain degrades albumin and DBP releasing 25(OH)D (Chen *et al.*, 1997; Yamane *et al.*, 2002). It is unknown whether the other forms of vitamin D are transported into the human placenta (dashed arrows). CYP27A1, CYP2J2, and CYP3A4 convert vitamin D into 25(OH)D, which is converted into active $1,25(OH)_2D$ via CYP27B1. 25(OH)D and $1,25(OH)_2D$ are broken down by CYP24A1. $1,25(OH)_2D$ binds the VDR-RXRα heterodimer and exerts vitamin D-mediated effects on gene transcription (Zhu and DeLuca, 2012). 25(OH)D is thought to be transported into the fetal circulation (Bouillon, 1981). However, it is currently unknown whether vitamin D or $1,25(OH)_2D$ are also transported into the fetal circulation from the placenta. All genes in red will be measured in placentas from the SWS.

3.1.4. Further possible effects of vitamin D on placental mRNA expression

Vitamin D may have wide ranging effects on placental mRNA expression and function. Furthermore, other systems operating within the placenta are likely to influence the availability and impact of vitamin D. A small proportion of these factors will be investigated. Specifically, mRNA expression of *LPL*, *PMCA1*, *DNMT1*, *DNMT3a* and *DNMT3b* will be measured, and a more detailed analysis of methylation at the promoter region of the *TAT1* VDRE will also be conducted. A summary of how these genes may be influenced by and influence placental vitamin D handling is shown in Figure 3.3.

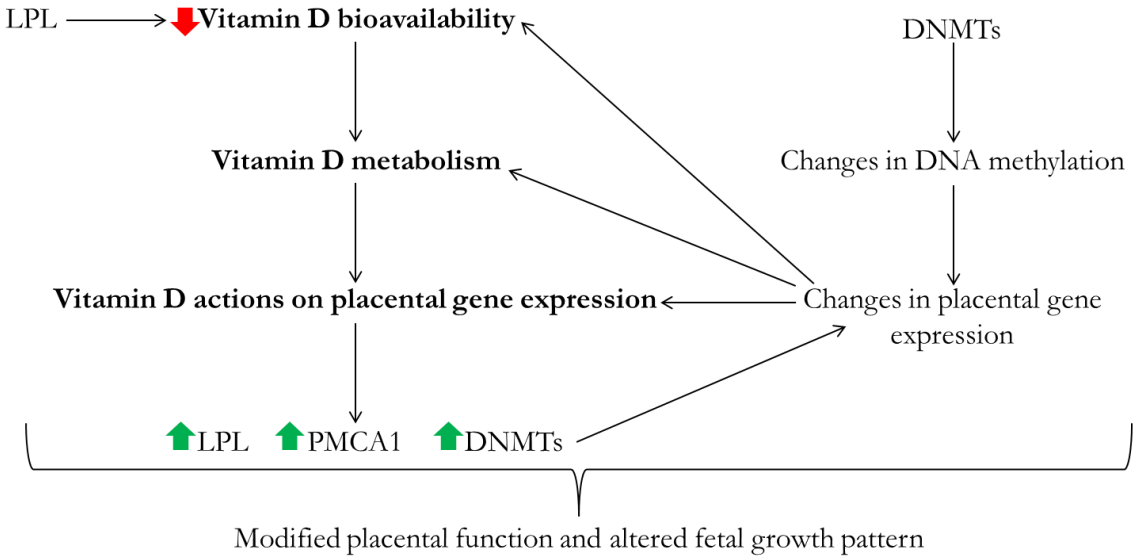

Figure 3.3: Placental vitamin D handling may influence and be influenced by other systems within the placenta. In other tissues vitamin D has been shown to result in or has been associated with increased mRNA expression of *DNMT*s, *LPL* and *PMCA1*. LPL expression may also regulate bioavailability of vitamin D through generating fatty acids which interact with albumin and megalin, while DNMTs could result in more profound changes in placental mRNA expression in response to vitamin D and may also regulate expression of placental vitamin D metabolising enzymes.

As discussed in Chapter 1 section 1.7.2, LPL may mediate vitamin D bioavailability as it results in the release of free fatty acids which also bind albumin and are consequently transported via megalin (Hammad, 2000; van der Vusse, 2009; Dube *et al.*, 2012). This could compete against megalin-mediated uptake of vitamin D. In addition, active vitamin D may itself mediate LPL activity, as culture of an adipocyte cell line with $1,25(OH)_2D$ resulted in increased LPL mRNA and activity (Vu *et al.*, 1996; Querfeld *et al.*, 1999).

PMCA1 will be investigated due to the close links between vitamin D and calcium transport. Calcium transport is important for fetal skeletal development (Kovacs, 2014). Due to time restrictions it was not possible to explore a wider range of calcium transporters. PMCA1 was focussed on as this transporter is key for mediating calcium efflux from the placenta into the fetal circulation. PMCA1 mRNA and protein increased in response to $1,25(OH)_2D$ in duodenal, kidney and osteoblast cells (Glendenning *et al.*, 2000, 2001; Walters *et al.*, 2007; Balesaria *et al.*, 2009), but not in rat cochlea cells (Yamauchi *et al.*, 2010). This could be a result of the differing cell type. Placental PMCA1 expression was reduced in pre-eclampsia (Hache *et al.*, 2011). To the best of our knowledge, placental *PMCA1* expression has not been investigated in human placenta in relation to maternal vitamin D levels.

Altered DNA methylation within the placenta has been linked with placenta-related pathologies, including IUGR and pre-eclampsia (Maccani and Marsit, 2009; Nelissen *et al.*, 2011). Recent evidence has linked vitamin D with changes in DNA methylation (Rawson *et al.*, 2012; Tapp *et al.*, 2013; Zhu *et al.*, 2013; Zhou *et al.*, 2014b). However the precise mechanism that links vitamin D with DNA

methylation is currently unclear. DNA methylation is catalysed by DNMTs, therefore vitamin D-mediated alterations to DNMT expression provides one potential mechanism linking vitamin D with altered methylation levels. Expression and activity of the DNMTs may also be important for proper placental function as deficiency of the oocyte-specific *DNMT1* transcript in mice resulted in abnormal placental morphology, lipid accumulation within the placenta and impaired X chromosome inactivation (McGraw *et al.*, 2013; Himes *et al.*, 2015). Furthermore, in humans altered expression of DNMTs has been shown in placentas from early pregnancy loss (Yin *et al.*, 2012) and pre-eclampsia (Zhuang *et al.*, 2014). Placental DNMTs may also be important for fetal growth as altered expression has been associated with altered embryonic growth patterns in mice (McGraw *et al.*, 2013; Himes *et al.*, 2015), reduced survival in sheep (Ptak *et al.*, 2013) and *DNMT3a* genotype was associated with birth weight in humans (Haggarty *et al.*, 2013). These studies highlight the important role of DNMT-mediated methylation within the placenta. If vitamin D were to impact *DNMT* expression, the consequences of maternal vitamin D deficiency for placental function could therefore be wide ranging. Furthermore, as expression of vitamin D-related genes has been shown to be regulated by methylation (Novakovic *et al.*, 2009; Marik *et al.*, 2010; Hobaus *et al.*, 2013; Fetahu *et al.*, 2014), DNMT expression may impact placental handling of vitamin D. In addition, associations between placental gene expression and DNMT mRNA expression may provide evidence to indicate which genes are more likely to be affected by methylation within the placenta.

3.1.5. Aims

1. Does placental amino acid transporter expression correlate with maternal 25(OH)D or DBP levels in samples from the SWS?
2. Are methylation levels at CpGs within a potential VDRE in the *TAT1* promoter region associated with *TAT1* mRNA expression? Do methylation levels associate with maternal anthropometry or fetal outcomes in samples from the SWS?
3. Do placental genes involved in vitamin D-related functions associate with measures of maternal size and body composition before or during pregnancy in samples from the SWS?
4. Do placental genes involved in vitamin D-related functions associate with measures of neonatal and infant size and body composition in samples from the SWS?
5. Does DNMT expression associate with expression of placental vitamin D and amino acid transporter genes?

3.2. Methods

qrt-PCR was used to measure mRNA expression of vitamin D-related and amino acid transporter genes in placentas from the SWS. mRNA expression levels were tested for associations with maternal vitamin D levels, anthropometry and lifestyle factors to investigate influences on placental mRNA expression. mRNA expression levels were also tested for associations with fetal, neonatal and childhood anthropometry and body composition measures to investigate the impact of placental mRNA expression on fetal and infant development. These studies were conducted according to the guidelines in the Declaration of Helsinki, and the Southampton and South West Hampshire Research Ethics Committee approved all procedures. Written informed consent was obtained from all participating women and by parents or guardians with parental responsibility on behalf of children.

3.2.1. SWS

PCR and methylation analysis was carried out on placental samples from the SWS (General Methods 2.2.1). A cohort of 102 placentas was selected from 300 collected in total, based on the availability of neonatal DXA data. The SWS recruited non-pregnant Southampton women aged 20-34 and those who became pregnant were followed throughout pregnancy. Maternal, fetal and infant measurements were taken by the SWS Study Group as described in section 2.2.1. Briefly, maternal anthropometry measurements, including BMI, sum of skinfold thickness, fat mass, calf circumference, ratio of subscapular/triceps skinfold thicknesses, mid-upper arm circumference, arm muscle area and height, were taken by trained research nurses at study entry, and at 11 and 34 weeks of gestation in those who became pregnant. Women were interviewed about their diet, physical activity, social circumstances and lifestyle. Maternal venous blood samples were also taken at 11 and 34 weeks of gestation and were used to measure 25(OH)D and DBP levels. 25(OH)D at 11 weeks of gestation was measured via HPLC and tandem mass spectrometry, while DBP and 25(OH)D at 34 weeks of gestation were measured by radioimmunoassay. Free 25(OH)D at 34 weeks of gestation was calculated by the ratio of total 25(OH)D to DBP levels.

Fetal anthropometry measurements were taken by ultrasound at 11, 19 and 34 weeks of gestation. Following delivery, neonatal anthropometry measurements and placental samples (see section 2.2.3) were taken. Placental weight was recorded and 10 samples from each placenta were collected by Dr R. Lewis using a random sampling grid and snap frozen within 30 min of delivery. The samples from each placenta were pooled and ground together in a frozen tissue press.

Children were followed up at 4 and 6 years of age (Inskip *et al.*, 2006)General methods 2.2.1). Neonatal and childhood measurements recorded were birth weight, head circumference, abdominal

circumference, crown-heel length, subscapular skinfold thickness and mid-upper arm circumference. A subset of infants underwent DXA measurements within 2 weeks after birth to analyse body composition, including lean and fat mass and bone indices; BA, BMC and BMD. At this time the child's length and height were also measured.

3.2.2. mRNA expression

RNA was extracted using the mirVana™ miRNA isolation kit as described in section 2.5.2. RNA quality was determined through bioanalyser measurements. cDNA was synthesised from 0.5 μg RNA using M-MLV rt as described in Chapter 2 section 2.5.4. NECs were synthesised at the same time as cDNA.

qrt-PCR was carried out to determine expression levels of genes of interest as described in section 2.5.8 using Roche UPL and Primer Design Perfect Probe assays. Primers are displayed in Table 3.1 and Table 3.2. Primers could not be designed that covered all transcript variants for *DNMT3a*, so this gene was eliminated from investigation. For each qrt-PCR assay, samples (4 ng) were run alongside a standard curve, CV controls, NECs and NTCs in triplicate (Table 2.6). UPL qrt-PCR cycling conditions were 95°C for 10 min; 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 s; and 50°C for 30 s, with data collection at the 72°C step. Perfect Probe target gene qrt-PCR cycling conditions were 95°C for 10 min; 50 cycles of 95°C for 10 s and 60°C for 60 s; and 50°C for 30 s, with data collection at the 60°C step. Perfect Probe HKG qrt-PCR cycling conditions were 95°C for 10 min; 50 cycles of 95°C for 10 s, 50°C for 30 s and 72°C for 15 s; and 50°C for 30 s, with data collection at the 50°C step. Cp values were determined by the second derivative method and were converted to DNA concentration (ng/5 μl) using a standard curve (see General methods 2.5.8). qrt-PCR assays for amino acid transporters were carried out by Dr P. Day and Dr J. Cleal. Expression levels of three HKGs (*ATP5B*, *TOP1* and *UBC*) were measured. These HKGs were selected as they have previously been shown to have stable expression in this cohort of placenta samples (Cleal *et al.*, 2009). Expression level of genes of interest was normalised to the geometric mean of the three HKGs.

Table 3.1: Primers and probes used for amino acid transporter genes.

Gene	Accession number	Primer sequence (5'-3')	UPL number	Amplicon (bp)
4F2hc	NM_001012662.2,	F: TGGTTCTCCACTCAGGTTGA	49	1213 – 1275
	NM_001012664.2,	R: CAGCCAAAACTCCAGAGCAT		1024 – 1086
	NM_001013251.2,			801 – 863
	NM_002394.5			1210 – 1272
ASCT1	NM_001193493.1,	F: TTTGCGACAGCATTTGCTAC	78	1189 – 1248
	NM_003038.4	R: GCACTTCATCATAGAGGGAAGG		1289 – 1348
ASCT2	NM_001145144.1,	F: GAGGAATATCACCGGAACCA	43	114 – 179
	NM_001145145.1,	R: AGGATGTTCATCCCCTCCA		304 – 369
	NM_005628.2			1250 – 1315
EAAT1	NM_001289939.1,	F: TTGAACTGAACTTCGGACAAATTA	76	1594 – 1666
	NM_001289940.1,	R: ATTCCAGCTGCCCCAATACT		1396 – 1468
	NM_004172.4			1732 – 1804
EAAT2	NM_001195728.2,	F: AAAATGCTCATTCTCCCTCTAATC	78	498 – 572
	NM_001252652.1,	R: GCCACTAGCCTTAGCATCCA		563 – 637
	NM_004171.3			860 – 934
EAAT3	NM_004170.5	F: AGTTGAATGACCTGGACTTGG	9	1384 – 1447
		R: GCAGATGTGGCCGTGATAC		
EAAT4	NM_001272087.1,	F: TGCAGATGCTGGTGTTACCT	19	1701 – 1765
	NM_001272088.1,	R: GTTGTCCAGGGATGCCATA		529 – 593
	NM_005071.2			307 – 371
EAAT5	NM_001287595.1,	F: CGCCCAGGTCAACAACTAC	9	1536 – 1604
	NM_001287597.1,	R: GCTGCAGTGGCTGTGATACT		1143 – 1211
	NM_006671.5			1359 – 1427
LAT1	NM_003486.5	F: GTGGAAAAACAAGCCCAAGT	25	1503 – 1573
		R: GCATGAGCTTCTGACACAGG		
LAT2	NM_012244.3,	F: TTGCCAATGTCGCTTATG	17	1591 – 1677
	NM_001267037.1,	R: GGAGCTTCTCTCCAAAAGTCAC		375 – 461
	NM_182728.2			529 – 615
LAT3	NM_001198810.1,	F: GCCCTCATGATTGGCTCTTA	29	623 – 642
	NM_003627.5	R: CCGGCATCGTAGATCAGC		794 – 864
LAT4	NM_001284498.1,	F: ACAAGTATGGCCCGAGGAA	3	673 – 742
	NM_152346.2	R: GCAATCAGCAAGCAGGAAA		471 – 540
SNAT1	NM_001077484.1,	F: ATTTTGGGACTCGCCTTTG	47	1034 – 1110
	NM_001278387.1,	R: AGCAATGTCACTGAAGTCAAAAGT		1496 – 1572
	NM_001278388.1,			1258 – 1334
	NM_001278389.1,			523 – 599
	NM_001278390.1,			1236 – 1312
	NM_030674.3			1373 – 1449
SNAT2	NM_018976.4	F: CCTATGAAATCTGTACAAAAGATTGG	9	1836 – 1939
		R: TTGTGTACCCAATCCAAAACAA		
SNAT4	NM_001143824.1,	F: TGTTCTGGTCATCCTTGTGC	29	1602 – 1697
	NM_018018.4	R:AAAACTGCTGGAAGAATAAAAATCAG		1794 – 1889
TAT1	NM_018593.4	F: GGTGTGAAGAAGGTTTATCTACAGG	6	1238 – 1329
		R: AGGGCCCCAAAGATGCTA		
y⁺LAT1	NM_001126105.2,	F: ACACTGCCGTGAGAACCTG	72	162 – 237
	NM_001126106.2	R: AGGAGAGGAAACCCTTCACC		268 – 343
y⁺LAT2	NM_001076785.2,	F: GCTGTGATCCCCCATACCT	66	786 – 862
	NM_003983.5	R: GGCACAGTTCACAAATGTCAG		699 – 775

F = forward primer, R = reverse primer.

Table 3.2: Primers and probes used for vitamin D-related and other genes.

Gene	Accession number	Primer sequence (5'-3')	UPL number	Amplicon (bp)
		Vitamin D genes		
Cubilin	NM_001081.3	F: GGACAATGTCAGAATAGCTTCGT R: CAGTGGCTAGCAGGGCTTT	10	8624 – 8696
CYP2J2	NM_000775.2	F: ATGGCCCTCTACCCAGAAAT R: TGGTGTAGGGCATGGACTC	66	984 – 1092
CYP3A4	NM_001202855.2, NM_017460.5	F: GATGGCTCTCATCCCAGACTT R: AGTCCATGTGAATGGGTTCC	2	107 – 202 107 – 202
CYP24A1	NM_001128915.1, NM_000782.4	F: GAAAGAATTGTATGCTGCTGTCA R: CACATTAGACTGTTTGCTGTCGT	78	1337 – 1408 1337 – 1408
CYP27A1	NM_000784.3	F: CTATGGGCCGTTCACCAC R: CTCATTGAAAGCATCCGTATAGAG	38	863 – 974
CYP27B1	NM_000785.3	F: CATTCATGTGGGTGACTATATTATCC R: AAAGAATTTGGCTCTGGGAACT	Perfect Probe	1334 – 1435
Legumain	NM_001008530.2, NM_005606.6	F: GGAAACTGATGAACACCAATGA R: GGAGACGATCTTACGCACTGA	25	1311 – 1423 1193 – 1305
Megalin	NM_004525.2	F: TTGTTTTGATGCCTCTGATGA R: AGCTAGGCATGTTCGCTCAG	34	8877 – 8937
RXRα	NM_001291920.1, NM_001291921.1, NM_002957.5	F: ACATGCAGATGGACAAGACG R: TCGAGAGCCCCTTGGAGT	26	1917 – 1994 1708 – 1785 1245 – 1322
VDR	NM_001017535.1, NM_001017536.1, NM_000376.2	F: GACGCCCACCATAAGACCTA R: CCACCATCATTCACACGAACT	Perfect Probe	691 – 770 960 – 1039 569 – 648
		Calcium transporter genes		
PMCA1	NM_001001323.1, NM_001682.2	F: CCATAGTATCATTGGGCCTTTC R: CTTCTTCCTCCCCAACAGAA	75	534 – 620 534 – 620
		Lipid genes		
LPL	NM_000237.2	F: GTGGCCGAGAGTGAGAACA R: GGAAGGAGTAGGTCTTATTTGTGG	13	1478 – 15
		Methylation genes		
DNMT1	NM_001130823.1, NM_001379.2	F: CGATGTGGCGTCTGTGAG R: TGTCCTTGCAGGCTTTACATT	66	2182 – 2225 2134 – 2197
DNMT3b	NM_001207055.1, NM_001207056.1, NM_006892.3, NM_175848.1, NM_175849.1, NM_175850.2	F: CGATGGCTATCAGTCTTACTGC R: CAGGCACTCCACACAGAAAC	74	1548 – 1647 1446 – 1545 1734 – 1833 1674 – 1773 1674 – 1773 1653 – 1752

F = forward primer, R = reverse primer.

rt-PCR was carried out on primer sets that did not amplify any product in placental tissue to ensure that lack of amplification was as a result of an absence of gene expression. rt-PCR was run on placental tissue and a positive control tissue in which the target gene was known to be highly expressed, as described in Section 2.5.5. PCR products were run on 4% agarose gels and visualised with UV light (see 2.5.6) to determine the presence or absence of product amplification.

3.2.3. Methylation analysis

The percentage DNA methylation of specific CpGs within the *TAT1* promoter region was measured.

DNA was extracted from 100 SWS placenta samples using the QuickPick™ SML gDNA kit as described in General methods 2.6.2.

Bisulfite conversion was carried out on the extracted DNA to enable unmethylated and methylated cytosines to be distinguishable during subsequent pyrosequencing. 1 µg of DNA was bisulfite converted using the EZ-96 DNA Methylation Gold Kit™ as described in 2.6.4.

Assay Design: A section of the *TAT1* DNA sequence starting 2000 bp upstream of the first exon and running through to the start of that first exon was retrieved from Ensembl (Cambridge, UK) and analysed using MatInspector for potential transcription factor binding sites. SNPs were identified using Ensembl and primers were designed to amplify specific regions of interest using Pyromark® Assay Design. CpGs were numbered from the start of the first exon. Two CpGs were identified due to their proximity to a potential VDRE; CpGs -1062 and -1041. CpG -1041 lies within the VDRE, whilst CpG -1062 lies 13 bp upstream. Figure 3.4 shows a section of the bisulfite converted *TAT1* DNA sequence beginning -1579 bp from the start of the first exon with CpGs -1062 and -1041 and primers highlighted (Table 3.3).

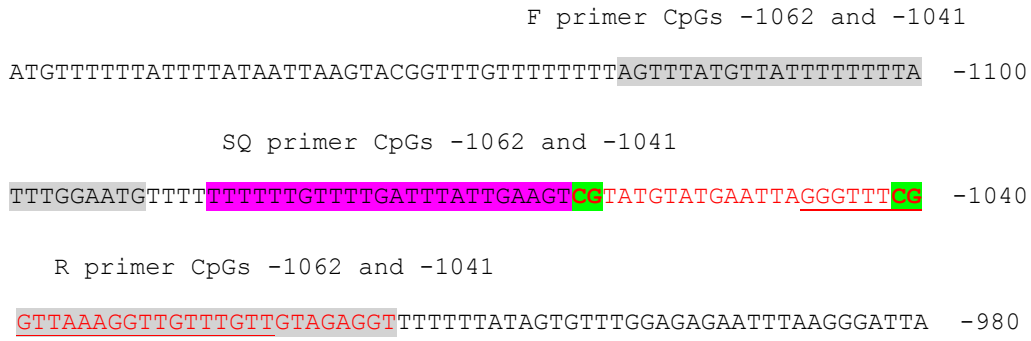

Figure 3.4: Bisulfite converted DNA sequence of *TAT1* beginning 1159 bp from the start of the first exon. CpGs are highlighted in green. No SNPs were identified in this region. CpGs -1062 and -1041 are in bold. The nuclear receptor subfamily 2 (VDR is included in this family) response element is underlined. Forward (F) and reverse (R) primers are indicated in grey highlight and sequencing (SQ) primers in pink highlight. The sequence that was analysed by pyrosequencing is shown in red text.

Table 3.3: Primer sequences for pyrosequencing of *TAT1* CpGs -1062 and -1041.

Primer sequence (5' – 3')	Biotinylated	Amplicon (bp)
F: AGTTTATGTTATTTTTTTTTATTTGGAATGT		-1119 – -1015
R: ACCTCTACAACAAACAACCTTTAAC	Y	
SQ: TTTTTTGTTTTGATTTATTGAAGT		

F = forward primer, R = reverse primer, SQ = sequencing primer.

PCR: The target region was amplified in bisulfite converted placental DNA (66 ng) using PCR as described in General methods 2.6.5. Sample DNA was amplified alongside NTCs and controls of known methylation level. Due to issues with positive NTCs the number of PCR cycles was reduced from 45 to 37. Cycling conditions were 95°C for 3 min; 37 cycles of 94°C for 15 s, 51°C for 15 s, and 72°C for 15 s; and a cooling step of 72°C for 1 min. A selection of samples from each PCR plate,

including NTCs, were run on a 2% agarose gel (Figure 3.5) to check for product amplification and the absence of contamination before pyrosequencing the PCR product.

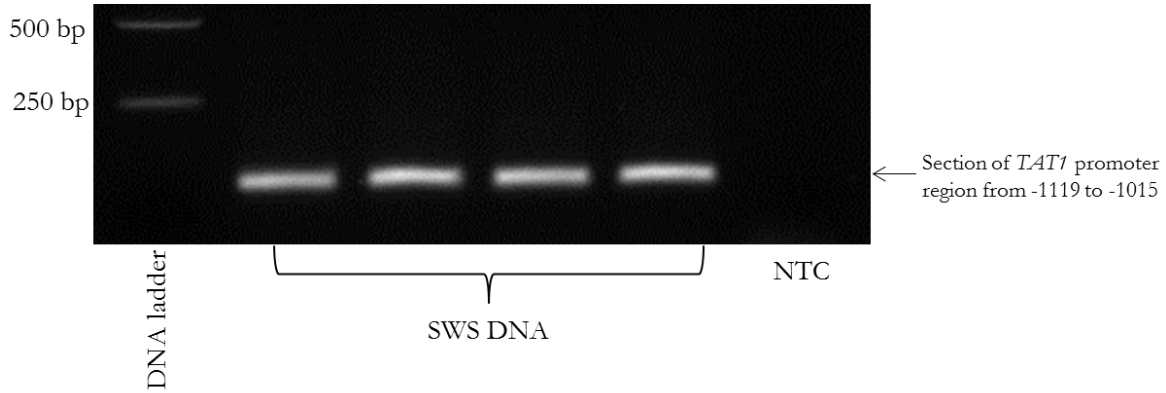

Figure 3.5: Representative PCR gel image for pyrosequencing PCR. Product (105 bp) was amplified using CpG -1062 and -1041 primers.

Pyrosequencing was carried out on the PCR product as described in General methods 2.6.6, to give a measure of percentage methylation at the selected CpGs. Briefly, 10 µl of biotinylated PCR product was immobilised onto 2 µl streptavidin sepharose beads with 38 µl Pyromark® Binding Buffer, ddH_2O added to a final volume of 80 µl and the mixture shaken for 5 min. A Pyromark® Q96 HS Plate was prepared with 417 nmol/l sequencing primer and 11.5 µl Pyromark® Annealing Buffer per well. The biotinylated PCR product bound to the sepharose beads was then cleaned using the Pyromark® Q96 Vacuum Prep Worktable through 70% ethanol, 136 mmol/l Denaturation Solution and 1 X Wash Buffer for 5 s each before being released into the Pyromark® Q96 HS Plate. The PSQ plate was heated at 80°C for 2 min for primer annealing. Pyrosequencing was performed using dNTPs and Pyromark® Gold Q96 enzyme and substrate mixtures on a PyroMark MD pyrosequencer.

3.2.4. Data analysis

mRNA expression and methylation data were tested for normal distribution and log transformed or square rooted if necessary. Maternal data that were not normally distributed were transformed logarithmically. Pearson's correlation coefficient (r_p) was used to determine partial correlations adjusted for sex and gestational age between placental mRNA or methylation levels, neonatal body composition and maternal factors using IBM® SPSS® Statistics Version 20. The partial correlation between placental mRNA expression and maternal vitamin D measures was also adjusted for potential confounding factors: maternal sum of skinfold thickness, walking speed, parity and smoking during pregnancy. Data are presented as r values or mean + SEM and significant results ($p < 0.05$) and trends ($p < 0.1$) are presented. Data were not adjusted for multiple comparisons due to the observational nature of the study. Furthermore, due to the collinearity among many of the outcomes, such as the measures of fetal

and neonatal size, adjusting for multiple comparisons was deemed inappropriate (Schulz and Grimes, 2005).

Sample size

Previous data from the SWS has been used to provide estimates for the sample sizes needed to give appropriate power for investigating associations with placental gene expression. Data from a study which investigated PMCA mRNA expression in SWS placental samples (Martin *et al.*, 2007) were used by Dr P. Day for power calculations which showed that for correlation studies to obtain an r value of 0.3 with a power of 0.8 and a p value of 0.05 a sample size of 85 would be required. For the SWS studies in this thesis a sample size of 102 was used, as it was decided to use as many samples as possible that fit on one qrt-PCR plate in triplicate alongside the necessary controls and standards. In addition, as power calculations are based on the variation within the data set, retrospective power calculations were also conducted on the gene expression measures used in this thesis using Sigma Plot version 12.5. These retrospective power calculations showed that within this data set for an r value of 0.3 and a p value of 0.05 there is a power of 0.82, and for an r value of 0.4 and a p value of 0.05 there is a power of 0.97.

3.3. Results

3.3.1. Characteristics of the SWS cohort

The mean age (SEM) of the 102 mothers at the birth of their child was 30.9 (0.39) years. 37.9% of the pregnancies studied were primiparous. 97% of the women were of white European ethnicity. Of the 102 placentas from the SWS studied here, 53 of the infants were male and 49 were female. The mean (SEM) birth weight for males was 3547 (57.1) g with 95% between the 33rd and 51st centile based on UK growth charts. The mean (SEM) birth weight for females was 3455 (70.0) g with 95% between the 36th and 59th centile. The mean (SEM) placental weight was 470 (9.6) g.

As comparisons between placental mRNA expression and a large number of maternal and infant characteristics were undertaken, only significant results and trends are presented here and the full data set is displayed in Appendix 3.

3.3.2. Maternal plasma vitamin D, DBP and placental mRNA expression

EAAT1, *EAAT4 EAAT5* and *CYP3A4* mRNA was not detected in human placental tissue. rt-PCR for *CYP3A4* confirmed expression in liver tissue and non-expression in human placenta (Figure 3.6).

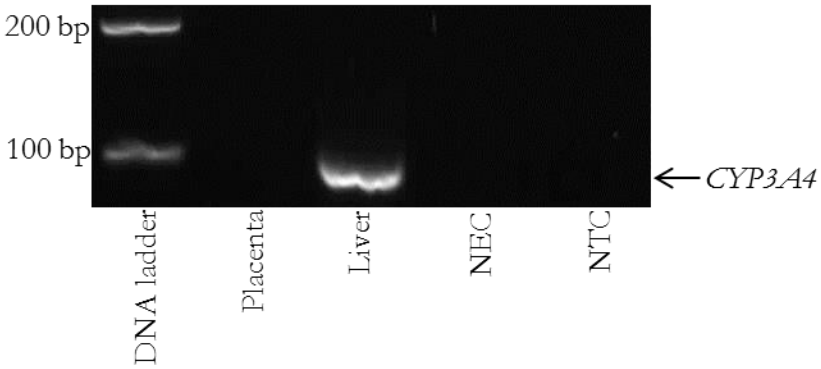

Figure 3.6: rt-PCR revealed no detectable expression of *CYP3A4* mRNA in placenta. Expression was clearly detected in liver tissue. Product size = 96 bp.

Maternal plasma 25(OH)D levels at 11 and 34 weeks and DBP levels at 34 weeks of gestation were tested for correlations with placental mRNA expression. 11 week plasma 25(OH)D levels were measured for 74 of the 102 women, the mean (SEM) 25(OH)D levels were 61.2 (3.0) nmol/l with a range of 20-128 nmol/l. 34 week plasma 25(OH)D levels were measured in 91 of the 102 women and DBP levels in 85 of the 102 women. The mean (SEM) 25(OH)D levels were 71.7 (3.4) nmol/l with a range of 20-158 nmol/l. The mean (SEM) DBP levels were 562.2 (8.7) mg/dl with a range of 416-857 mg/dl. A proxy measure of free 25(OH)D at 34 weeks of gestation was attained by calculating the ratio

of 25(OH)D to DBP. 26.8% of the women were taking vitamin D supplements of 400 iu/day. The mean vitamin D intake (from Food Frequency Questionnaires and data on supplements) from the 98 available (out of 102) women's diets was 3.5 μg/day (ranging from 13 – 9.0 μg/day).

Amino acid transporters

Maternal 34 week 25(OH)D correlated positively with *ASCT1*, *LAT3* and *y+LAT1* and negatively with *SNAT1* mRNA expression. Maternal DBP levels correlated positively with *4F2hc*, *EAAT3*, *LAT1* (trend), *LAT3*, *LAT4*, *SNAT1*, *SNAT2*, *TAT1*, and *y+LAT2* mRNA expression. Free 25(OH)D levels were positively associated with *ASCT1*, *LAT3* and *y+LAT1* mRNA expression. Free 25(OH)D levels also showed negative associations with *LAT1* and *SNAT1*, and negative trends with *LAT4* and *4F2hc* mRNA expression. No correlations were observed between early pregnancy 25(OH)D levels and placental amino acid transporter mRNA expression (Table 3.4). When the correlations were adjusted for maternal confounding factors (maternal sum of skinfold thickness, walking speed, parity and smoking during pregnancy) all correlations were still present except for relationships between 25(OH)D and *ASCT1*(trend), and DBP with *LAT3* and *TAT1* (trends), which were no longer significant. The trend observed between 34 week free 25(OH)D and *LAT4* expression was also no longer present. The adjusted data also showed a positive association between DBP and *LAT1* mRNA (Table 3.4). mRNA expression levels were also correlated with *CYP24A1* mRNA as a potential biomarker for active vitamin D levels within the placenta. mRNA expression of *ASCT2, TAT1* (trend) and *y+LAT2* (trend) showed positive associations with *CYP24A1* mRNA expression. *EAAT3* and *LAT1* both showed significant negative associations with placental mRNA expression of *CYP24A1*.

Vitamin D genes

Maternal 11 week 25(OH)D associated positively with *CYP27A1* and *CYP27B1*(trend) mRNA expression. Maternal DBP levels correlated positively with *legumain* mRNA expression. 34 week free 25(OH)D levels associated positively with *CYP2J2* (trend) and negatively with *legumain* (trend) *mRNA* expression. When the correlation was adjusted for maternal confounding factors (maternal sum of skinfold thickness, walking speed, parity and smoking during pregnancy) all correlations were still present, apart from the trend between *legumain* and 34 week free 25(OH)D. In addition, a trend between *VDR* expression and 11 week 25(OH)D was observed, and the association between *CYP2J2* and 34 week free 25(OH)D was significant at the $p < 0.05$ level (Table 3.5). mRNA expression of *CYP24A1* was positively associated with expression of *cubilin*, *CYP27A1* and *megalin* (Table 3.5).

Other genes

DNMT3b showed a positive trend for associations with maternal 11 and 34 week 25(OH)D, while *LPL* mRNA expression was positively associated with 34 week free 25(OH)D. When the correlation was adjusted for maternal confounding factors (maternal sum of skinfold thickness, walking speed,

parity and smoking during pregnancy) all correlations were still present, apart from the trend between *DNMT3b* and 34 week 25(OH)D levels. The association between *DNMT3b* and 11 week 25(OH)D, and *CYP2J2* and 34 week free 25(OH)D was significant at the $p < 0.05$ level (Table 3.5). mRNA expression of *CYP24A1* was positively associated with expression of *PMCA1, DNMT1* and *DNMT3b* (Table 3.5).

Table 3.4: Associations between relative mRNA expression of placental amino acid transporter genes and maternal 25(OH)D and DBP levels.

Gene		11 week 25(OH)D levels (nmol/l)	34 week 25(OH)D levels (nmol/l)	34 week DBP levels (mg/dl)	34 week free 25(OH)D (nmol/l)	11 week 25(OH)D levels (nmol/l)	34 week 25(OH)D levels (nmol/l)	34 week DBP levels (mg/dl)	34 week free 25(OH)D (nmol/l)	*CYP24A1* mRNA expression
		Adjusted for sex and GA				Adjusted for sex, GA and maternal confounding factors				
4F2hc	r	0.06	-0.12	**0.25**	*-0.21*	0.05	-0.08	**0.23**	*-0.21*	-0.10
	p	0.64	0.26	**0.02**	*0.06*	0.65	0.48	**0.04**	*0.07*	0.31
	n	71	88	**82**	*80*	71	88	**82**	*75*	100
ASCT1	r	0.01	**0.23**	0.06	**0.27**	0.05	*0.20*	0.11	**0.23**	-0.07
	p	0.96	**0.03**	0.62	**0.01**	0.70	*0.07*	0.33	**0.046**	0.47
	n	71	**88**	82	**80**	71	*88*	82	**75**	100
ASCT2	r	0.07	0.04	*0.18*	-0.03	0.08	0.05	0.17	0.03	**0.27**
	p	0.58	0.74	*0.095*	0.82	0.53	0.63	0.14	0.80	**0.01**
	n	71	88	*82*	80	71	88	82	75	**100**
EAAT2	r	0.17	0.12	-0.07	0.03	0.20	0.06	-0.01	-0.02	0.06
	p	0.16	0.26	0.53	0.80	0.10	0.56	0.91	0.90	0.54
	n	71	88	82	80	71	88	82	75	100
EAAT3	r	0.19	0.09	**0.30**	0.02	0.18	0.12	**0.29**	0.06	0.10
	p	0.11	0.39	**0.01**	0.87	0.13	0.24	**0.01**	0.59	0.31
	n	70	87	**81**	80	70	87	**81**	75	100
LAT1	r	0.03	-0.14	*0.21*	**-0.23**	0.04	-0.17	**0.23**	**-0.26**	**-0.20**
	p	0.78	0.19	*0.06*	**0.04**	0.71	0.12	**0.04**	**0.02**	**0.04**
	n	71	88	*82*	**80**	71	88	**82**	**75**	**100**
LAT2	r	0.001	-0.08	0.18	-0.11	0.01	-0.07	0.17	-0.09	**-0.25**
	p	0.99	0.44	0.10	0.32	0.90	0.54	0.14	0.42	**0.01**
	n	69	86	81	80	69	86	81	75	**99**
LAT3	r	0.02	**0.31**	**0.22**	**0.32**	0.05	**0.37**	*0.22*	**0.37**	0.03
	p	0.89	**0.003**	**0.04**	**0.003**	0.70	**0.003**	*0.05*	**0.001**	0.78
	n	71	**88**	**82**	**80**	71	**88**	*82*	**75**	100
LAT4	r	0.06	-0.12	**0.28**	*-0.20*	0.03	-0.13	**0.28**	-0.17	-0.11
	p	0.60	0.25	**0.01**	*0.08*	0.80	0.26	**0.01**	0.12	0.29
	n	71	88	**82**	*80*	71	88	**82**	75	100
SNAT1	r	0.09	**-0.23**	**0.25**	**-0.31**	0.09	*-0.20*	**0.23**	**-0.30**	0.03

	p	0.44	**0.03**	**0.02**	**0.004**	0.45	*0.07*	**0.01**	**0.008**	0.79
	n	71	**88**	**82**	**80**	71	*88*	**82**	**75**	100
SNAT2	r	0.13	0.01	**0.23**	0.05	0.14	0.04	**0.23**	0.05	-0.13
	p	0.27	0.96	**0.03**	0.69	0.23	0.70	**0.04**	0.64	0.21
	n	71	88	**82**	80	71	88	**82**	75	100
SNAT4	r	0.09	0.14	0.08	0.09	0.12	0.12	0.12	0.09	0.07
	p	0.45	0.19	0.45	0.43	0.34	0.30	0.29	0.45	0.47
	n	71	88	82	80	71	88	82	75	100
TAT1	r	-0.02	0.07	**0.23**	0.02	-0.02	0.14	0.12	0.09	*0.19*
	p	0.87	0.50	**0.03**	0.99	0.87	0.21	0.10	0.42	*0.06*
	n	70	87	**81**	80	70	87	81	75	*100*
y⁺LAT1	r	0.01	**0.31**	0.03	**0.33**	0.02	**0.36**	0.02	**0.40**	0.16
	p	0.92	**0.003**	0.81	**0.002**	0.88	**0.001**	0.99	**0.001**	0.11
	n	71	**88**	82	**80**	71	**88**	82	**75**	100
y⁺LAT2	r	0.10	0.04	**0.26**	-0.02	0.14	-0.08	**0.33**	-0.06	*0.17*
	p	0.41	0.73	**0.02**	0.86	0.26	0.94	**0.003**	0.61	*0.096*
	n	71	88	**82**	80	71	88	**82**	75	*100*

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics. GA = gestational age.

Table 3.5: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE promoter methylation and maternal 25(OH)D and DBP levels.

Gene		11 week 25(OH)D levels (nmol/l)	34 week 25(OH)D levels (nmol/l)	34 week DBP levels (mg/dl)	34 week free 25(OH)D (nmol/l)	11 week 25(OH)D levels (nmol/l)	34 week 25(OH)D levels (nmol/l)	34 week DBP levels (mg/dl)	34 week free 25(OH)D (nmol/l)	*CYP24A1* mRNA expression
		Adjusted for sex and GA				Adjusted for sex, GA and maternal confounding factors				
Vitamin D genes										
Cubilin	r	0.05	-0.14	-0.05	-0.13	0.03	-0.13	-0.09	-0.10	**0.27**
	p	0.69	0.19	0.66	0.25	0.80	0.23	0.46	0.38	**0.006**
	n	70	86	80	78	64	81	75	74	**100**
CYP2J2	r	0.02	0.17	-0.04	*0.20*	0.07	0.16	-0.03	**0.23**	0.02
	p	0.86	0.11	0.72	*0.08*	0.58	0.16	0.83	**0.045**	0.85
	n	70	86	80	*78*	64	81	75	**74**	100
CYP24A1	r	0.15	-0.15	-0.11	-0.18	0.12	-0.13	-0.16	-0.13	
	p	0.22	0.18	0.34	0.11	0.34	0.25	0.16	0.28	
	n	70	86	80	78	64	81	75	74	
CYP27A1	r	**0.25**	0.08	-0.05	0.09	**0.26**	0.11	-0.10	0.18	**0.37**
	p	**0.04**	0.44	0.68	0.45	**0.03**	0.32	0.40	0.13	**0.001**
	n	**69**	85	79	77	**64**	80	74	73	**99**
CYP27B1	r	*0.21*	0.14	-0.03	0.19	*0.23*	0.08	0.07	0.08	-0.04
	p	*0.08*	0.20	0.78	0.11	*0.07*	0.50	0.57	0.48	0.73
	n	*67*	82	78	76	*62*	77	73	72	91
Legumain	r	0.05	-0.02	**0.27**	*-0.22*	0.03	-0.01	**0.26**	-0.17	0.01
	p	0.71	0.83	**0.02**	*0.06*	0.84	0.94	**0.03**	0.15	0.94
	n	70	86	**80**	*78*	64	81	**75**	74	100
Megalin	r	0.11	-0.14	-0.03	-0.16	0.09	-0.11	-0.09	-0.11	**0.38**
	p	0.35	0.21	0.80	0.16	0.50	0.33	0.45	0.34	**0.001**
	n	69	85	79	78	64	81	75	74	**100**
RXRα	r	0.17	0.04	-0.01	0.04	0.19	0.05	-0.02	0.05	0.003
	p	0.17	0.71	0.91	0.73	0.13	0.67	0.86	0.70	0.98
	n	70	86	80	78	64	81	75	74	100
VDR	r	-0.20	-0.08	-0.05	-0.04	*-0.21*	-0.08	-0.06	-0.04	-0.02
	p	0.10	0.44	0.64	0.76	*0.09*	0.50	0.59	0.75	0.87

	n	70	86	80	78	*64*	81	75	74	100
Calcium transporter genes										
PMCA1	r	0.17	0.13	0.01	0.13	0.17	0.14	-0.01	0.17	**0.30**
	p	0.16	0.24	0.91	0.25	0.16	0.20	0.97	0.15	**0.003**
	n	69	85	79	78	64	81	75	74	**100**
Lipid genes										
LPL	r	-0.12	0.15	-0.09	**0.31**	-0.06	0.17	-0.09	**0.30**	-0.13
	p	0.32	0.16	0.41	**0.007**	0.64	0.13	0.44	**0.008**	0.19
	n	68	84	78	**76**	63	79	73	**72**	98
Methylation genes										
DNMT1	r	0.14	0.17	-0.05	0.13	0.15	0.13	-0.02	0.15	**0.22**
	p	0.23	0.11	0.64	0.58	0.22	0.24	0.83	0.19	**0.03**
	n	70	86	80	78	64	81	75	74	**100**
DNMT3b	r	*0.22*	*0.20*	-0.03	0.16	**0.25**	0.18	-0.002	0.16	**0.29**
	p	*0.07*	*0.06*	0.77	0.15	**0.04**	0.11	0.99	0.16	**0.004**
	n	*68*	*84*	78	76	**63**	79	73	72	**98**
***TAT1* VDRE promoter methylation**										
CpG -1062	r	-0.08	0.01	0.04	-0.01	-0.05	-0.06	0.10	-0.06	*-0.18*
	p	0.50	0.96	0.69	0.95	0.70	0.61	0.37	0.59	*0.07*
	n	68	86	80	79	63	81	75	75	*97*
CpG -1041	r	-0.14	-0.11	-0.11	-0.02	-0.15	-0.10	-0.14	-0.01	-0.03
	p	0.24	0.32	0.34	0.90	0.22	0.39	0.24	0.95	0.74
	n	68	86	80	79	63	81	75	75	97

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics. GA = gestational age.

3.3.3. Methylation of *TAT1* and *TAT1* mRNA expression

Methylation levels of two CpGs that lie within or near a predicted VDRE within the *TAT1* promoter region were measured. Pyrosequencing revealed an average methylation level of 44% at CpG -1062 and 85% at CpG -1041. Methylation at CpGs -1062 and -1041 was not associated with *TAT1* mRNA expression, r = 0.07 and -0.05, respectively (Figure 3.7a and b). Methylation of the two CpGs positively correlated with each other (r = 0.25, p = 0.01; Figure 3.7c).

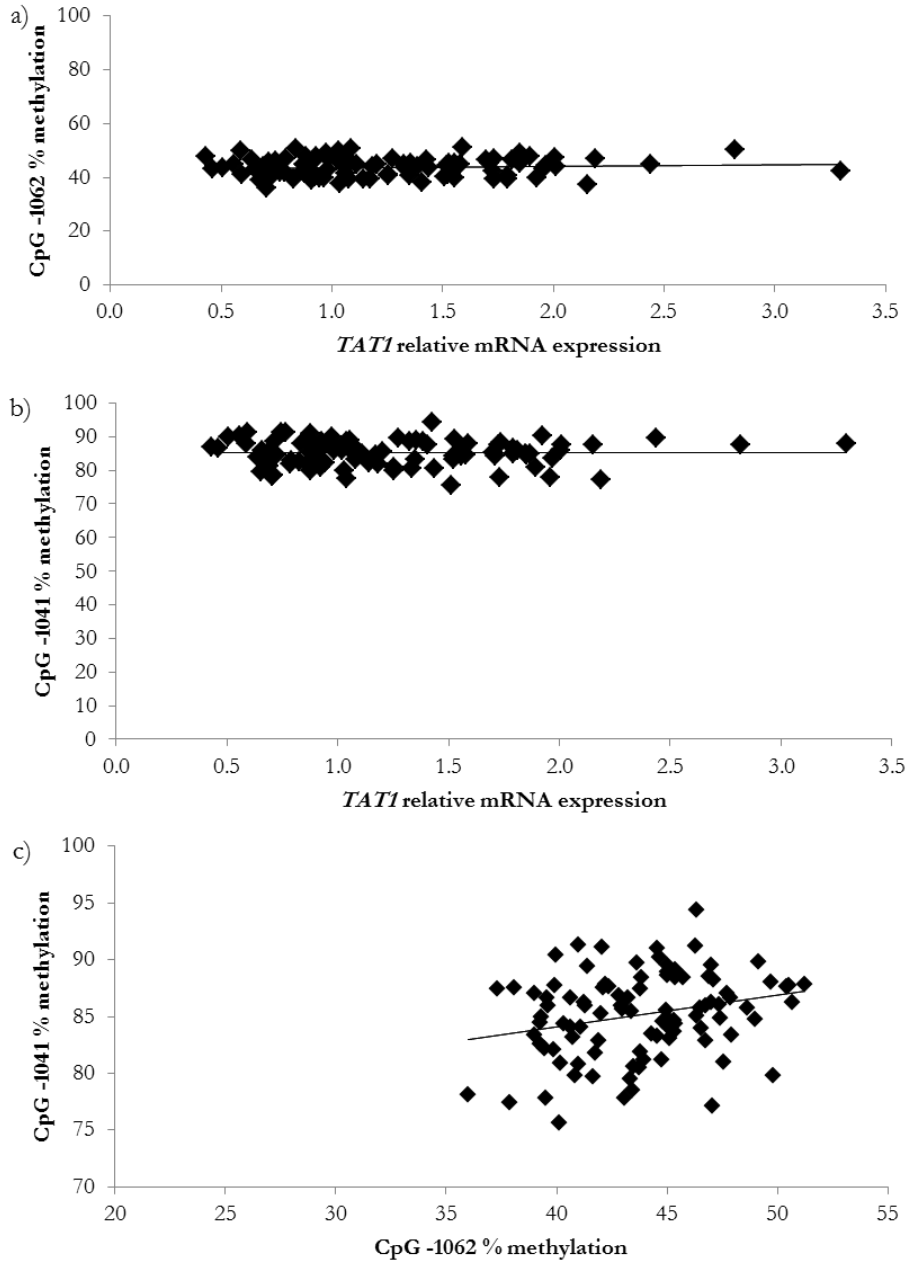

Figure 3.7: Percentage methylation levels at CpG -1062 and CpG -1041. a) Correlation between CpG -1062 methylation and *TAT1* relative mRNA expression. b) Correlation between CpG -1041 methylation and *TAT1* relative mRNA expression. c) Correlation between CpG -1041 methylation and CpG -1062 methylation. Data presented as percentage methylation against *TAT1* mRNA expression. n = 99.

3.3.4. Maternal anthropometry, lifestyle and placental vitamin D-related gene expression

Pre-pregnancy

Placental mRNA expression of specific vitamin D genes showed associations with maternal anthropometry prior to pregnancy. Maternal BMI associated negatively with *CYP27B1* (trend) expression, while both BMI and fat mass associated positively with *CYP24A1* (trend) mRNA expression. The ratio of subscapular to triceps skinfold thickness showed a positive correlation with placental *VDR* expression. Mid-upper arm circumference and arm muscle area showed positive associations with *CYP24A1* expression, while *CYP27B1* mRNA showed a negative trend with mid-upper arm circumference. Maternal height was negatively correlated with *CYP2J2* expression. Percentage methylation at CpG -1062 in the *TAT1* promoter was negatively associated with a number of the maternal pre-pregnancy anthropometry measures, including BMI, fat mass, and mid-upper arm and calf circumference (Table 3.6).

Table 3.6: Associations between relative mRNA expression of placental vitamin D-related genes, *TAT1* VDRE promoter methylation and pre-pregnancy maternal anthropometry.

Gene		BMI	Fat (kg)	Sum of skinfold thickness (mm)	Subscapular/ triceps ratio skinfold thicknesses (mm)	Mid-upper arm circ (cm)	Arm muscle area (cm^2)	Calf circ (cm)	Height (cm)
Vitamin D genes									
CYP2J2	r	-0.10	-0.13	-0.07	0.16	-0.15	-0.11	-0.17	**-0.23**
	p	0.35	0.21	0.47	0.11	0.15	0.31	0.10	**0.03**
	n	96	96	95	95	96	96	95	**96**
CYP24A1	r	*0.17*	*0.19*	0.15	0.15	**0.24**	**0.33**	0.12	0.15
	p	*0.09*	*0.06*	0.13	0.13	**0.02**	**0.001**	0.26	0.15
	n	*96*	*96*	95	95	**96**	**96**	95	96
CYP27B1	r	*-0.18*	-0.15	-0.17	0.12	*-0.18*	-0.07	-0.15	0.11
	p	*0.09*	0.16	0.12	0.28	*0.09*	0.53	0.16	0.30
	n	*89*	89	88	88	*89*	89	89	89
Megalin	r	0.03	0.06	0.07	0.04	0.13	*0.18*	0.05	0.03
	p	0.80	0.55	0.50	0.72	0.21	*0.07*	0.64	0.74
	n	96	96	95	95	96	*96*	95	96
VDR	r	0.10	0.05	0.06	**0.23**	0.03	0.03	0.04	-0.09
	p	0.32	0.63	0.58	**0.02**	0.75	0.77	0.72	0.37
	n	96	96	95	**95**	96	96	95	96
***TAT1* VDRE promoter methylation**									
CpG -1062	r	**-0.25**	**-0.24**	*-0.19*	-0.02	**-0.25**	*-0.18*	**-0.25**	-0.08
	p	**0.01**	**0.02**	*0.07*	0.82	**0.02**	*0.09*	**0.02**	0.41
	n	**95**	**95**	*94*	94	**95**	*95*	**94**	95
CpG -1041	r	0.06	0.02	-0.01	*0.18*	0.01	0.15	0.07	0.08
	p	0.53	0.84	0.98	*0.08*	0.92	0.15	0.50	0.45
	n	95	95	94	*94*	95	95	94	95

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Associations were also observed between some pre-pregnancy maternal lifestyle factors and placental vitamin D-related gene expression. Frequency of strenuous exercise showed a negative association with *legumain* mRNA (trend). Dietary prudence score was positively associated with *CYP27A1* mRNA, whilst high energy diet score was negatively associated with *CYP24A1* mRNA expression (Table 3.7).

Table 3.7: Associations between relative mRNA expression of placental vitamin D-related genes and pre-pregnancy maternal lifestyle.

Gene		Frequency of strenuous exercise	Dietary prudence	High energy diet
CYP24A1	r	-0.01	-0.01	**-0.21**
	p	0.90	0.89	**0.04**
	n	97	97	**97**
CYP27A1	r	0.11	**0.25**	0.06
	p	0.30	**0.02**	0.54
	n	96	**96**	96
Legumain	r	*-0.17*	0.05	-0.01
	p	*0.09*	0.65	0.94
	n	*97*	97	97

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

During pregnancy

CYP24A1 mRNA showed positive correlations with arm muscle area in both early and late pregnancy, and with mid-upper arm circumference in early (trend) and late pregnancy. 11 week arm muscle area showed a negative trend with *CYP27B1* expression, while 34 week mid-upper arm circumference showed a positive association with *cubilin* mRNA (trend). Maternal age at delivery showed a positive association with *CYP27A1* expression and a trend for a positive correlation with *CYP2J2* and *DNMT3b* mRNA levels. Percentage methylation at CpG -1062 in the *TAT1* promoter region showed a negative association with 34 week arm muscle area and 34 week mid-upper arm circumference (trend) (Table 3.8). Only one association was observed with maternal diet during pregnancy; a trend ($p = 0.09$) for a positive relationship between high energy diet score and percentage methylation at CpG -1062 in the *TAT1* promoter region ($r = 0.20$, $n = 73$).

Table 3.8: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE methylation and maternal anthropometry during pregnancy.

		11 week gestation		34 week gestation		Delivery
Gene		**Mid-upper arm circ (cm)**	**Arm muscle area (cm^2)**	**Mid-upper arm circ (cm)**	**Arm muscle area (cm^2)**	**Age at delivery (years)**
Vitamin D genes						
Cubilin	r	0.10	0.03	*0.19*	0.14	0.13
	p	0.42	0.80	*0.07*	0.18	0.20
	n	72	72	*90*	90	97
CYP2J2	r	-0.13	-0.09	-0.12	-0.09	*0.17*
	p	0.28	0.43	0.25	0.38	*0.09*
	n	72	72	90	90	*97*
CYP24A1	r	*0.21*	**0.33**	**0.24**	**0.26**	0.03
	p	*0.08*	**0.004**	**0.02**	**0.01**	0.77
	n	*72*	**72**	**90**	**90**	97
CYP27A1	r	0.02	0.05	0.07	0.13	**0.26**
	p	0.84	0.70	0.50	0.22	**0.009**
	n	71	71	89	89	**96**
CYP27B1	r	-0.19	-0.19	*-0.19*	-0.07	0.11
	p	0.12	0.11	*0.08*	0.53	0.28
	n	68	68	*68*	84	90
Methylation genes						
DNMT3b	r	0.08	0.07	0.07	0.07	*0.17*
	p	0.51	0.58	0.54	0.53	*0.09*
	n	70	70	88	88	*95*
***TAT1* VDRE promoter methylation**						
CpG -1062	r	-0.13	-0.13	*-0.18*	**-0.23**	0.05
	p	0.27	0.26	*0.09*	**0.03**	0.64
	n	70	70	*89*	**89**	96

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

3.3.5. Fetal, neonatal and infant growth and placental gene expression

Fetal

Placental *VDR* mRNA expression was negatively associated with both 34 week abdominal circumference and 19-34 week conditional abdominal circumference. A positive trend was observed with *LPL* expression and 19-34 week abdominal circumference. 11 week abdominal circumference showed a positive trend with *megalin* mRNA, while 19 week abdominal circumference showed a positive trend with *CYP24A1* expression. 11-19 week conditional abdominal circumference showed a positive trend with *legumain* mRNA. Methylation levels at *TAT1* CpG -1041 were negatively associated with 11 week and 11-19 week conditional abdominal circumference z score, while methylation at CpG -1062 showed a trend for a positive association with 34 week abdominal circumference z score (Table 3.9).

Table 3.9: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE methylation and fetal abdominal circumference measurements throughout gestation.

Gene		11 week Royston abdominal circ z-score	19 week Royston abdominal circ z-score	34 week Royston abdominal circ z-score	11-19 week conditional Royston abdominal circ z-score	19-34 week conditional Royston abdominal circ z-score
Vitamin D genes						
CYP24A1	r	-0.11	*-0.23*	-0.21	-0.05	-0.94
	p	0.54	*0.09*	0.12	0.79	0.50
	n	30	*53*	54	30	53
Legumain	r	0.15	0.21	0.21	*0.35*	0.05
	p	0.40	0.13	0.12	*0.05*	0.73
	n	30	53	54	*30*	53
Megalin	r	*0.31*	-0.02	0.05	0.15	0.07
	p	*0.09*	0.84	0.73	0.40	0.64
	n	*30*	53	54	30	53
VDR	r	-0.25	-0.03	**-0.32**	-0.49	**-0.35**
	p	0.17	0.83	**0.02**	0.79	**0.01**
	n	30	53	**54**	30	**53**
Lipid genes						
LPL	r	-0.26	-0.20	0.12	-0.15	*0.26*
	p	0.16	0.15	0.39	0.41	*0.05*
	n	30	53	54	30	*53*
***TAT1* VDRE promoter methylation**						
CpG -1062	r	0.11	0.09	*0.25*	0.22	0.19
	p	0.57	0.54	*0.07*	0.24	0.17
	n	29	53	*54*	29	53
CpG -1041	r	**-0.38**	-0.20	-0.07	**-0.37**	0.08
	p	**0.04**	0.14	0.60	**0.04**	0.54
	n	**29**	53	54	**29**	53

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Crown-heel length at 11 weeks of gestation was positively correlated with *megalin* and *DNMT1* (trend) mRNA. Head circumference at 11 weeks of gestation was negatively associated with methylation of CpG -1041 in the *TAT1* promoter, while at 34 weeks of gestation head circumference associated positively with *CYP24A1* and *legumain* expression. 19-34 week conditional head circumference was positively associated with *CYP24A1*, and showed trends for positive associations with *CYP27A1*, *legumain* and *PMCA1*. *VDR* expression showed a negative association with 34 week head circumference, and 19-34 week conditional head circumference, as well as with 34 week femur length and a negative trend was observed with 19-34 week conditional femur length. 34 week femur length also showed a positive correlation with *legumain* mRNA, and a positive trend with *cubilin*. 19-34 week conditional femur length showed a positive correlation with *cubilin*, a positive trend with *CYP24A1* and a negative trend with *CYP27B1* mRNA levels (Table 3.10).

Table 3.10: Associations between relative mRNA expression of placental vitamin D-related genes, other genes, *TAT1* VDRE methylation and fetal measurements throughout gestation.

Gene		11 week Royston crown-heel length z-score	11 week Royston head circ z-score	34 week Royston head circ z-score	19-34 week conditional Royston head circ z-score	34 week Royston femur length z-score	19-34 week conditional Royston femur length z-score
Vitamin D genes							
Cubilin	r	0.03	-0.07	0.18	0.20	*0.23*	**0.27**
	p	0.82	0.69	0.18	0.15	*0.08*	**0.046**
	n	47	32	54	53	*54*	**53**
CYP24A1	r	-0.16	0.02	**0.31**	**0.44**	0.15	*0.23*
	p	0.28	0.92	**0.02**	**0.001**	0.27	*0.09*
	n	47	32	**54**	**53**	54	*53*
CYP27A1	r	-0.08	-0.02	0.18	*0.23*	-0.01	-0.03
	p	0.59	0.93	0.18	*0.09*	0.96	0.81
	n	46	32	53	*52*	53	52
CYP27B1	r	0.003	0.17	-0.01	-0.11	-0.19	*-0.26*
	p	0.99	0.35	0.97	0.45	0.17	*0.06*
	n	46	31	53	52	53	*52*
Legumain	r	0.04	0.22	**0.29**	*0.26*	**0.30**	0.19
	p	0.80	0.21	**0.03**	*0.06*	**0.03**	0.16
	n	47	32	**54**	*53*	**54**	53
Megalin	r	**0.29**	0.21	0.22	0.19	0.000	0.01
	p	**0.045**	0.24	0.10	0.17	0.99	0.96
	n	**47**	32	54	53	54	53
VDR	r	-0.18	-0.20	**-0.33**	**-0.42**	**-0.28**	*-0.26*
	p	0.21	0.25	**0.01**	**0.001**	**0.04**	*0.05*
	n	47	32	**51**	**53**	**54**	*53*
Calcium transporter genes							
PMCA1	r	0.10	-0.02	0.17	*0.27*	-0.03	0.03
	p	0.49	0.91	0.20	*0.05*	0.82	0.82
	n	47	32	54	*53*	54	53
Methylation genes							
DNMT1	r	*0.24*	0.18	0.16	0.12	0.04	0.03
	p	*0.099*	0.32	0.23	0.38	0.78	0.83
	n	*47*	32	54	53	54	53
***TAT1* VDRE promoter methylation**							
CpG -1041	r	-0.15	**-0.42**	-0.14	-0.08	-0.13	-0.07
	p	0.33	**0.02**	0.27	0.59	0.36	0.61
	n	46	**31**	53	53	54	53

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Neonatal

Placental *VDR* mRNA expression showed negative associations with placental weight, birth weight (trend) and neonatal lean and fat mass. *PMCA1* expression levels were positively associated with placental weight, birth weight and neonatal fat mass. Fat mass also showed a negative association with *RXRa* expression, while birth weight showed positive trends with expression of *CYP24A1*, *cubilin* and *megalin*. Lean mass also showed a positive trend with *cubilin* mRNA (Table 3.11). Neonatal head circumference showed positive correlations with placental mRNA expression of *CYP24A1*, *cubilin*, *CYP27A1* and *PMCA1*, as well as a negative trend with *RXRa*. Crown-heel length showed a positive correlation with *cubilin* mRNA, and a negative correlation with *VDR* mRNA, as well as a negative trend with *CYP27B1*. Subscapular skinfold thickness and mid-upper arm circumference showed

positive associations with *PMCA1* expression. Mid-upper arm circumference also showed a negative trend with *RXRa* mRNA. *DNMT3b* mRNA showed a trend for a negative association with subscapular skinfold thickness. mRNA expression of *megalin* showed a trend for a negative association with percentage BMC (Table 3.11).

Table 3.11: Associations between relative mRNA expression of placental vitamin D-related genes and other genes and placental and neonatal measurements.

Gene		Placental weight (g)	Birth weight (kg)	Head circ (cm)	Crown-heel length (cm)	Subscapular skinfold thickness (mm)	Mid-upper arm circ (cm)	Lean mass (g)	Fat mass (g)	% BMC
Vitamin D genes										
Cubilin	r	0.16	*0.18*	**0.22**	**0.21**	0.01	0.08	*0.19*	-0.03	-0.08
	p	0.12	*0.07*	**0.03**	**0.04**	0.89	0.44	*0.07*	0.80	0.46
	n	95	*96*	**96**	**96**	96	96	*96*	96	96
CYP24A1	r	0.15	*0.19*	**0.26**	0.01	0.16	0.10	0.08	0.08	0.07
	p	0.15	*0.06*	**0.01**	0.95	0.12	0.34	0.46	0.44	0.47
	n	95	*96*	**96**	96	96	96	96	96	96
CYP27A1	r	0.12	0.14	**0.21**	0.03	0.12	0.09	0.02	0.05	-0.16
	p	0.23	0.16	**0.04**	0.75	0.24	0.37	0.88	0.61	0.13
	n	94	95	**95**	95	95	95	95	95	95
CYP27B1	r	-0.10	-0.11	0.01	*-0.20*	-0.14	-0.04	-0.02	-0.16	0.01
	p	0.36	0.30	0.95	*0.06*	0.19	0.74	0.87	0.14	0.94
	n	88	89	89	*89*	89	89	89	89	89
Megalin	r	0.14	*0.20*	0.11	0.11	0.13	0.07	0.11	0.07	*-0.17*
	p	0.16	*0.05*	0.30	0.29	0.19	0.49	0.29	0.49	*0.098*
	n	95	*96*	96	96	96	96	96	96	*96*
RXRα	r	-0.16	-0.13	*-0.18*	-0.10	-0.16	*-0.17*	-0.10	*-0.19*	0.02
	p	0.11	0.21	*0.08*	0.31	0.12	*0.09*	0.31	*0.07*	0.88
	n	95	96	*96*	96	96	*96*	96	*96*	96
VDR	r	**-0.22**	*-0.19*	-0.11	**-0.22**	-0.13	-0.15	**-0.24**	**-0.22**	-0.02
	p	**0.03**	*0.06*	0.27	**0.03**	0.19	0.13	**0.02**	**0.03**	0.82
	n	**95**	*96*	96	**96**	96	96	**96**	**96**	96
Calcium transporter genes										
PMCA1	r	**0.26**	**0.28**	**0.26**	0.08	**0.31**	**0.21**	0.14	**0.21**	-0.10
	p	**0.01**	**0.01**	**0.01**	0.44	**0.002**	**0.04**	0.18	**0.04**	0.35
	n	**95**	**96**	**96**	96	**96**	**96**	96	**96**	96
Methylation genes										
DNMT3b	r	0.07	0.12	0.09	-0.05	*0.18*	0.01	-0.01	0.08	-0.04
	p	0.52	0.24	0.36	0.65	*0.09*	0.89	0.91	0.42	0.68
	n	93	94	94	94	*94*	94	94	94	94

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Infant

Placental *VDR* mRNA expression showed negative correlations with weight, lean and fat mass at 4 years of age. *RXRa* also showed negative associations with fat mass, weight (trend) and height (trend). A negative trend was also observed between fat mass at 4 years of age with *CYP27B1* and *DNMT3b* mRNA expression and *TAT1* CpG -1041 methylation levels. 4 year height was positively associated with *LPL* mRNA expression levels. *LPL* expression also showed positive trends with weight and lean mass. *CYP27A1* showed a positive trend with lean mass at 4 years of age, while lean mass at 6 years of age showed a trend for a negative association with *CYP27B1*. Fat mass at 6 years of age was positively associated with *CYP24A1* and *CYP2J2* (trend) mRNA expression (Table 3.12).

Table 3.12: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE methylation and infant anthropometry at 4 and 6 years of age.

		4 years of age				6 years of age
Gene		**Weight (kg)**	**Fat mass (kg)**	**Lean mass (kg)**	**Height (m)**	**Fat mass (kg)**
Vitamin D genes						
CYP2J2	r	-0.91	0.06	-0.16	-0.11	*0.28*
	p	0.52	0.73	0.31	0.45	*0.07*
	n	51	41	41	51	*43*
CYP24A1	r	0.10	0.07	0.16	0.01	**0.34**
	p	0.48	0.67	0.30	0.96	**0.02**
	n	51	41	41	51	**43**
CYP27A1	r	0.02	-0.14	*0.30*	-0.10	-0.07
	p	0.90	0.37	*0.05*	0.47	0.64
	n	50	40	*40*	50	43
CYP27B1	r	-0.14	*-0.27*	0.06	-0.05	**-0.31**
	p	0.33	*0.08*	0.71	0.74	**0.04**
	n	50	*42*	42	50	**42**
RXRα	r	*-0.27*	**-0.34**	-0.19	*-0.26*	-0.09
	p	*0.05*	**0.03**	0.23	*0.07*	0.55
	n	*51*	**41**	41	*51*	43
VDR	r	**-0.32**	**-0.35**	**-0.42**	-0.13	-0.15
	p	**0.02**	**0.02**	**0.01**	0.35	0.32
	n	**51**	**41**	**41**	51	43
Lipid genes						
LPL	r	*0.23*	0.09	*0.27*	**0.29**	0.12
	p	*0.095*	0.56	*0.09*	**0.04**	0.44
	n	*50*	40	*40*	**50**	43
Methylation genes						
DNMT3b	r	-0.09	*-0.30*	0.15	-0.02	-0.05
	p	0.52	*0.05*	0.33	0.91	0.77
	n	50	*40*	40	50	43
***TAT1* VDRE promoter methylation**						
CpG -1041	r	-0.20	*-0.27*	-0.15	-0.14	-0.13
	p	0.16	*0.09*	0.33	0.33	0.38
	n	51	*41*	41	51	44

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Some correlations were observed between measures of bone size and structure at 4 years of age and placental mRNA expression. *LPL* expression was positively associated with BA, BMC and BMD. *VDR* mRNA also showed a positive association with BMD. Both *CYP27A1* and *PMCA1* showed

strong negative correlations (r = 0.42) with percentage BMC and negative trends with total BA. Methylation levels at CpG -1041 in the *TAT1* VDRE showed a trend for a positive association with percentage BMC (Table 3.13).

Table 3.13: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE methylation and infant bone measures at 4 years of age.

Gene		Total BA (cm^2)	Total BMC (g)	% BMC (without heads)	Total Prentice BMD (g)
Vitamin D genes					
CYP27A1	r	*-0.31*	-0.17	**-0.42**	0.001
	p	*0.05*	0.30	**0.01**	0.99
	n	*37*	37	**42**	37
VDR	r	-0.14	-0.05	0.14	**0.35**
	p	0.40	0.75	0.36	**0.03**
	n	38	38	43	**38**
Calcium transporter genes					
PMCA1	r	*-0.27*	-0.23	**-0.42**	-0.22
	p	*0.09*	0.16	**0.004**	0.18
	n	*38*	38	**43**	38
Lipid genes					
LPL	r	**0.37**	**0.45**	0.06	**0.35**
	p	**0.02**	**0.004**	0.69	**0.03**
	n	**37**	**37**	42	**37**
***TAT1* VDRE promoter methylation**					
CpG -1041	r	-0.21	-0.15	*0.27*	0.22
	p	0.19	0.36	*0.07*	0.18
	n	39	39	*43*	39

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

3.3.6. Placental *DNMT* expression and placental nutrient transporter gene expression

Expression of *DNMT1* was positively associated with mRNA expression of the amino acid transporters, *TAT1* and *y⁺LAT2*, and showed positive trends with *EAAT2*, *EAAT3* and *y⁺LAT2*. *DNMT3b* expression was also positively associated with *EAAT2*, *EAAT3*, *y⁺LAT1* and *y⁺LAT2*, as well as *ASCT2* (Table 3.14). mRNA expression of both DNMTs was positively correlated with mRNA levels of *cubilin*, *CYP24A1*, *megalin* and *PMCA1*. *DNMT3b* was also positively associated with *CYP27A1* and *RXRa* mRNA expression. Expression of the DNMTs was highly correlated with each other (Table 3.14). DNMT expression was not associated with methylation levels at either of the CpGs investigated in the *TAT1* promoter region.

Table 3.14: Associations between relative mRNA expression of placental DNMT expression with placental gene expression.

		DNMT1	*DNMT3b*			*DNMT1*	*DNMT3b*
Amino acid transporter genes				**Vitamin D and calcium transporter genes**			
ASCT2	r	0.16	**0.25**	***Cubilin***	r	**0.37**	**0.25**
	p	0.10	**0.01**		p	**0.001**	**0.01**
	n	100	**98**		n	**100**	**98**
EAAT2	r	*0.17*	**0.29**	***CYP24A1***	r	**0.22**	**0.29**
	p	*0.09*	**0.004**		p	**0.03**	**0.004**
	n	*100*	**98**		n	**100**	**98**
EAAT3	r	*0.17*	**0.26**	***CYP27A1***	r	0.16	**0.28**
	p	*0.09*	**0.01**		p	0.11	**0.01**
	n	*100*	**98**		n	99	**97**
TAT1	r	**0.20**	0.09	***Megalin***	r	**0.71**	**0.40**
	p	**0.049**	0.37		p	**0.001**	**0.001**
	n	**100**	98		n	**100**	**98**
y⁺LAT1	r	*0.20*	**0.22**	***PMCA1***	r	**0.70**	**0.51**
	p	*0.05*	**0.03**		p	**0.001**	**0.001**
	n	*100*	**98**		n	**100**	**98**
y⁺LAT2	r	**0.31**	**0.33**	***RXRα***	r	0.15	**0.35**
	p	**0.002**	**0.001**		p	0.13	**0.001**
	n	**100**	**98**		n	100	**98**
Methylation genes							
DNMT1	r		**0.57**	***DNMT3b***	r	**0.57**	
	p		**0.001**		p	**0.001**	
	n		**98**		n	**98**	

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

3.4. Discussion

This study has shown that selected placental amino acid transporter expression is associated with maternal vitamin D and DBP levels. Furthermore, placental expression of genes involved in transport and metabolism of vitamin D showed associations with fetal and infant growth. This leads to the possibility that maternal vitamin D status and placental handling of vitamin D may influence amino acid transport by the placenta and subsequent fetal growth.

3.4.1. *CYP3A4* is not expressed in placental tissue

CYP3A4 was investigated for its role in the 25-hydroxylation of vitamin D to form 25(OH)D, as if the placenta can produce 25(OH)D this could provide another source of 25(OH)D for the placenta and fetus. *CYP3A4* mRNA expression was not detected and published data are inconsistent as to whether CYP3A4 is expressed in placental tissue. Some studies show no mRNA expression (Nishimura *et al.*, 2003; Bieche *et al.*, 2007), while others have reported expression at the mRNA and enzyme activity level (Maezawa *et al.*, 2010). Further studies have revealed weak mRNA and protein expression but no detection of enzymatic activity (Hakkola *et al.*, 1996a; Hakkola *et al.*, 1996b). The studies which did not detect *CYP3A4* expression utilised pooled cDNA stocks, whilst those which detected *CYP3A4* used human placental tissues collected. Within the human placental samples, there was a wide variation in *CYP3A4* detection reported, with a higher proportion of samples showing no detection (Hakkola *et al.*, 1996a; Hakkola *et al.*, 1996b). Our data have also arisen from pooled samples, created from placental cDNA stocks collected in our laboratory, suggesting that when samples are pooled the result is no detection of the enzyme as it is not expressed at detectable levels in the majority of the sample population. Other CYP enzymes also hydroxylate vitamin D, namely, CYP27A1 and CYP2R1, therefore the lack of *CYP3A4* expression in the placenta in regards to vitamin D metabolism is unlikely to affect placental function.

3.4.2. Maternal plasma vitamin D, DBP and placental mRNA expression

Associations were observed between a number of placental amino acid transporter genes and maternal vitamin D and DBP levels. These associations could represent an effect of vitamin D in mediating placental mRNA expression of these transporters. As amino acid transport is important for fetal growth (Jansson *et al.*, 2006; Cleal *et al.*, 2011), and maternal vitamin D has been associated with fetal growth (Bowyer *et al.*, 2009; Hart *et al.*, 2015) this could mean that vitamin D modulates fetal growth partly through alterations to placental amino acid transport. The effects seen on transporter expression are on both placental membranes, and cover the transport of the entire range of amino acids (Figure 3.8).

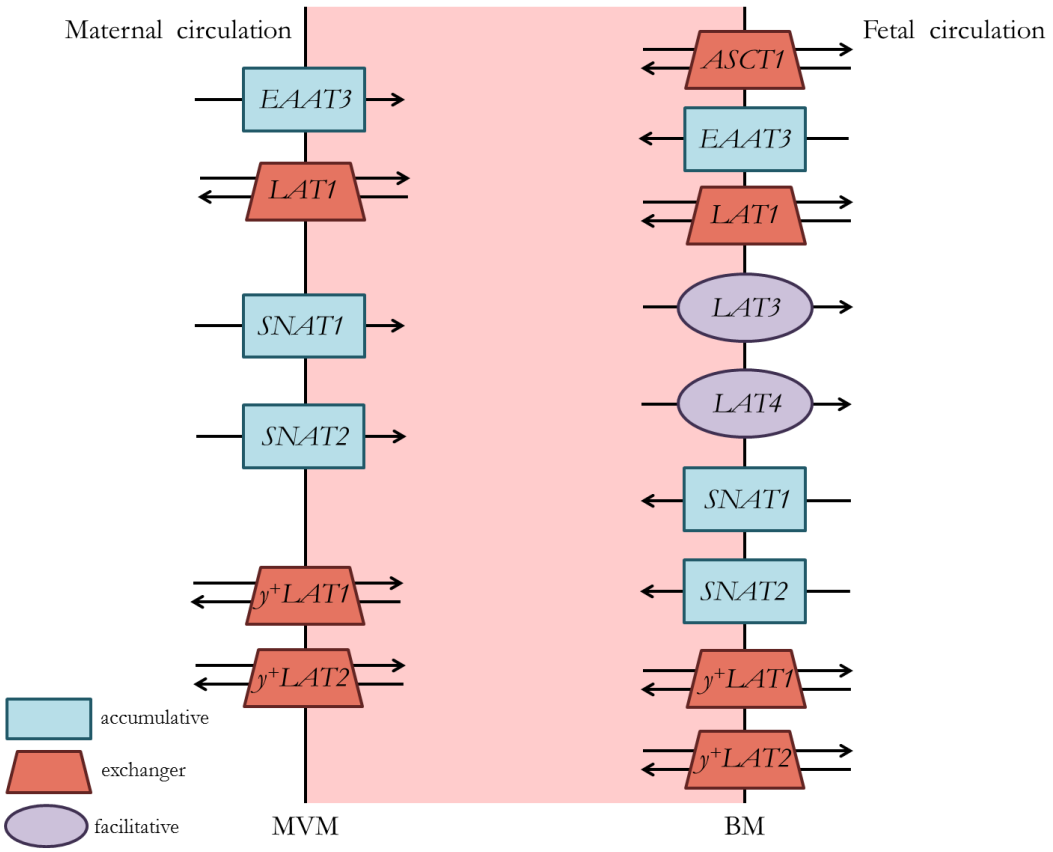

Figure 3.8: Amino acid transporters associated with maternal vitamin D and DBP levels.

When bound to the VDR, 1,25$(OH)_2$D effects gene transcription, therefore the associations between maternal vitamin D and DBP with amino acid transporter mRNA may be direct effects of vitamin D action. This is supported by the fact that some amino acid transporters, including *LAT3* and *ASCT1* have VDREs in their promoter regions (Wang *et al.*, 2005). Alternatively, the associations may be indirect either due to the effect of vitamin D on another gene or on epigenetic processes, such as DNA methylation and histone acetylation (Fetahu *et al.*, 2014). When vitamin D is bound to the VDR, the co-activators recruited are also histone acetyl transferases (Karlic and Varga, 2011) which promote the formation of an active chromatin state that is favourable for gene transcription. If the amino acid transporters studied lie within these regions of chromatin, this could provide an alternative mechanism for the effect of vitamin D on their expression. In this study a positive association was observed between maternal 25(OH)D and *DNMT3b* mRNA, therefore it is possible that some associations observed are a result of increased DNMT activity affecting methylation status of the target gene or its co-activators or repressors. Negative associations between maternal 25(OH)D levels and mRNA expression of amino acid transporters such as *SNAT1* and *LAT1* suggest that a nVDRE may exist within their promoter regions, as has been demonstrated for *CYP27B1* (Turunen *et al.*, 2007). Alternatively, the activated VDR may block the cAMP response element within the promoter of these genes blocking transcription. Indeed, both *SNAT1* and *4F2hc* have previously been shown to be positively regulated by cAMP (Gochenauer and Robinson, 2001; Ogura *et al.*, 2007).

Factors such as maternal obesity, smoking and activity levels influence vitamin D status (Bodnar *et al.*, 2007a; Brock *et al.*, 2010; Zhao *et al.*, 2012; Bjorn Jensen *et al.*, 2013; Vimaleswaran *et al.*, 2013; Gill *et al.*, 2014) and may also influence placental function. For example, an association between maternal muscle mass and placental system A amino acid transport activity was also seen in this cohort (Lewis *et al.*, 2010a). Following adjustment for these factors, associations between *ASCT2* and *TAT1* with maternal DBP were lost. These transporters may therefore be regulated by aspects of maternal body composition, such as fat mass, that are also associated with vitamin D status, rather than being regulated by vitamin D itself.

Maternal DBP levels were associated with the expression of twice as many amino acid transporters as 25(OH)D, indicating that delivery of vitamin D to the placenta may be important. Transport of DBP bound 25(OH)D into the placenta via megalin may be the rate limiting step in determining the effects of vitamin D within the placenta. However, an alternative explanation could be that DBP along with megalin and cubilin are transporting other molecules into the placenta that regulate amino acid transporter expression. DBP is also known to bind both monounsaturated and saturated fatty acids (Speeckaert *et al.*, 2006), leading to the possibility that the associations could be driven by maternal lipid levels. Currently there are no data available for serum lipids from this cohort. Some transporter genes showed a negative association with maternal free 25(OH)D and a positive association with DBP supporting the idea that DBP may mediate vitamin D availability to the placenta. However, this was not consistently observed, suggesting that the relationship between vitamin D and placental amino acid transport is complex and one mechanism is not likely to explain all the associations observed.

No correlations were present between placental amino acid transporter expression and maternal 25(OH)D levels at 11 weeks of gestation. This is likely due to the fact that at this early point in gestation the placenta would not be delivering nutrition to the fetus, as the fetus would still be relying on histiotrophic nutrition from the uterine glands. Interestingly, correlations were observed between 11 week 25(OH)D levels with *CYP27B1*, *CYP27A1* and *DNMT3b* expression. This could suggest that the placenta is already sensing the maternal environment and adjusting its transcriptome accordingly. Both correlations with vitamin D metabolising enzymes were positive, suggesting the placenta may be adapting its enzymatic function to deal with the higher level of vitamin D.

CYP24A1 mRNA expression was used as a potential biomarker for active vitamin D levels within the placenta, as *CYP24A1* is known to be up-regulated by $1,25(OH)_2D$ (Turunen *et al.*, 2007). Placental *CYP24A1* expression was positively associated with a number of amino acid transporter, vitamin D and methylation related genes, including *TAT1 cubilin*, *megalin*, and *DNMT1*. These associations could represent regulation of the genes by active vitamin D levels within the placenta. However, without measures of $1,25(OH)_2D$ this idea of *CYP24A1* as a biomarker cannot be confirmed. Alternatively, the

correlations between mRNA expressions could represent regulation of the genes by common factors. *CYP24A1* mRNA levels were not associated with maternal inactive vitamin D or DBP during pregnancy, therefore if *CYP24A1* is a marker of active vitamin D this suggests that placental uptake and metabolism of vitamin D may be more important in generating the activated vitamin D rather than maternal 25(OH)D levels. Alternatively, the lack of association between maternal 25(OH)D and DBP with various members of the vitamin D pathway could imply that the traditional negative feedback loop which exists in tissues such as the kidney is not active within the placenta.

While this thesis is focussed on the potential effect of vitamin D on the placenta and the effects on fetal growth via the placenta it should also be recognised that vitamin D transported across the placenta could also act directly on the fetus to effect fetal growth. VDR expressed in the fetus could respond to vitamin D transported across the placenta, and as discussed in section 1.6.2 the fetus also expresses CYP27B1 and can therefore generate its own source of $1,25(OH)_2D$ from 25(OH)D transported by the placenta. *VDR*$^{+/-}$ fetuses cannot respond to active vitamin D, and these fetuses have been shown to have reduced body weight as well as reduced mineralised bone volume compared to *VDR*$^{+/+}$ fetuses (Rummens *et al.*, 2003), suggesting that vitamin D is important for fetal growth and bone development. However, the placenta is also composed of fetal tissue, therefore it is currently unclear whether these effects are due to placental or fetal VDR or a combination of both. Some effects of vitamin D on fetal development are likely to be mediated via direct effects on the fetus; conditional knockouts of VDR in either the placenta or fetus would help to ascertain what actions of vitamin D are direct effects on the fetus and what effects are mediated by the placenta.

3.4.3. *TAT1* promoter methylation

TAT1 mRNA expression was not regulated by methylation at the specific sites tested, as seen for specific CpGs in placental GLUT transporters (Novakovic *et al.*, 2013). *TAT1* mRNA expression was positively associated with expression of *DNMT1*, therefore the transporter may be regulated by methylation at sites not studied here. In human placenta methylation of some GLUT transporters and global methylation increases during gestation (Novakovic *et al.*, 2011b; Novakovic *et al.*, 2013). Regulation of mRNA expression by methylation within the placenta is dynamic and changes throughout gestation, therefore methylation of *TAT1* could regulate mRNA expression at earlier time points in gestation. Alternatively, the region studied could be a regulatory region for another gene or could regulate *TAT1* expression within fetal tissue.

The average methylation levels at the two CpGs measured are different despite being only 20 bp apart; 44% compared to 85%. This suggests that these methylation levels may be tightly regulated, although it is unclear what may be regulating methylation at these CpGs, as methylation levels were not associated

with mRNA expression of either of the DNMTs studied. The high methylation level at CpG -1041 could prevent VDR binding and activating *TAT1* transcription and could explain the lack of association between vitamin D and *TAT1* mRNA expression.

Methylation at CpG -1062 in the *TAT1* promoter region was negatively associated with several measures of maternal fat mass before pregnancy, suggesting that maternal body composition before pregnancy can alter placental epigenetic status. This highlights the importance of the pre-conceptional maternal environment. Previous research has correlated maternal factors during pregnancy with placental DNA methylation status (Novakovic and Saffery, 2012) but to our knowledge this is the first demonstration that the pre-conceptional maternal environment may alter placental epigenetics. Pre-conceptional maternal environment could therefore alter placental epigenetic status, which may contribute to differences in placental function and the development of pregnancy-specific conditions related to placental methylation (Anderson *et al.*, 2015).

Methylation at CpG -1041 within the *TAT1* promoter region showed a negative correlation with total fat mass at 4 years of age, suggesting that placental epigenetics could impact upon child development. A similar finding for *RXRa* methylation in cord tissue has been described in the same cohort. *RXRa* methylation negatively associated with percentage BMC at 4 years (Harvey *et al.*, 2014c). These studies provide evidence that placental epigenetics can impact fetal and childhood development, which may have implications for disease in later life.

3.4.4. Maternal anthropometry, lifestyle and placental vitamin D-related gene expression

Expression of *CYP24A1* showed associations with measures of maternal size and body composition before and during pregnancy. The association between measures of maternal muscle mass and placental *CYP24A1* expression suggest that with a higher maternal muscle mass *CYP24A1* is more highly expressed within the placenta, possible resulting in the degradation of larger amounts of vitamin D within the placenta. This could result in a reduction in vitamin D transfer to the fetus, and a reduction in vitamin D-mediated effects within the placenta, such as the potential impact on amino acid transport to the fetus. Vitamin D levels have previously been reported to be increased in those undertaking regular vigorous exercise (Brock *et al.*, 2007), therefore it is possible that the relationship between *CYP24A1* and muscle mass was mediated via an effect of exercise on vitamin D levels. However, *CYP24A1* expression was not associated with the level of strenuous exercise undertaken in this cohort, suggesting that the association with muscle mass is independent of exercise. *CYP24A1* is highly methylated within the placenta (Novakovic *et al.*, 2009), it would therefore be interesting to see if the increased mRNA expression associated with a higher maternal muscle mass is a result of a reduction in *CYP24A1* methylation within the placenta.

3.4.5. Fetal, neonatal and infant growth and placental mRNA expression

The expression of genes involved in vitamin D transport and metabolism showed consistent correlations with several measures of fetal and infant growth. *CYP27B1* and *VDR* showed negative associations with growth while *CYP24A1* showed positive associations. *CYP24A1* mRNA expression is increased in response to active vitamin D as part of a negative feedback loop to tightly control active vitamin D levels, while *CYP27B1* expression is down-regulated in response to active vitamin D to reduce conversion from inactive vitamin D (Turunen *et al.*, 2007). The associations of the mRNA expression of these genes with fetal growth could imply a role of active vitamin D levels in fetal growth (Figure 3.9). Studies show positive associations with maternal vitamin D and birth weight (Harvey *et al.*, 2014a). Our data suggests *CYP24A1* levels are representative of the active vitamin D levels in the placenta that are available to the fetus to influence its growth in a positive manner. *CYP24A1* mRNA levels could represent a biomarker for active vitamin D levels. Conversely a reduction in *CYP27B1* expression in response to active vitamin D levels means this gene is inversely representative of active vitamin D levels and therefore relates negatively to fetal growth.

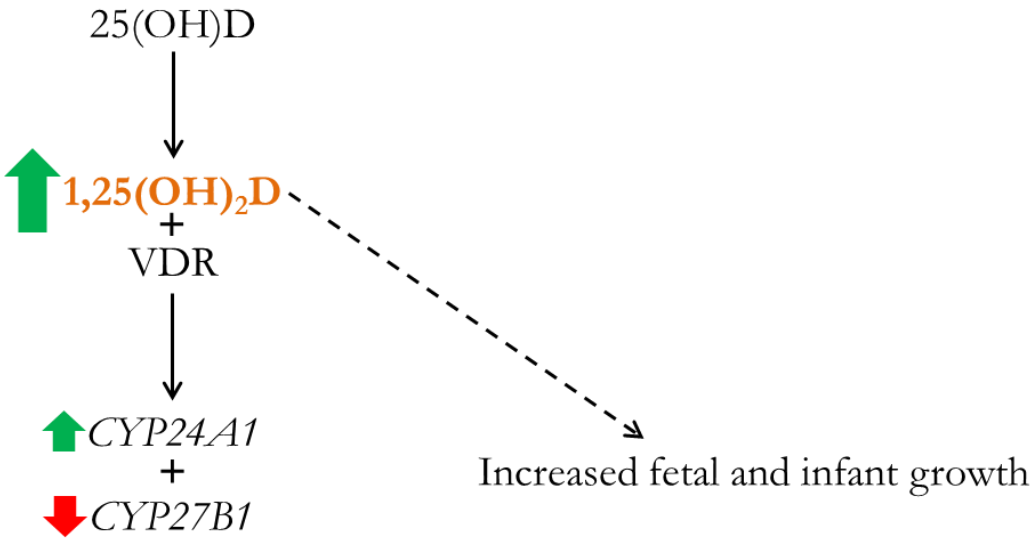

Figure 3.9: Potential relationship between active vitamin D levels, placental *CYP27B1* and *CYP24A1* expression, and fetal and infant growth.

Placental *VDR* mRNA expression was negatively associated with fetal growth and infant body composition at birth and 4 years of age. Again, *VDR* relates negatively to vitamin D levels (11 weeks of gestation). Negative associations were also observed with *RXRa* and neonatal and infant measurements. The VDR/RXRα signalling pathway may therefore be operating within the placenta which is affecting placental function and subsequent fetal growth. This could occur through an effect on placental amino acid transporters or other genes which contain VDREs. RXRα can heterodimerise with other nuclear receptors to initiate transcription, such as retinoic acid receptors (Chawla *et al.*, 2001), therefore other signalling pathways could also be involved in the observed associations.

The limited studies relating placental VDR expression and fetal growth suggest a positive relationship. Placental *VDR* mRNA expression was reduced in FGR pregnancies (Nguyen *et al.*, 2015), whilst higher VDR protein expression associated positively with longer bone length in fetuses of pregnant adolescents (Young *et al.*, 2014). In our data set *VDR* mRNA associated negatively with femur length which differed from observations in Young *et al.* (2014). This could be because we measured mRNA whereas they measured protein. Alternatively age differences may explain the inconsistency; women in our study had a mean age of 30 years, whereas the average age of mothers in Young *et al.* (2014) was 17 years. *VDR* genotype can modify the relationship between maternal vitamin D levels and fetal growth (Morley *et al.*, 2009). Our study included largely Caucasian women, whilst Young *et al.* (2014) had a higher proportion of African American women, therefore differences in *VDR* genotype between the populations could also underlie the discrepancies in the data between these studies.

Megalin, cubilin, *legumain* and *CYP27A1* showed some positive associations with measures of fetal, neonatal and infant size. These associations, and those seen with the other genes involved in vitamin D transport and metabolism, suggest that placental handling of vitamin D, particularly active vitamin D, may play a role in determining fetal growth outcomes. None of the vitamin D associated genes showed significant associations with measures of infant bone mass at birth suggesting that placental vitamin D transport and metabolism had little impact on fetal bone development. However, measures of bone size at 4 years, but not 6 years of age associated negatively with *CYP27A1* and positively with *VDR* and *LPL*. LPL could be producing higher levels of fatty acids within the placenta that are influencing placental function or fetal growth.

PMCA1 associated positively with many measures of fetal growth and negatively with percentage BMC and BA at 4 years of age. This seems counterintuitive due to the necessity of calcium for bone formation. However, this single transporter is part of a much wider placental calcium transport system. For example, *PMCA3* mRNA expression within another subset of samples from the same cohort was positively associated with BA and BMC of the neonate (Martin *et al.*, 2007). To get a better picture of the effect of calcium transport of fetal development we need to interrogate a much wider range of transporters.

3.4.6. Associations between placental DNMT expression and placental nutrient transporter gene expression

A number of placental genes involved in amino acid transport or vitamin D processes were positively associated with *DNMT1* and/or *DNMT3b* mRNA. DNA methylation is thought to negatively effect mRNA expression; through preventing the binding of co-activators and transcription factors to their binding sites within the promoter region (Bird, 2002). We would therefore expect to see inverse

correlations between DNMT and target gene expression, where increased DNMT would increase DNA methylation resulting in a decreased target mRNA expression. DNMT activity may however methylate the gene within a negative response element preventing the down-regulation of transcription via this site. Alternatively, methylation of specific transcriptional repressors may occur, resulting in a lifting of transcriptional repression. *DNMT3b* associated with mRNA expression of twice the number of genes than *DNMT1*, which could represent particular importance of *de novo* methylation in regulation of placental mRNA expression which could result from the initial establishment of epigenetic marks (Okano *et al.*, 1999). It was postulated that vitamin D may mediate mRNA expression via changes in DNMT expression and *DNMT3b* mRNA was associated with maternal 25(OH)D levels. However, only a few genes were associated with both maternal 25(OH)D and DNMT expression, suggesting that the majority of potentially vitamin D-mediated effects observed here are not due to *DNMT3b* expression changes.

3.4.7. Limitations

This study uses a well characterised human cohort to investigate potential relationships between vitamin D and placental nutrient transport. However, a few limitations should be acknowledged, Firstly, the small sample size should be noted, particularly for the infant measures at 4 and 6 years. The smaller numbers may result in less power to detect associations. This may partly explain why there were not many associations detected between placental mRNA expression and anthropometric measures at these ages. Secondly, the study is limited by the current serum measurements which have been made in this cohort. Measurement of other related compounds would help investigate the associations observed more thoroughly; for example $1,25(OH)_2D$ and lipid levels in maternal serum. In addition, the current measure of free 25(OH)D is an estimation based on the ratio of 25(OH)D to DBP levels. This method gives an approximation of free 25(OH)D but does not take into account albumin bound 25(OH)D. For a more accurate measure of free 25(OH)D serum albumin measures within the participants are also required. Thirdly, the difference in timing between maternal blood sampling for vitamin D and DBP levels and the timing of the placenta sampling should be noted. The final maternal blood sample was taken at 34 weeks of gestation. This is 6 weeks before the expected delivery of the child and placenta, therefore the assumption is made that the maternal levels remain relatively constant until delivery.

This study used a large number of statistical tests therefore some of the significant results observed may be due to chance. Adjustment for multiple comparisons was deemed inappropriate due to the observational nature of the study and the collinearity among outcomes, such as the numerous measures of fetal size. Results obtained from this study on fetal and maternal factors need to be confirmed within other human cohorts, while data on vitamin D levels and mRNA expression could

be confirmed using *ex vivo* studies. The data presented are correlations, and in an observational study such as this it is not possible to determine whether the associations observed are causative even though data have been adjusted for potential confounding factors. Some of the associations presented have r values around 0.2 indicating a 20% correlation. These lower level correlations highlight the complex nature of the system we are investigating. In reality, a range of factors will impact placental mRNA expression and function. Nevertheless, the data provide a basis for generating hypotheses which can be tested with in-depth mechanistic studies.

The pyrosequencing approach to investigate DNA methylation levels is a targeted approach and due to the high numbers of CpGs within the *TAT1* promoter region it was not possible to measure methylation levels at all the CpGs. Therefore, CpGs were identified for pyrosequencing based on the predicted transcription factor binding sites that they were associated with. This approach means that only a small proportion of CpGs were analysed. For example, the first 1000 bp of the sequence of interest contains eleven CpGs, of which we have pyrosequenced only two. The pyrosequencing approach also only targets a specific gene. Methylation arrays can give a much broader picture of the methylation status using a large number of probes. However, these probes are pre-determined, therefore the areas targeted cannot be specified. However, a methylation array could be used to identify genes with variable methylation levels that could then be investigated more thoroughly using pyrosequencing. Further mechanistic studies would be required to investigate whether methylation changes within the placenta are causative of changes on infant development.

3.4.8. Future work

The correlations observed in this human cohort study have raised many interesting questions that will be investigated further throughout the course of this thesis. These include:

1. How is vitamin D transported into the placenta from the maternal circulation? Does DBP play a key role in this transport?
2. Vitamin D and DBP showed many positive associations with expression levels of placental amino acid transporters. Do these correlations represent a direct effect of vitamin D on the transporter expression?
3. *CYP24A1* expression is increased by $1,25(OH)_2D$. In this cohort *CYP24A1* expression was associated positively with a number of measures of fetal and infant growth, including birth weight and head circumference. Could *CYP24A1* expression be a biomarker of active vitamin D levels within the placenta?

Future work involving this cohort could include measurement of active vitamin D levels within the maternal plasma. If measures of both inactive and active vitamin D were compared we could get an idea of the level of vitamin D metabolism within the placenta. Furthermore, active vitamin D levels could be tested for correlations with placental mRNA expression. This may prove useful in establishing whether vitamin D is directly regulating amino acid transporter expression and for the investigation of whether *CYP24A1* could be a biomarker for active vitamin D.

Measurement of fatty acid levels could also be interesting, as a number of vitamin D genes were associated with measures of maternal size before and during pregnancy, particularly *CYP24A1*. Measurement of fatty acid levels may help to elucidate the nature of these relationships. Furthermore, as DBP can also bind fatty acids, this may help elucidate whether associations with DBP represent vitamin D-specific effects.

The infants in the SWS continue to be followed up. Data on 10 year infants are currently being collected by the SWS Study Group. When this data collection is complete it would be interesting to test infant size at 10 years for correlations with the placental mRNA expression to see if any of the associations with fetal and neonatal growth re-emerge.

Sex differences in placental gene expression and developmental programming have been described (Rosenfeld, 2015; discussed further in 7.4.1). The data in this chapter have been adjusted for sex, however, it would be interesting to separate the cohort into male and female offspring and examine whether the associations differ based on the sex of the offspring.

3.4.9. Conclusions

Maternal vitamin D status has previously been linked with fetal growth and development, as has placental amino acid transport. The associations presented here between maternal vitamin D and DBP levels and placental amino acid transporter expression suggest that vitamin D may impact fetal growth through an effect on placental amino acid transport. Furthermore, correlations between DBP and amino acid transporter expression suggest that vitamin D transport into the cell via megalin and cubilin may be a rate limiting factor in determining the effects of maternal vitamin D on placental function. Associations between genes involved in the vitamin D pathway and maternal factors suggest that maternal body composition both before and during pregnancy may impact the placental handling of vitamin D. Furthermore, expression levels of genes related to vitamin D showed associations with specific measures of fetal and infant size, including fat mass. This implies that placental vitamin D transport and metabolism may have long lasting impacts for the child (Figure 3.10).

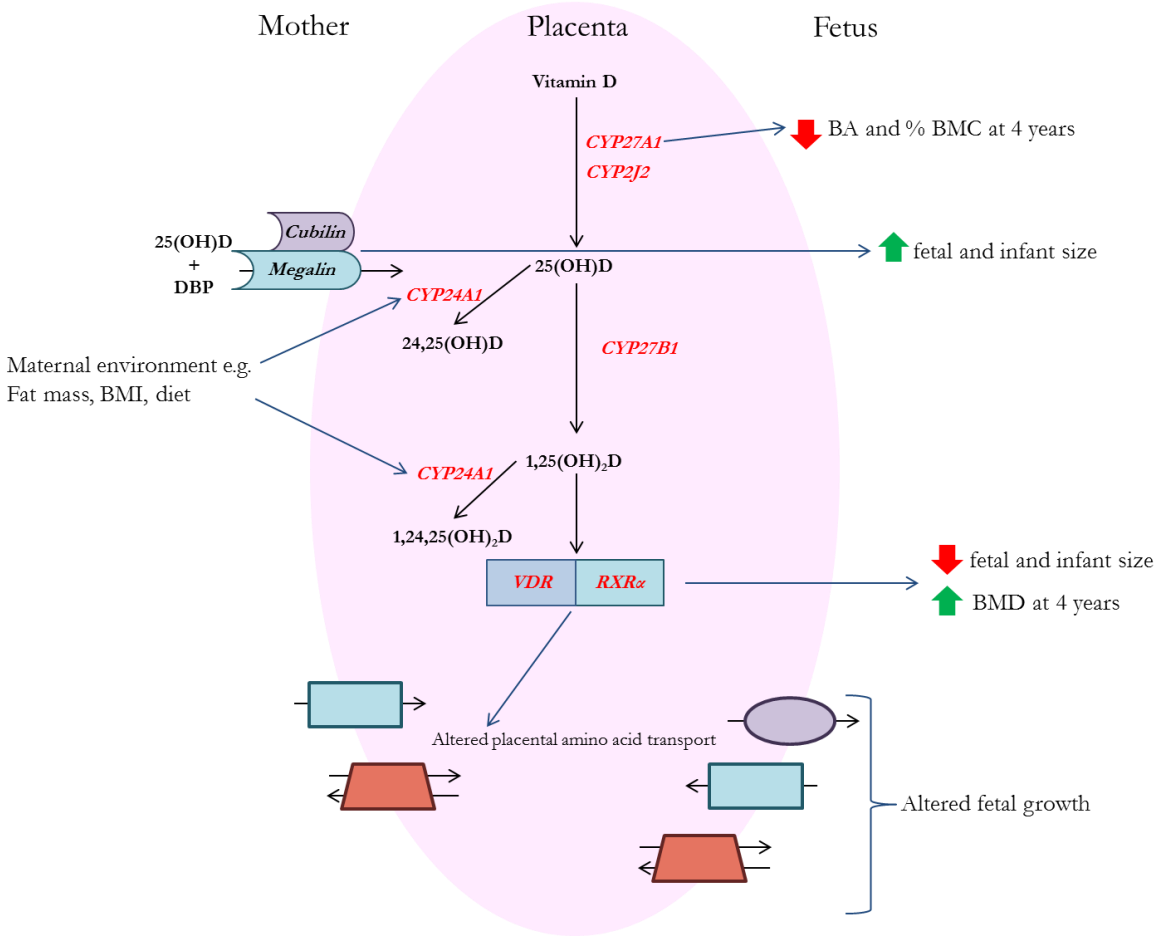

Figure 3.10: Summary of the major associations between the maternal environment, placental vitamin D mRNA expression and fetal outcomes. Maternal factors including BMI and fat mass were strongly associated with *CYP24A1* expression within the placenta. Maternal vitamin D and DBP levels were associated with mRNA expression of a number of placental amino acid transporters and expression of amino acid transporters within the placenta has previously been associated with fetal growth. *VDR* and *RXRa* were associated with reduced fetal and infant size but increased BMD at 4 years. *CYP27A1* was negatively associated with measures of bone development at 4 years, while *cubilin* and *megalin* expression were positively associated with measures of fetal and infant size. These associations suggest that maternal vitamin D may impact placental nutrient transport to the fetus but also show that transport and metabolism of vitamin D within the placenta may play an important role in fetal development. Black lines indicate vitamin D signalling and metabolism, while blue lines indicate associations with placental gene expression and maternal and fetal factors in the SWS.

Chapter 4:
Placental cell models: Characterisation and effects of vitamin D on mRNA expression

4.1. Introduction

Vitamin D may impact upon fetal growth through effects on placental function. Vitamin D is also thought to interact with epigenetic mechanisms of mRNA expression. These two separate but linked mechanisms could alter placental mRNA expression, impacting upon placental nutrient transport, which could ultimately alter fetal growth. In order to investigate the effect of vitamin D on placental mRNA expression a suitable cell culture model is needed.

4.1.1. Current placental cell models

To investigate human placental function, an appropriate model is required that resembles the role of the placenta as a transporting epithelium. Placentation in animals varies from that in humans meaning that animal models are not always appropriate for these studies (Carter, 2007). The ideal systems to study placental function are human placental primary trophoblast culture, villous explants or villous fragment culture. However, these methods are reliant on the availability of fresh human placental tissue. Furthermore, primary placental cells do not proliferate in culture which limits experimentation (Orendi *et al.*, 2011). Villous explants also pose disadvantages as the syncytiotrophoblast of the explant dies off and then regrows (Siman *et al.*, 2001). It is unclear what cell type this new syncytiotrophoblast is. Due to these issues, it was decided to pursue a cell culture model to study human placental nutrient transport.

A vast number of placental cell models are available. These vary in the starting material and the method of immortalisation, creating major differences between the cell lines (King *et al.*, 2000). Further variation in cell lines arises as the placenta is a heterogeneous organ composed of villous cytotrophoblast, stroma, extravillous cytotrophoblast, syncytiotrophoblast and endothelial cells (Sullivan, 2004). The placental syncytiotrophoblast forms the major transporting epithelium of the placenta (Cleal and Lewis, 2008); therefore this is the placental cell population that we wish to model.

It is clear from the literature that characterisation of placental cell lines is lacking (King *et al.*, 2000; Shiverick *et al.*, 2001; Sullivan, 2004). While efforts are being made to characterise the available cell lines, some of the criteria for characterisation are questionable. Placental syncytiotrophoblast models are characterised by hCG secretion (King *et al.*, 2000), which is also a common feature of cancer cells (Heyderman *et al.*, 1985), meaning characterisation of choriocarcinomas is difficult. Due to the questions over characterisation of cell lines as truly placental, two potential cell lines of epithelial origin will be investigated and characterised prior to experiments on placental function. The cells investigated will be the BeWo choriocarcinoma and the HEK293 kidney cell lines.

BeWo

The BeWo choriocarcinoma cell line is the most extensively used cell model for human placenta. BeWo cells were classified as placental based on the secretion of hCG (Pattillo *et al.*, 1971), but this may not be a reliable indicator for placental origins of a cancerous cell line. BeWo cells have previously been used to study some placental amino acid transporters (Fang *et al.*, 2006; Jones *et al.*, 2006) but not the facilitated amino acid transporters. Some components of the vitamin D signalling pathway have previously been investigated in BeWo cells in comparison to placenta with lower *VDR* in BeWo cells (Pospechova *et al.*, 2009). Unlike other choriocarcinoma cell lines, the BeWo cell line can be promoted to syncytialise and form a multinucleated cell layer, similar to the placental syncytiotrophoblast (Wice *et al.*, 1990), so this provides another potential model to investigate.

HEK293

HEK293 cells form an epithelial transport layer similar to the placental syncytiotrophoblast meaning they may provide an appropriate model. Expression of *VDR*, *CYP27B1* and *CYP24A1* has been demonstrated in HEK293 cells. Furthermore, reduced *CYP27B1* and increased *CYP24A1* mRNA expression has been shown in response to culture with $1,25(OH)_2D_3$ (Turunen *et al.*, 2007), suggesting that HEK293 cells respond in a classical manner to vitamin D. HEK293 cells are also commonly transfected with transporter genes to study transport (Khunweeraphong *et al.*, 2012; Kovacs *et al.*, 2012; Nishimura *et al.*, 2014; Jebbink *et al.*, 2015), raising the possibility of using transfection to introduce transporter genes if our genes of interest are not naturally expressed.

4.1.2. Methylation

DNA methylation is generally associated with repression of mRNA expression (Bird, 2002). Differences in methylation levels between cell types has been described (Novakovic *et al.*, 2011a). In particular cancer cells are reported to show aberrant methylation (Kulis and Esteller, 2010). These variations in DNA methylation levels between cell types could result in differences in expression of key genes, therefore it is important to characterise the response to methylation in the two cell models being considered. Vitamin D has been linked with altered methylation status (Rawson *et al.*, 2012; Tapp *et al.*, 2013; Zhu *et al.*, 2013; Zhou *et al.*, 2014b) and expression of vitamin D genes is regulated by methylation (Novakovic *et al.*, 2009; Zhou *et al.*, 2014b). Therefore the placental response to maternal vitamin D may partly be mediated by methylation which in turn may be modified by environmental influences. These pathways of vitamin D and DNA methylation may converge to adapt placental function in light of maternal influences (Figure 4.1).

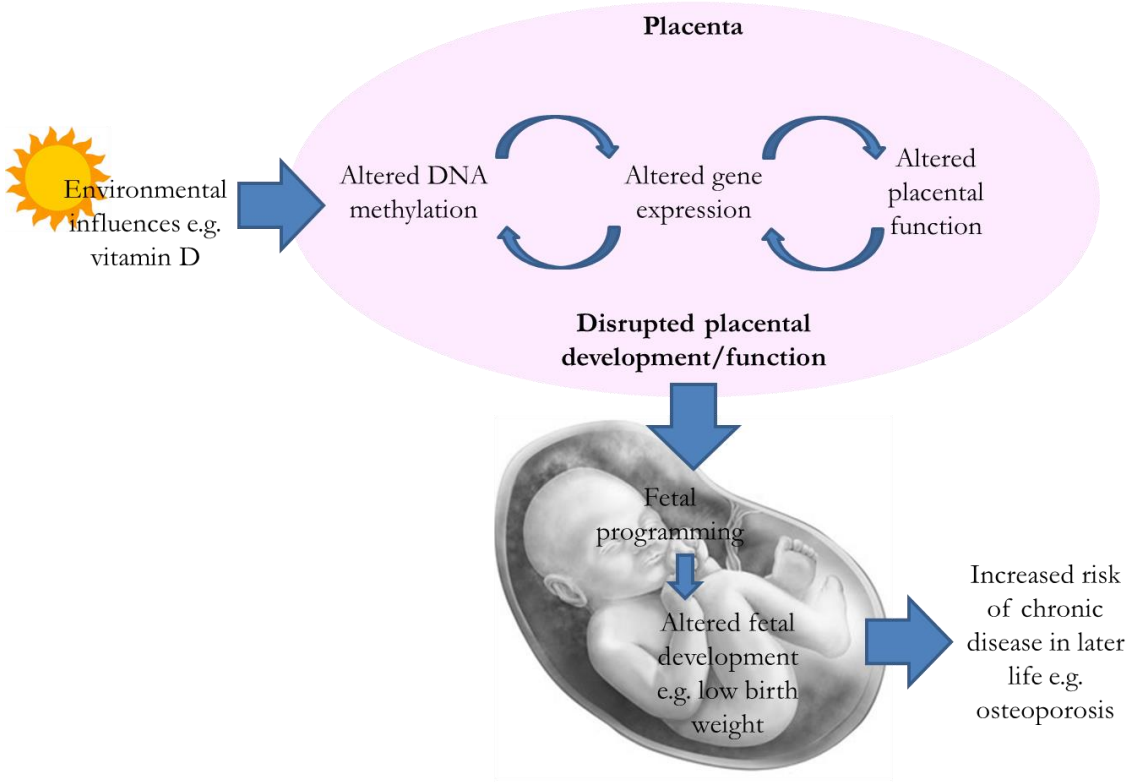

Figure 4.1: Potential influences of vitamin D and DNA methylation on placental function. Environmental factors, such as maternal vitamin D levels, may alter DNA methylation within the placenta. This could then result in changes to mRNA expression, resulting in alterations to placental function. The adaptations in placental function may propagate further adaptations within the placenta. These placental adaptations could result in abnormal or disrupted placental development and function, which could result in fetal programming. Fetal development may be altered in response to the placental limitations, resulting in consequences such as low birth weight. These effects could lead to predisposition to chronic disease in adult life.

While epigenetic modification of some vitamin D-related genes within the placenta has been demonstrated (Novakovic *et al.*, 2009), methylation status of placental nutrient transporters is unclear. Methylation status of some placental glucose transporters associated with expression, while for other glucose transporters there was no association between methylation and expression. (Novakovic *et al.*, 2013), suggesting that regulation is transporter-specific. There are no current data on methylation status of placental amino acid transporter genes. Gene expression of some placental amino acid transport and vitamin D-related genes associated with DNMT mRNA expression in placentas from the SWS (Chapter 3), suggesting that they may be regulated by methylation. Investigation of expression of these genes in response to removal of methylation will provide further evidence as to whether expression of these genes is regulated by methylation.

4.1.3. Vitamin D

Data from the SWS (Chapter 3) revealed associations between maternal 25(OH)D levels and mRNA expression of specific amino acid transporters within the placenta. This has raised the possibility that maternal vitamin D levels could alter placental nutrient transport. Furthermore, if vitamin D does alter

placental nutrient transport is this via a direct effect on transporter mRNA expression? Sequence analysis has revealed that some placental amino acid transporters, including *TAT1*, contain potential VDREs within their promoter regions, strengthening the argument that these genes may be directly regulated by vitamin D.

Observational studies have described associations between maternal vitamin D levels and fetal growth (Harvey *et al.*, 2008; Bowyer *et al.*, 2009; Mahon *et al.*, 2010). Placental amino acid transport is also related to fetal growth (Cleal *et al.*, 2011) and reductions in placental amino acid transport have been shown to occur prior to FGR (Jansson *et al.*, 2006). What is currently unknown is what may cause the reductions in placental expression of amino acid transporters. The link between vitamin D and fetal growth, alongside the associations observed between maternal vitamin D and placental amino acid transporter expression leads to the possibility that maternal vitamin D levels may be one factor regulating amino acid transporter expression within the placenta.

4.1.4. Aims

The aim of this investigation is to establish a suitable cell culture model to study vitamin D transport and the effects of vitamin D on placental nutrient transporter expression. Specifically, the aims of this investigation are:

1. To characterise mRNA expression in HEK293 and BeWo cell lines and assess their suitability as a model for the placental syncytiotrophoblast.
2. To investigate the impact of methylation on genes involved in placental nutrient transport and vitamin D handling in HEK293 and BeWo cell lines.
3. To investigate the impact of inactive and active vitamin D on expression of genes involved in the placental response to vitamin D and nutrient transporters in a suitable placental cell model.

4.2. Methods

BeWo and HEK293 cell lines were investigated for their potential as a placental syncytiotrophoblast model. Suitability as a good model was assessed through, rt-PCR, qrt-PCR and western blotting. Transfection of cells was tested to produce expression of genes of interest. The effect of removing methylation on target gene expression was also investigated in the cell lines. Following establishment of a suitable cell culture model the response to vitamin D was assessed.

4.2.1. SWS: Placental cells

As a comparison for mRNA expression in HEK293 and BeWo cells, pooled stocks of SWS placental cDNA were created. Protein was also extracted from SWS placental samples to compare amino acid transporter expression against BeWo and HEK293. These studies were conducted according to the guidelines in the Declaration of Helsinki, and the Southampton and South West Hampshire Research Ethics Committee approved all procedures. Written informed consent was obtained from all participating women.

4.2.2. Cell culture

BeWo and HEK293 cells were cultured as described in General methods 2.3.2 in a 1:1 mixture of DMEM and Ham's F12 containing 10% FBS, 100 iu/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine. BeWo cells were plated at 2.5 x 10^5, while HEK293 cells were plated at 1.2-2 x 10^5 per 32 mm well. RNA or protein was extracted at ~80% confluency. Primary cytotrophoblast cells were cultured and RNA extracted by Dr J. Cleal as described in section 2.3.2.

Three independent cell culture experiments were carried out with conditions in triplicate for each experiment. 24 h after plating, DMEM/Ham's F12 media was removed and media was replaced with media containing a specific concentration of vitamin D or AZA, a DNMT inhibitor. BeWo and HEK293 cells were cultured with 7 μmol/l AZA or control media (DMEM/Ham's F12) to investigate the removal of methylation on target gene expression (n = 9). HEK293 cells were also cultured with 25(OH)D (3 nmol/l, 10 nmol/l, 50 nmol/l and 100 nmol/l), 1,25$(OH)_2$D (0.1 nmol/l, 1 nmol/l, 10 nmol/l and 50 nmol/l), ethanol vehicle control or control media to investigate the effect of vitamin D on target gene expression. After 48 h, cell culture media was removed, and RNAzol® was added to the well. Cells were scrapped into RNAzol®, transferred to an eppendorf and stored at -80ºC until RNA extraction. Numbers for vitamin D experiments reduced following RNA extraction due to some poor quality RNA, resulting in final numbers of: 9 for 10 nmol/l 25(OH)D and 1 nmol/l 1,25$(OH)_2$D, 8 for

3 nmol/l 25(OH)D, 100 nmol/l 25(OH)D, 0.1 nmol/l 1,25$(OH)_2$D, 50 nmol/l 1,25$(OH)_2$D, ethanol control and media control, 7 for 10 nmol/l 1,25$(OH)_2$D, and 6 for 50 nmol/l 25(OH)D.

4.2.3. mRNA expression

RNA was extracted with RNAzol® as previously described (General methods 2.5.2). RNA quality was determined through NanoDrop™ measurements and gel electrophoresis. cDNA was synthesised from 200 ng RNA using the Promega method as described in Chapter 2 section 2.5.4. NECs were synthesised at the same time as cDNA.

rt-PCR was used to determine whether specific genes are expressed in the BeWo and HEK293 cell lines. rt-PCR reactions were as described in Chapter 2 section 2.5.5 and primer pairs are displayed in Table 4.1. PCR products were run on 1-3% agarose gels to visualise the presence (or absence) of the gene of interest and PCR products were purified and sent for sequencing to GATC Biotech (sections 2.5.6 and 2.5.7). Products from all primer sets have been verified by DNA sequencing apart from *CYP2J2*, *CYP2R1*, *CYP3A4*, *CYP24A1*, *CYP27A1* and *CYP27B1*.

qrt-PCR was used to compare mRNA expression levels of genes between placenta (n = 10), BeWo (n = 10) and HEK293 (n = 9) samples, and to investigate mRNA expression changes in cell models in response to removal of methylation and vitamin D. qrt-PCR was carried out as described in section 2.5.8 using Roche UPL assays. Cycling conditions were 95°C for 10 min; 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 s; and 50°C for 30 s. Data were collected at the 72°C step. Primers are displayed in Table 4.2. For each qrt-PCR assay, samples (4 ng) were run alongside a standard curve, NECs and NTCs in triplicate (Table 2.6). Cp values were determined by the second derivative method and were converted to DNA concentration (ng/5 µl) using a standard curve (see General methods 2.5.8). In a few cases, the standard curve failed and $2^{-\Delta\Delta cp}$ was used to calculate gene expression levels (see General methods 2.5.8).

HKG expression and stability were analysed in the three cell types using the Perfect Probe geNorm HKG selection kit as described in Chapter 2 section 2.5.8. Cycling conditions were 95°C for 10 min; 50 cycles of 95°C for 15 s, 50°C for 30 s and 72°C for 15 s; and 50°C for 30 s. Data were collected at the 50°C step. Cp values for HKGs were determined by the second derivative method and were converted to DNA concentration (ng/5 µl) using a standard curve (see General methods 2.5.8). The genes tested were; *ATP5B*, *GAPDH*, *SDHA*, *TOP1* and *UBC*. *ATP5B*, *TOP1* and *UBC* were selected as these were used for normalisation in SWS placental samples. *SDHA* and *GAPDH* were selected as these have previously been shown to have lower expression stability in placental samples (Cleal et al.,

2009), allowing a broader range of HKGs to be studied. The most stable HKGs were established using qbase+ Software (see 2.5.8), and target gene expression was normalised to the geometric mean of appropriate HKGs.

Table 4.1: Primers used for rt-PCR.

Gene	Accession number	Primer sequence (5'-3')	Amplicon (bp)	Tm
Facilitated amino acid transporter genes				
LAT3	NM_001198810.1,	F: CCTGAGGAAGTCAATTACACG	962 – 1273	64°C
	NM_003627.5	R: CATCTTGTTCACAGCAGCCAT	791 – 1102	
LAT4	NM_001284498.1,	F: ATTGCGTACGGAGCAAGTAAA	738 – 1073	66°C
	NM_152346.2	R: CACCGAGTAGTCCATGTCCTC	536 – 871	
TAT1	NM_018593.4	F: GTGTCCATGCTGGAAACCTT	461 – 794	59°C
		R: TGACAATGCCATTCACCAGT		
Vitamin D genes				
Cubilin	NM_001081.3	F: ACTGTGAAGGGGGTTCTGTG	699 – 1003	60°C
		R: AGAACAGCCGCCGTTATTTA		
CYP2J2	NM_000775.2	F: ATGGCCCTCTACCCAGAAAT	984 – 1092	60°C
		R: TGGTGTAGGGCATGGACTC		
CYP2R1	NM_024514.4	F: CAGCCTCATCCGAGCTTC	167 – 262	59°C
		R: CCACAGTTGATATGCCTCCA		
CYP3A4	NM_001202855.2,	F: GATGGCTCTCATCCCAGACTT	107 – 202	55°C
	NM_017460.5	R: AGTCCATGTGAATGGGTTCC	107 – 202	
CYP24A1	NM_001128915.1,	F: TCATCATGGCCATCAAAACA	1111 – 1176	59°C
	NM_000782.4	R: GCAGCTCGACTGGAGTGAC	1111 – 1176	
CYP24A1	NM_001128915.1,	F: GCAGCCTAGTGCAGATTTCC	1283 – 1611	53°C
	NM_000782.4	R: CCAGAACTGTTGCCTTGTCA	1283 – 1611	
CYP27A1	NM_000784.3	F: GGCTGGAGTGGACACGAC	1436 – 1540	60°C
		R: ACCACACCCACCACTTCCT		
CYP27B1	NM_000785.3	F: CGAGAAGGACCTGGAGTCTG	1001 – 1438	61°C
		R: CGAAAAGAATTTGGCTCTGG		
DBP	NM_000583.3,	F: TCCAAATGCTGTGAGTCTGC	1131 – 1466	59°C
	NM_ 001204306.1,	R: TTCACCAAGGCTTTTTAGGG	1002 – 1337	
	NM_001204307.1		994 – 1329	
Megalin	NM_004525.2	F: AACCAAGGTCAACCGCATAG	1668 – 2011	61°C
		R: GGGAGCCTCCATGAACTACA		
RXRα	NM_001291920.1,	F: CCTTTCTCGGTCATCAGCTC	1031 – 1382	61°C
	NM_002957.5	R: TGTCAATCAGGCAGTCCTTG	359 – 710	
VDR	NM_001017535.1,	F: CCAGTTCGTGTGAATGATGG	748 – 1131	61°C
	NM_001017536.1,	R: GTCGTCCATGGTGAAGGACT	1017 – 1400	
	NM_00376.2		626 – 1009	
Calcium transporter genes				
PMCA3	NM_001001344.2	F: CTTCGTCTTGGACCTGAAGC	1773 – 2036	62°C
	NM_021949.3	R: GGCTCGATGATCTTCCTCAC	1773 – 2036	

F = forward primer, R = reverse primer.

Table 4.2: Primers and probes used for qrt-PCR for amino acid transporters, vitamin D and calcium transporter genes.

Gene	Accession number	Primer sequence (5'-3')	UPL number	Amplicon (bp)
		Amino acid transporter genes		
4F2hc	NM_001012662.2,	F: TGGTTCTCCACTCAGGTTGA	49	1213 – 1275
	NM_001012664.2,	R: CAGCCAAAACTCCAGAGCAT		1024 – 1086
	NM_001013251.2			801 – 863
	NM_002394.5			1210 – 1272
ASCT1	NM_001193493.1,	F: TTTGCGACAGCATTTGCTAC	78	1189 – 1248
	NM_003038.4	R: GCACTTCATCATAGAGGGAAGG		1289 – 1348
ASCT2	NM_001145144.1,	F: GAGGAATATCACCGGAACCA	43	114 – 179
	NM_001145145.1,	R: AGGATGTTCATCCCCTCCA		304 – 369
	NM_005628.2			1250 – 1315
b0+	NM_001126335.1,	F: CCTCCTCAAATCGTTGTGAAA	70	553 – 629
	NM_001243036.1,	R: CGCACGCTCCAGTGAGTTC		648 – 724
	NM_014270.4			639 – 715
EAAT1	NM_001289939.1,	F: TTGAACTGAACTTCGGACAAATTA	76	1594 – 1666
	NM_001289940.1,	R: ATTCCAGCTGCCCCAATACT		1396 – 1468
	NM_004172.4			1732 – 1804
EAAT2	NM_001195728.2,	F: AAAATGCTCATTCTCCCTCTAATC	78	498 – 572
	NM_001252652.1,	R: GCCACTAGCCTTAGCATCCA		563 – 637
	NM_004171.3			860 – 934
EAAT3	NM_004170.5	F: AGTTGAATGACCTGGACTTGG	9	1384 – 1447
		R: GCAGATGTGGCCGTGATAC		
EAAT4	NM_001272087.1,	F: TGCAGATGCTGGTGTTACCT	19	1701 – 1765
	NM_001272088.1,	R: GTTGTCCAGGGATGCCATA		529 – 593
	NM_005071.2			307 – 371
EAAT5	NM_001287595.1,	F: CGCCCAGGTCAACAACTAC	9	1536 – 1604
	NM_001287597.1,	R: GCTGCAGTGGCTGTGATACT		1143 – 1211
	NM_006671.5			1359 – 1427
LAT1	NM_003486.5	F: GTGGAAAAACAAGCCCAAGT	25	1503 – 1573
		R: GCATGAGCTTCTGACACAGG		
LAT2	NM_012244.3,	F: TTGCCAATGTCGCTTATG	17	1591 – 1677
	NM_001267037.1,	R: GGAGCTTCTCTCCAAAAGTCAC		375 – 461
	NM_182728.2			529 – 615
LAT3	NM_001198810.1,	F: GCCCTCATGATTGGCTCTTA	29	623 – 642
	NM_003627.5	R: CCGGCATCGTAGATCAGC		794 – 864
LAT4	NM_001284498.1,	F: ACAAGTATGGCCCGAGGAA	3	673 – 742
	NM_152346.2	R: GCAATCAGCAAGCAGGAAA		471 – 540
SNAT1	NM_001077484.1,	F: ATTTTGGGACTCGCCTTTG	47	1034 – 1110
	NM_001278387.1,	R: AGCAATGTCACTGAAGTCAAAAGT		1496 – 1572
	NM_001278388.1,			1258 – 1334
	NM_001278389.1,			523 – 599
	NM_001278390.1,			1236 – 1312
	NM_030674.3			1373 – 1449
SNAT2	NM_018976.4	F: CCTATGAAATCTGTACAAAAGATTGG	9	1836 – 1939
		R: TTGTGTACCCAATCCAAAACAA		
SNAT4	NM_001143824.1,	F: TGTTCTGGTCATCCTTGTGC	29	1602 – 1697
	NM_018018.4	R:AAAACTGCTGGAAGAATAAAAATCAG		1794 – 1889
TAT1	NM_018593.4	F: GGTGTGAAGAAGGTTTATCTACAGG	6	1238 – 1329
		R: AGGGCCCCAAAGATGCTA		
xCT	NM_014331.3	F: TGTGGCCTACTTTACGACCA	17	1135 – 1211
		R: GCCGCTCAGAAAAGGTCA		
		Vitamin D genes		
CYP24A1	NM_001128915.1,	F: GAAAGAATTGTATGCTGCTGTCA	78	1337 – 1408
	NM_000782.4	R: CACATTAGACTGTTTGCTGTCGT		1337 – 1408
Megalin	NM_004525.2	F: TTGTTTTGATGCCTCTGATGA	34	8877 – 8937
		R: AGCTAGGCATGTTCGCTCAG		
RXRα	NM_001291920.1,	F: ACATGCAGATGGACAAGACG	26	1917 – 1994
	NM_001291921.1,	R: TCGAGAGCCCCTTGGAGT		1708 – 1785
	NM_002957.5			1245 – 1322
VDR	NM_001017535.1,	F: TCTGTGACCCTAGAGCTGTCC	43	928 – 1055
	NM_001017536.1,	R: TCCTCAGAGGTGAGGTCTCTG		1197 – 1324
	NM_000376.2			806 – 933
		Calcium transporter genes		
PMCA1	NM_001001323.1,	F: CCATAGTATCATTGGGCCTTTC	75	534 – 620

NM_001682.2	R: CTTCTTCCTCCCCAACAGAA	534 – 620

F = forward primer, R = reverse primer.

4.2.4. Protein expression

Protein was extracted from human placenta samples collected from the SWS as described in Chapter 2 section 2.7.2 and concentration was determined by BCA protein assay (section 2.7.3).

Western blotting was used to determine the presence or absence of a specific protein as described in Chapter 2 section 2.7.4. 40 μg of protein with DTT and Laemmli Sample Buffer was reduced for 10 min at 70°C and separated by SDS-PAGE in 4–20% Mini-PROTEAN® TGX™ gels. Samples were transferred to PVDF membrane, which was then blocked in 3% ECL Prime™ blocking solution and incubated in primary antibody overnight at 4°C. Antibodies to a human LAT4 peptide were raised in a rabbit and affinity purified by Thermo Scientific. LAT4 primary antibody was used at a dilution of 1:500. TAT1 primary antibody was used at a dilution of 1:150.

Following PBS-T washes, membranes were incubated with goat polyclonal to rabbit IgG HRP-conjugated secondary antibody at 1:50,000 for 2 hours at RT. Membranes were then washed again in PBS-T and protein bands visualised with ECL. Equal loading was demonstrated by incubating membranes with monoclonal anti-β-actin-HRP (1:10,000). For each western blot a negative control was run where a second membrane was probed with just the secondary antibody. Relative density for protein expression was quantified using ImageJ for both the target protein and β-actin.

4.2.5. Transfection

GFP-*TAT1* was prepared via transformation and purification with a Plasmid Maxiprep Kit (see section 2.3.3). BeWo cells were plated at 2.5 x 10^5 per well in 32 mm well plates with gelatin-coated coverslips. 24 h after plating, media was replaced and transfection reagent/DNA or transfection reagent/water mixture was added to the fresh media. A number of transfection reagents were trialled; FuGENE®, Lipofectamine® 2000 and Nanofectin. 12 h after transfection, media containing transfection reagent was removed and replaced with normal media. 24 and 48 h post-transfection cells were fixed in 10% formaldehyde and the nuclei stained with DAPI. Cells were visualised with the Axiovert 200 fluorescent microscope or the Leica SP5 confocal microscope, and transfection efficiency was calculated (see Chapter 2 section 2.3.4).

4.2.6. Syncytialisation

Forskolin was used to attempt syncytialisation of BeWo cells to create a multinucleated transport layer similar to the placental syncytiotrophoblast. Syncytialisation was assessed by hCG secretion and desmoplakin staining and was not achieved. Methods and results are displayed in Appendix 2.

4.2.7. Data analysis

All data were analysed in SPSS, and graphs were created in GraphPad Prism V6 or Excel. Unless otherwise stated, data are presented as mean + SEM. $p < 0.05$ was deemed statistically significant and $p < 0.1$ was considered a trend.

Comparison of mRNA and protein expression in HEK293, BeWo cells and placental tissue

qrt-PCR data were normalised to the geometric mean of appropriate HKGs. Relative protein expression data were adjusted for β-actin to give adjusted relative density. Data are presented as mean + SEM. Data were tested for normal distribution and log transformed or square rooted if not normally distributed. If data were still not normally distributed after log or square root transformation, non-parametric statistical tests were used. Data were tested for homogeneity of variance with the Levene statistic. For normally distributed data, mRNA or protein expression levels were compared using one way ANOVA. Tukey's multiple comparison test was used to identify which groups differed significantly in data sets with equal variance. For data with a significant Levene statistic, the Games-Howell test was used for post-hoc analysis. For non-normally distributed data, expression levels were compared with a Kruskal-Wallis test. For TAT1 protein expression, a t-test was used as there were only 2 groups to compare.

Effect of methylation on mRNA expression in HEK293 and BeWo cells

qrt-PCR data were normalised to *UBC* expression. A second HKG, *GAPDH*, was also run on the samples, but after analysis this HKG changed with AZA treatment, so was excluded from analysis. Data were tested for normal distribution and log or square root transformed if not normally distributed. Following transformation data were re-tested for normal distribution. Normally distributed data were analysed with a t-test. A Levene test for equality of variance was carried out and p values for the t-test were selected accordingly. Non-normally distributed data were analysed with a Mann Whitney U test.

Effect of vitamin D on mRNA expression in HEK293 cells

qrt-PCR data were normalised to the geometric mean of *ATP5B* and *SDHA*. Normalised data for each target gene were divided by the average normalised expression of that gene in media controls for each

experiment to give fold change in mRNA expression. Data are presented as mean fold change + SEM. Data were tested for normal distribution and log or square root transformed if not normally distributed. Transformed data were then re-tested for normal distribution. Data were tested for homogeneity of variance. If the Levene statistic was non-significant, indicating equal variance in the data set, data were analysed using one way ANOVA with Dunnett t post-hoc to compare treatment groups to media control. If the Levene statistic was significant, data were analysed using one way ANOVA with Dunnett T3 post-hoc test.

4.3. Results

4.3.1. Characterisation of BeWo and HEK293 cell lines in comparison to placental tissue

Comparison of housekeeping gene expression and stability in HEK293, BeWo cells and placental tissue

V values demonstrated that 2 or 3 HKGs were adequate to normalise target gene data for each cell type, as a V value below 0.15 represents the optimal number of HKGs (Figure 4.2).

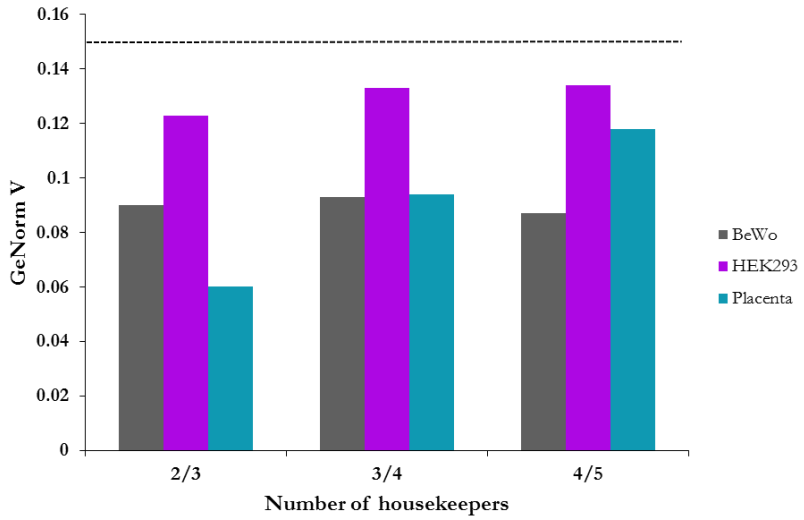

Figure 4.2: The number of genes required for normalisation. The levels of variation in average HKG stability with the sequential addition of each HKG to the equation (for calculating the normalization factor). Starting with the most stably expressed genes on the left with the inclusion of a 3rd, 4th, 5th gene etc. moving to the right. The graph indicates that the two most stable genes create a normalization factor which is not significantly altered by the addition of 3 more genes as they all have a V score below 0.15 (dashed line).

HKG stability differed between the three cell types (Figure 4.3). For placenta the most stably expressed HKGs were *GAPDH*, *UBC* and *ATP5B*, while for BeWo they were *SDHA*, *GAPDH* and *UBC*. For HEK293 *SDHA*, *ATP5B* and *GAPDH* were the most stably expressed genes.

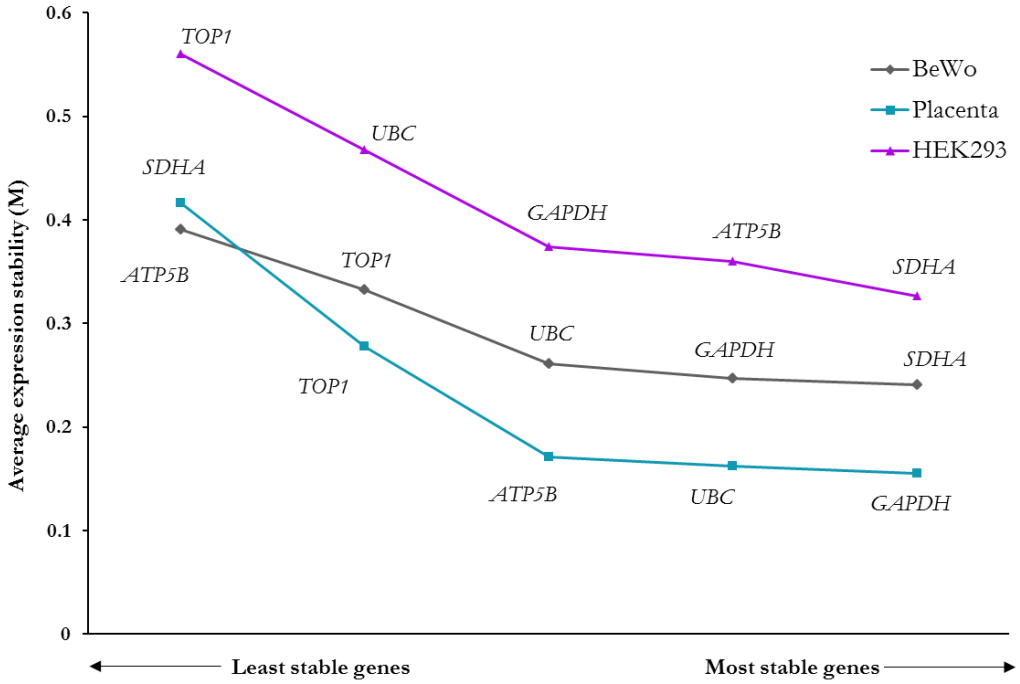

Figure 4.3: The most stably expressed HKGs. The average expression stability value (M) for each HKG ranked according to increasing stability with the most stable genes on the right.

mRNA expression levels of HKGs were investigated in the three cell types as levels needed to be similar to analyse target gene expression differences between the cells. Only *UBC* expression did not differ significantly between BeWo, HEK293 and placenta (Figure 4.4). HEK293 and placenta also did not differ significantly in their expression levels of *SDHA* and *TOP1*. As *GAPDH* expression in placenta is low, this was ruled out as a good HKG for placenta. To compare mRNA expression differences between the three cell types *UBC* was used, as the expression levels are similar. To compare mRNA expression within one cell type the most stable HKGs for that cell type were used.

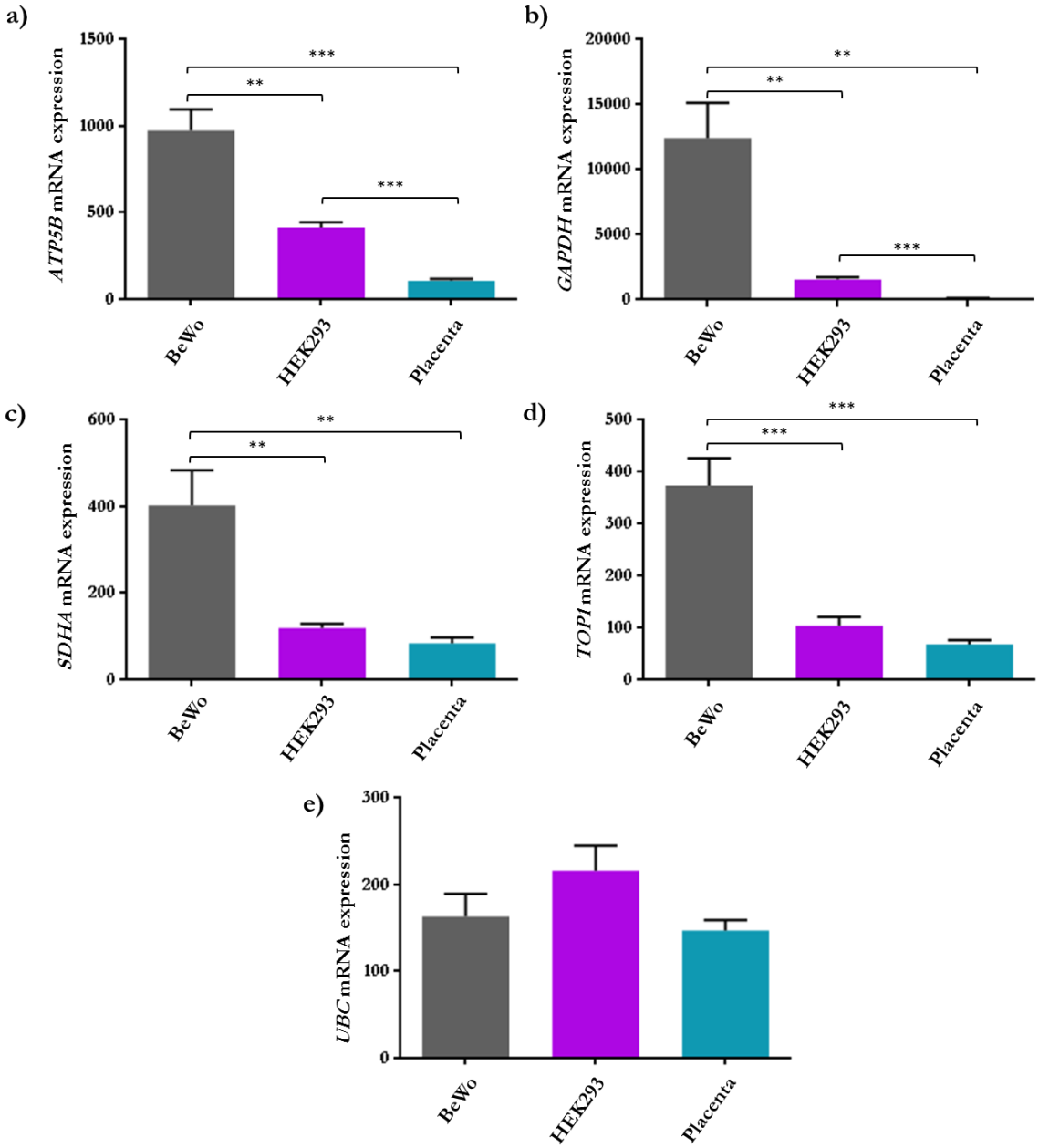

Figure 4.4: Comparison of mRNA expression levels (ng/5 µl) of HKGs in BeWo, HEK293 and placenta. a) *ATP5B* mRNA expression differed significantly in all three cell types. b) *GAPDH* mRNA expression differed significantly in all three cell types. c) *SDHA* mRNA expression differed significantly in BeWo cells compared to HEK293 and placenta. d) *TOP1* mRNA expression differed significantly in BeWo cells compared to HEK293 and placenta. e) *UBC* mRNA expression was the only HKG that did not differ significantly between the cell types. Data presented as mean + SEM. BeWo n = 10, HEK293 n = 9, placenta n = 10. ** $p < 0.01$ *** $p < 0.001$.

Comparison of vitamin D-related gene expression in placental tissue, HEK293 and BeWo cells

The assay for *CYP3A4* failed to amplify any product in BeWo, HEK293 and placenta. As described in Chapter 3, a positive control yielded *CYP3A4* expression demonstrating efficacy of the primer set. All five of the remaining vitamin D metabolising enzymes, *CYP2J2*, *CYP2R1*, *CYP24A1*, *CYP27A1* and *CYP27B1* were expressed in cytotrophoblast or placental cells. While only *CYP27A1* and *CYP27B1* were detected in BeWo cells. *CYP2J2* was detected in one of the two HEK293 samples tested. *CYP24A1* mRNA expression was also detected faintly in this sample. Due to the lack of product

separation between PCR product and primer dimers it is unclear whether *CYP2R1* and *CYP27A1* are expressed in HEK293 cells, but the slightly brighter bands observed in the HEK293 samples suggest *CYP27A1* may be expressed. Due to problems with DNA contamination the *CYP27B1* rt-PCR assay also amplified DNA, but did indicate expression of *CYP27B1* in HEK293 cells (Figure 4.5). Comparison of genes involved in vitamin D transport showed that *DBP* has a low mRNA expression in BeWo, HEK293 and placental cells, while *megalin* and *cubilin* mRNA was detected in cytotrophoblast, placenta and HEK293 but not BeWo cells (Figure 4.6). mRNA expression of the nuclear receptors *RXRa* and *VDR* was detected in BeWo, HEK293 cytotrophoblast and placenta (Figure 4.6). A summary of all the vitamin D genes tested is shown in Table 4.3.

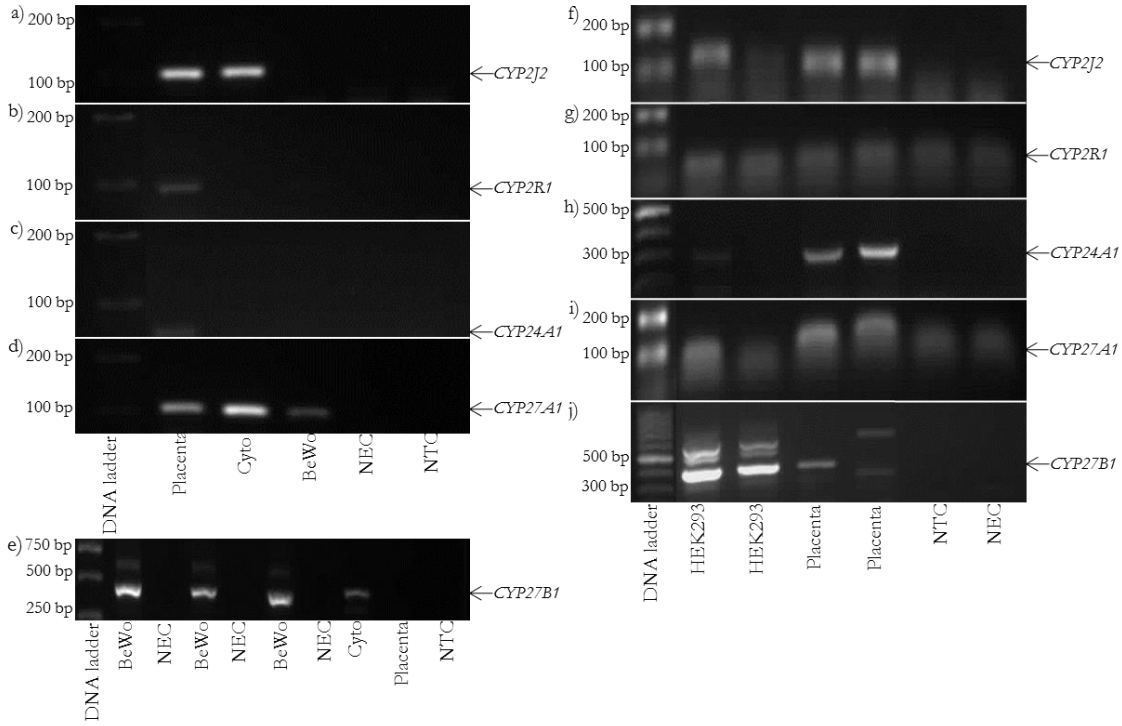

Figure 4.5: rt-PCR for expression of vitamin D metabolising enzymes in placenta, BeWo and HEK293 cells. rt-PCR images for BeWo: a) *CYP2J2,* product size 109 bp, b) *CYP2R1,* product size 96 bp, c) *CYP24A1,* product size 66 bp, d) *CYP27A1,* product size 105 bp and e) *CYP27B1,* product size 438 bp. rt-PCR images for HEK293: f) *CYP2J2,* product size 109 bp, g) *CYP2R1,* product size 96 bp, h) *CYP24A1,* product size 329 bp, i) *CYP27A1,* product size 105 bp and j) *CYP27B1,* product size 438 bp. Cyto = cytotrophoblast.

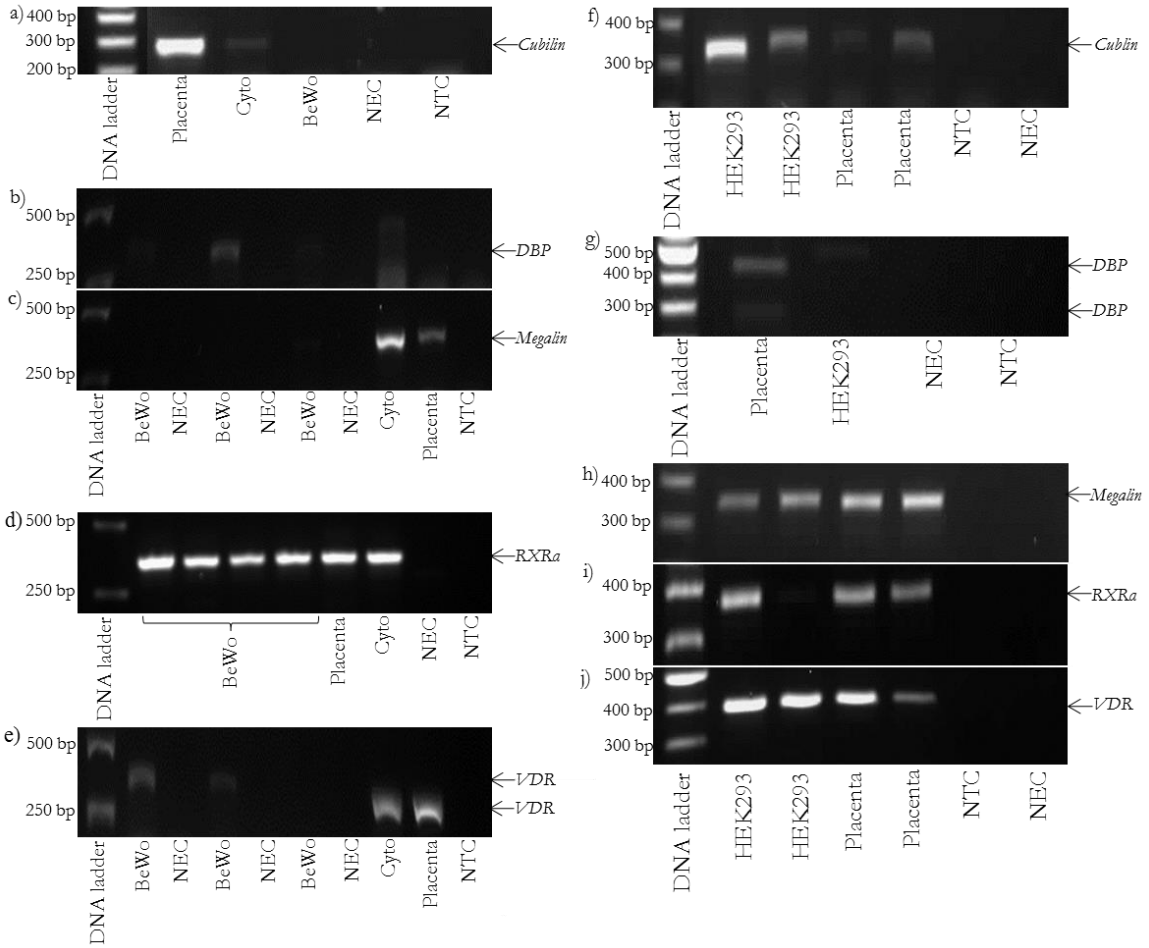

Figure 4.6: rt-PCR for expression of vitamin D transport and signalling genes in placenta, BeWo and HEK293 cells. rt-PCR images for BeWo: a) *cubilin*, product size 305 bp, b) *DBP,* product size 336 bp, c) *megalin,* product size 344 bp, d) *RXRa,* product size 352 bp and e) *VDR,* product size 384 bp. rt-PCR images for HEK293: f) *cubilin,* product size 305 bp, g) *DBP*, product size 336 bp, h) *megalin,* product size 344 bp, i) *RXRa,* product size 352 bp and j) *VDR,* product size 384 bp. Cyto = cytotrophoblast.

Table 4.3: Summary of vitamin D-related gene expression in BeWo cells, placenta and cytotrophoblast.

Gene	Placenta	Cytotrophoblast	BeWo	HEK293
Vitamin D metabolism enzymes				
CYP2J2	Y	Y	N	Y
CYP2R1	Y	N	N	Maybe
CYP3A4	N	N	N	N
CYP24A1	Y	N	N	Barely detected
CYP27A1	Y	Y	Y	Maybe
CYP27B1	N	Y	Y	Maybe
Vitamin D transport genes				
Cubilin	Y	Y	N	Y
DBP	N	Y	Some	Y
Megalin	Y	Y	N	Y
Vitamin D signalling genes				
RXRα	Y	Y	Y	Y
VDR	Y	Y	Some	Y

Comparison of calcium and facilitated amino acid transporter expression in HEK293, BeWo cells and placental tissue

rt-PCR on BeWo cells and placental tissue revealed mRNA expression of *PMCA3*, *LAT3*, *LAT4* and *TAT1* in both cell types (Figure 4.7; Table 4.4).

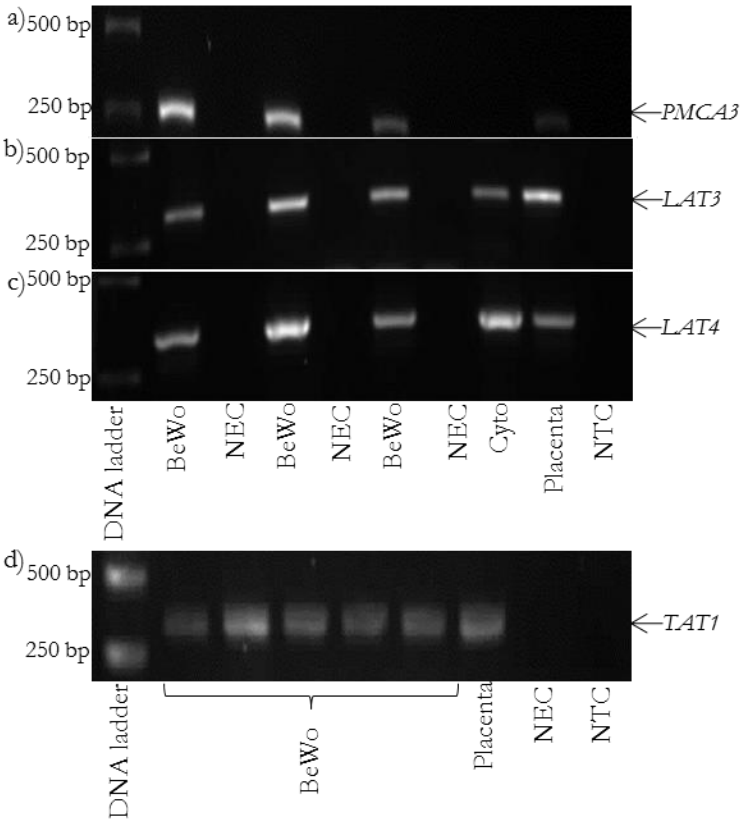

Figure 4.7: rt-*PCR for PMCA3, LAT3, LAT4* and *TAT1* in placenta and BeWo cells. rt-PCR images for a) *PMCA3*, product size 264 bp, b) *LAT3,* product size 312 bp, c) *LAT4,* product size 336 bp and d) *TAT1,* product size 334 bp. Cyto = cytotrophoblast.

Table 4.4: Summary of calcium and facilitated amino acid transporter mRNA expression in placenta, cytotrophoblast and BeWo cells.

Gene	Placenta	Cytotrophoblast	BeWo
Calcium transporter			
PMCA3	Y	N	Y
Facilitated amino acid transporters			
LAT3	Y	Y	Y
LAT4	Y	Y	Y
TAT1	Y	Y	Y

qrt-PCR analysis revealed that BeWo, HEK293 and placenta all differed significantly from each other in terms of *LAT3* and *LAT4* mRNA expression levels. BeWo and HEK293 cells expressed lower levels of *LAT4* mRNA than placenta cells. LAT4 protein expression was detected in all three cell types (see Appendix 4). *LAT3* mRNA was expressed at higher levels in HEK293 compared to placenta,

while in BeWo cells expression was lower than placenta. HEK293 and placenta did not differ in their mRNA expression of *TAT1*. However, TAT1 mRNA and protein expression was significantly lower in BeWo cells compared to placenta (Figure 4.8). Transfection of BeWo cells with GFP-*TAT1* was therefore attempted (Figure 4.9), however transfection efficiency was low. A range of transfection reagents were tested and alterations to the transfection reagent to DNA ratio were tested, however transfection efficiency was only 20% (see Appendix 5), so this was not pursued further.

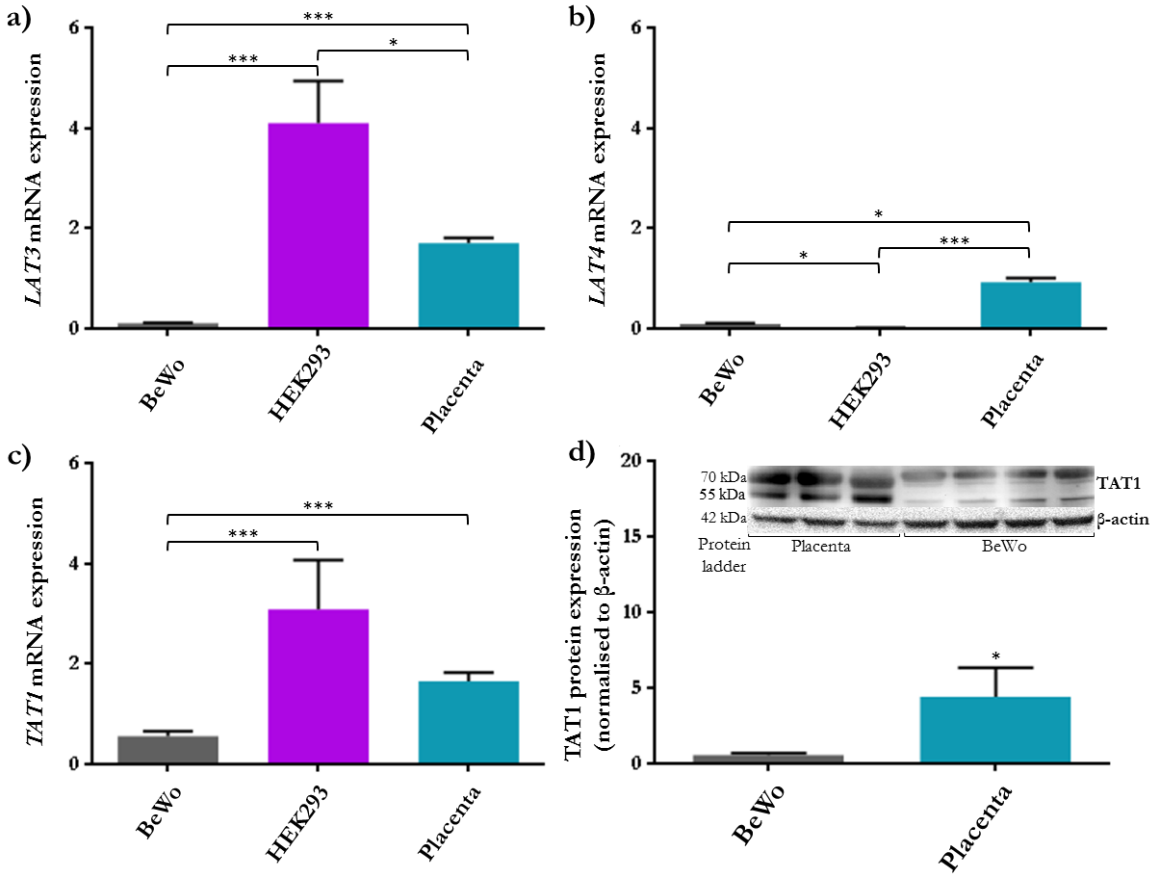

Figure 4.8: Comparison of relative mRNA expression levels of facilitated amino acid transporters in BeWo, HEK293 and placenta. a) *LAT3* mRNA expression differed significantly between the three cell types. b) *LAT4* mRNA expression differed significantly between the three cell types. c) *TAT1* mRNA expression differed significantly between BeWo and HEK293, and BeWo and placenta. d) TAT1 protein expression was significantly lower in BeWo cells compared to placenta. A representative western blot is shown (BeWo n = 5, placenta n = 5). Data presented as mean + SEM. BeWo n = 10, HEK293 n = 9, placenta n = 10. * $p < 0.05$, *** $p < 0.001$.

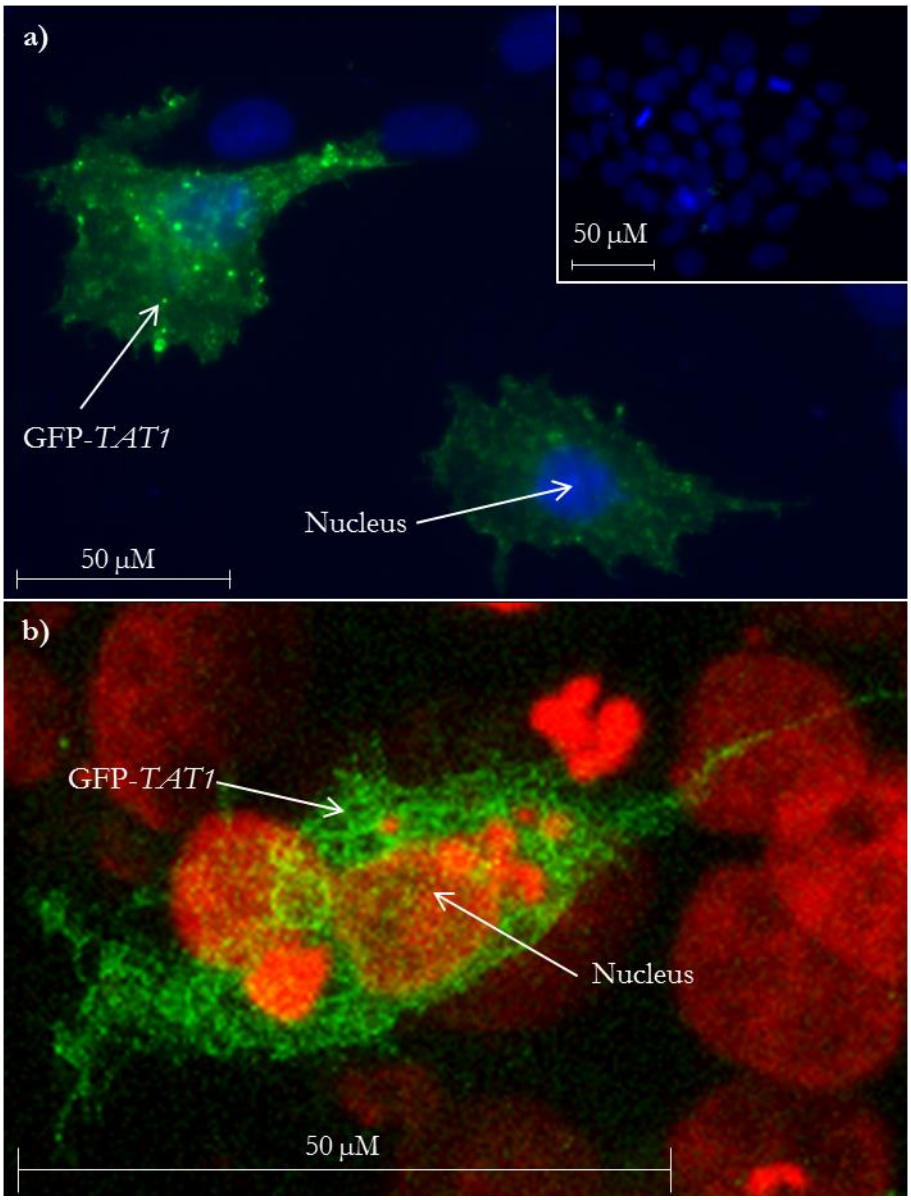

Figure 4.9: GFP-*TAT1* expression in BeWo cells. a) GFP-*TAT1* transfection of BeWo cells viewed under the Axiovert fluorescent microscope at x 40 magnification, DAPI stained nuclei are blue, and GFP-*TAT1* is green. Inset shows a transfection reagent control, where cells were transfected with transfection reagent minus the vector. b) GFP-*TAT1* transfection of BeWo cells viewed under the SP5 fluorescent confocal microscope at x 60 magnification. DAPI stained nuclei are red, and GFP-*TAT1* is green.

4.3.2. Effect of methylation on nutrient transporter and vitamin D-related mRNA expression in BeWo and HEK293 cells

HEK293 and BeWo cells were cultured with AZA to investigate the regulation of amino acid transporter and vitamin D-related gene expression by methylation.

Expression of accumulative amino acid transporters in response to removal of DNA methylation

EAAT2 mRNA was not detected in BeWo cells, and *EAAT4*, *EAAT5* and *SNAT4* were not detected in HEK293 cells. The standard curve failed for *EAAT1* and *EAAT5*, therefore mRNA expression of these genes was calculated by 2^(-ddct). *EAAT1* and *EAAT5* mRNA showed significant increases in response to AZA treatment in BeWo cells. *EAAT2* and *EAAT1* (trend, p = 0.08*)* mRNA expression was increased in HEK293 cells when DNA methylation was inhibited, while *EAAT3* mRNA was decreased in HEK293 and BeWo cells with removal of methylation. *SNAT1* showed increased mRNA expression with AZA treatment in both HEK293 and BeWo cells, as did *SNAT2* mRNA in HEK293 cells. *SNAT2* mRNA was not affected by inhibition of DNA methylation in BeWo cells, while *SNAT4* showed a trend for increased expression (p = 0.06; Figure 4.10 and Figure 4.11).

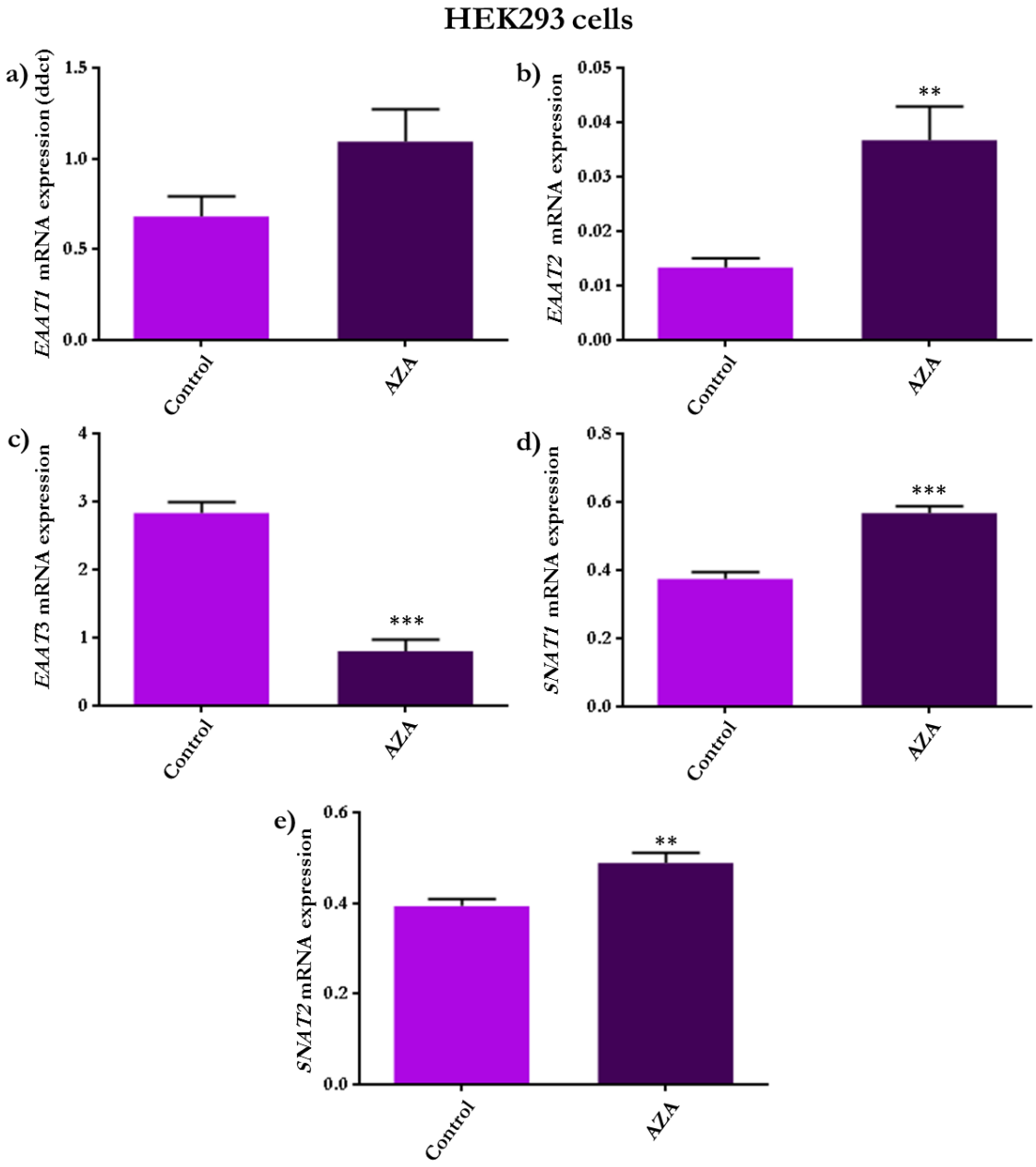

Figure 4.10: Relative mRNA expression of accumulative amino acid transporters in HEK293 cells in response to removal of methylation. a) *EAAT1* mRNA expression was not significantly altered by removal of methylation. b) *EAAT2* mRNA expression was significantly increased by removal of methylation. c) *EAAT3* mRNA expression was significantly decreased by removal of methylation. d) *SNAT1* mRNA expression was significantly increased by removal of methylation. e) *SNAT2* mRNA expression was significantly increased by removal of methylation. Data presented as mean + SEM. n = 9 for both control and AZA. ** $p < 0.01$, *** $p < 0.001$.

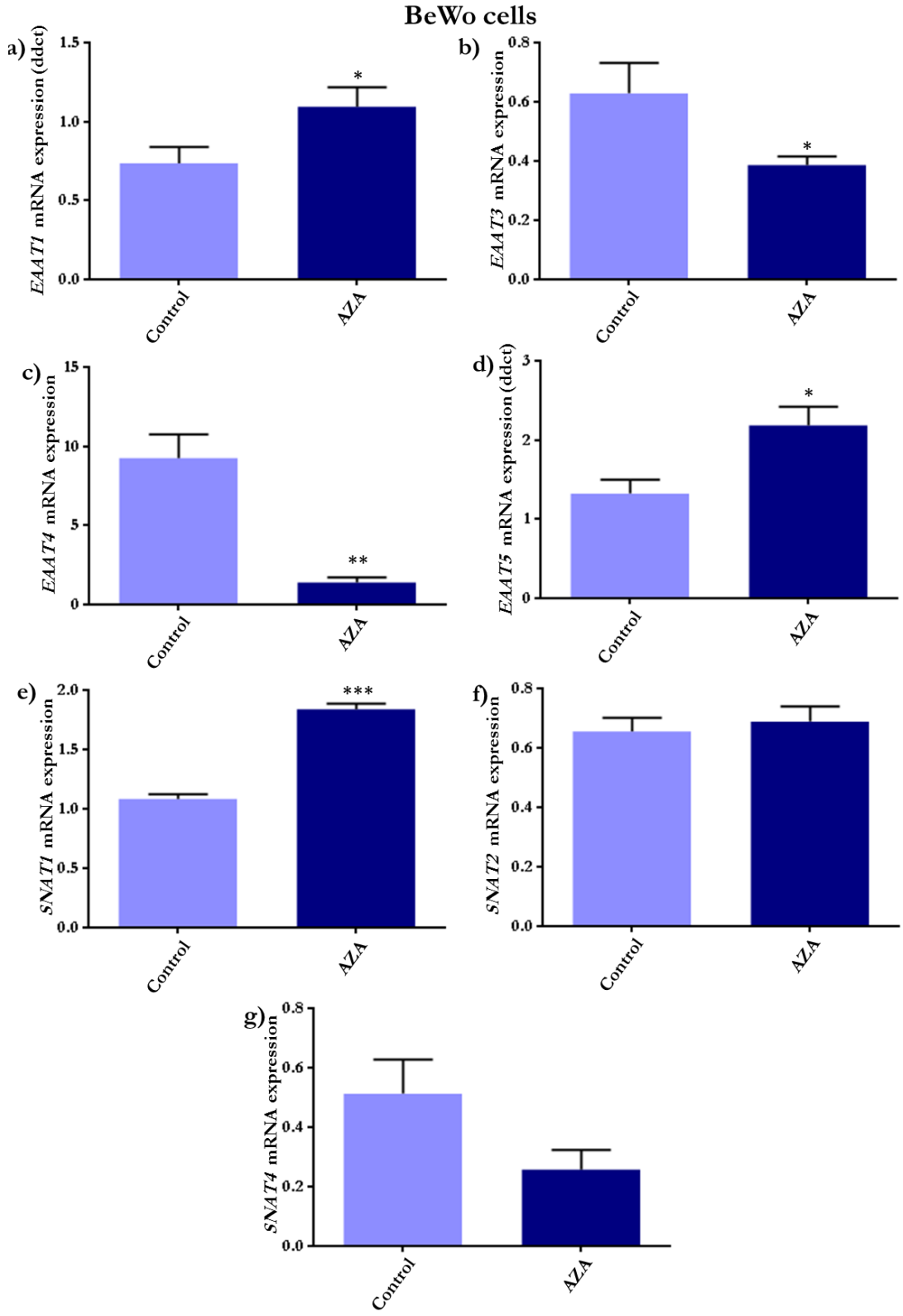

Figure 4.11: Relative mRNA expression of accumulative amino acid transporters in BeWo cells in response to removal of methylation. a) *EAAT1* mRNA expression was significantly increased with inhibition of methylation. b) *EAAT3* mRNA expression was significantly reduced with removal of DNA methylation. c) *EAAT4* mRNA expression significantly decreased with methylation inhibition. d) *EAAT5* mRNA expression significantly increased with AZA treatment. e) *SNAT1* mRNA expression significantly increased in response to removal of methylation. f) *SNAT2* mRNA expression was not affected by AZA treatment. g) *SNAT4* mRNA expression was not significantly affected by removal of methylation. Data presented as mean + SEM. n = 9 for control and AZA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Expression of amino acid exchangers in response to removal of DNA methylation

The exchanger b^{0+} was not detected in HEK293 cells. In the BeWo cell line b^{0+} mRNA expression was not altered by AZA treatment. *4F2hc*, *ASCT1* and *xCT* all showed increased mRNA expression in both cell lines in response to AZA treatment. *ASCT2* and *LAT2* both showed increased mRNA expression in BeWo cells in response to the removal of methylation, while their expression in HEK293 cells was not significantly altered. However, *LAT2* mRNA expression did show a trend for increased expression in HEK293 cells ($p = 0.08$). *LAT1* mRNA was increased in HEK293 with inhibition of methylation, but was not measured in BeWo cells (Figure 4.12 and Figure 4.13).

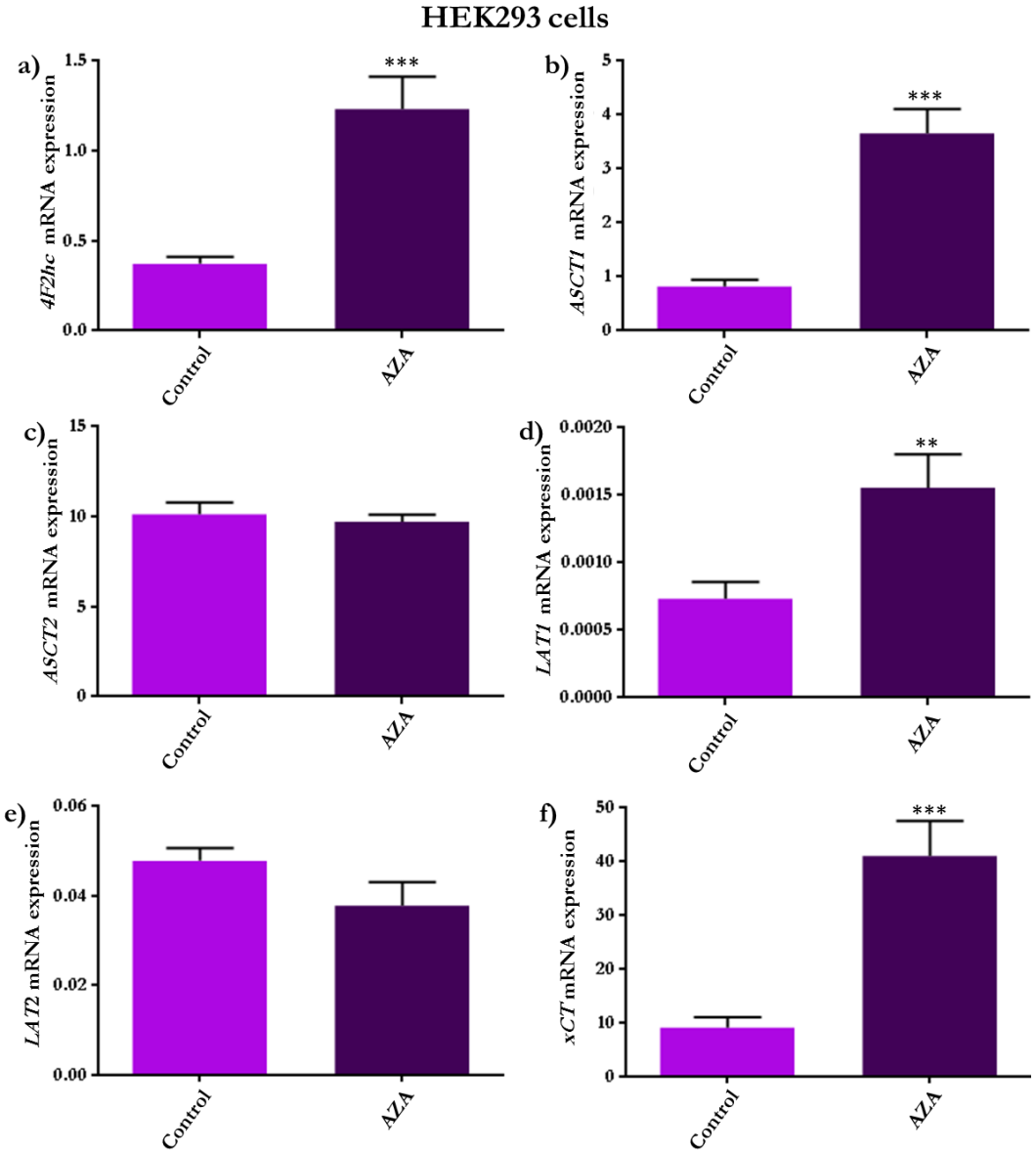

Figure 4.12: Relative mRNA expression of amino acid exchangers in HEK293 cells in response to removal of methylation. a) *4F2hc* mRNA expression significantly increased with inhibition of methylation. b) *ASCT1* mRNA significantly increased with removal of methylation. c) *ASCT2* mRNA was not affected by inhibition of methylation. d) *LAT1* mRNA significantly increased with AZA treatment. e) *LAT2* mRNA expression was not significantly affected by removal of methylation. f) *xCT* showed a large increase in mRNA expression with inhibition of DNA methylation. Data presented as mean + SEM. n = 9 for control and AZA. ** $p < 0.01$, *** $p < 0.001$.

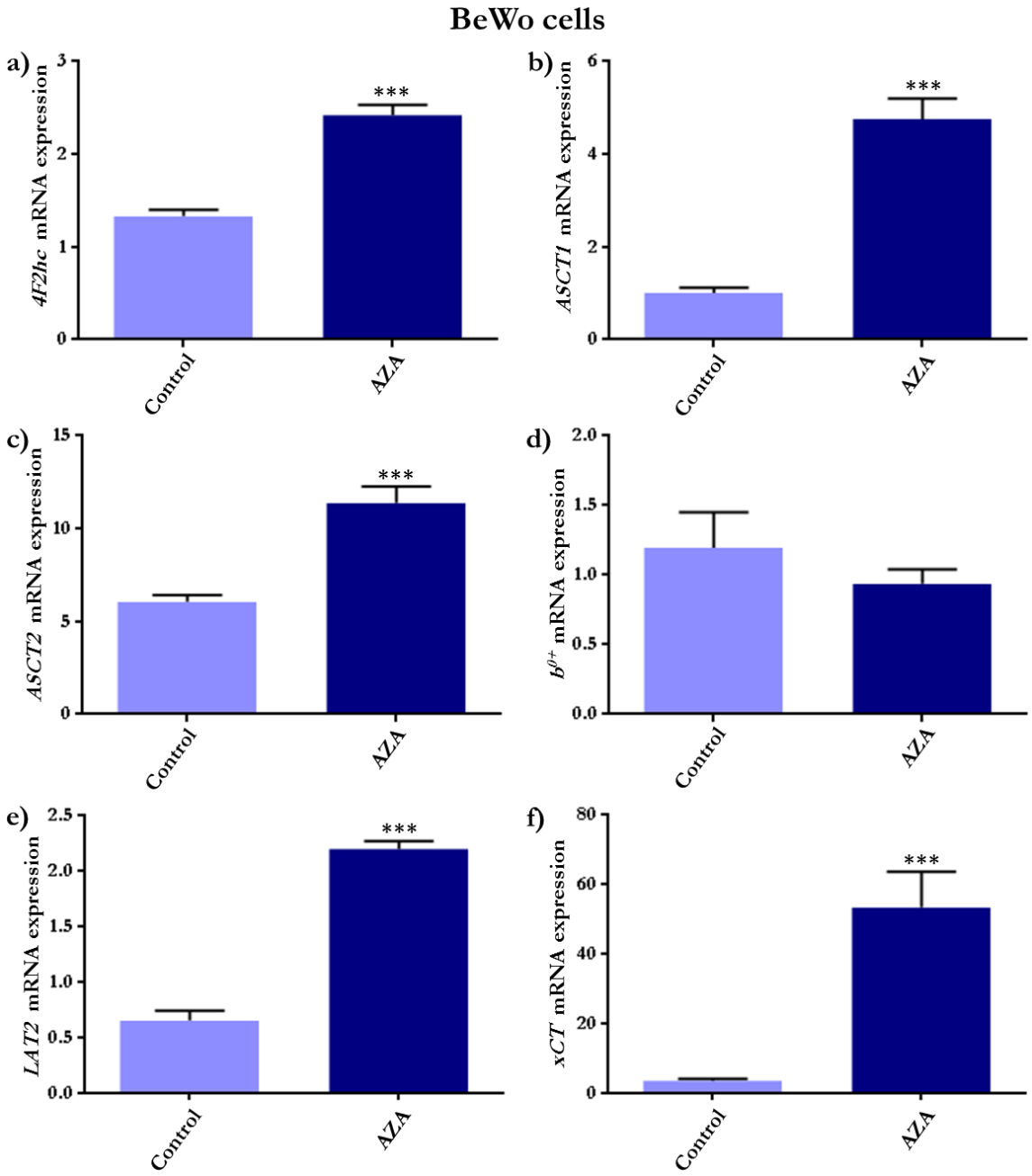

Figure 4.13: Relative mRNA expression of amino acid exchangers in BeWo cells in response to removal of methylation. a) *4F2hc* mRNA significantly increased with inhibition of methylation. b) *ASCT1* mRNA significantly increased with removal of methylation. c) *ASCT2* mRNA expression was increased with AZA treatment. d) b^{0+} mRNA expression was not altered by removal of methylation. e) *LAT2* mRNA significantly increased with inhibition of methylation. f) *xCT* showed a large increase in mRNA expression with removal of DNA methylation. Data presented as mean + SEM. n = 9 for control and AZA. *** $p < 0.001$.

Expression of facilitated amino acid transporters in response to removal of DNA methylation

LAT3 and *LAT4* mRNA expression was increased in both cell lines with AZA treatment. *TAT1* responded to the removal of methylation in a cell-specific manner; mRNA was reduced in HEK293 cells but in BeWo cells expression was unchanged (Figure 4.14 and Figure 4.15).

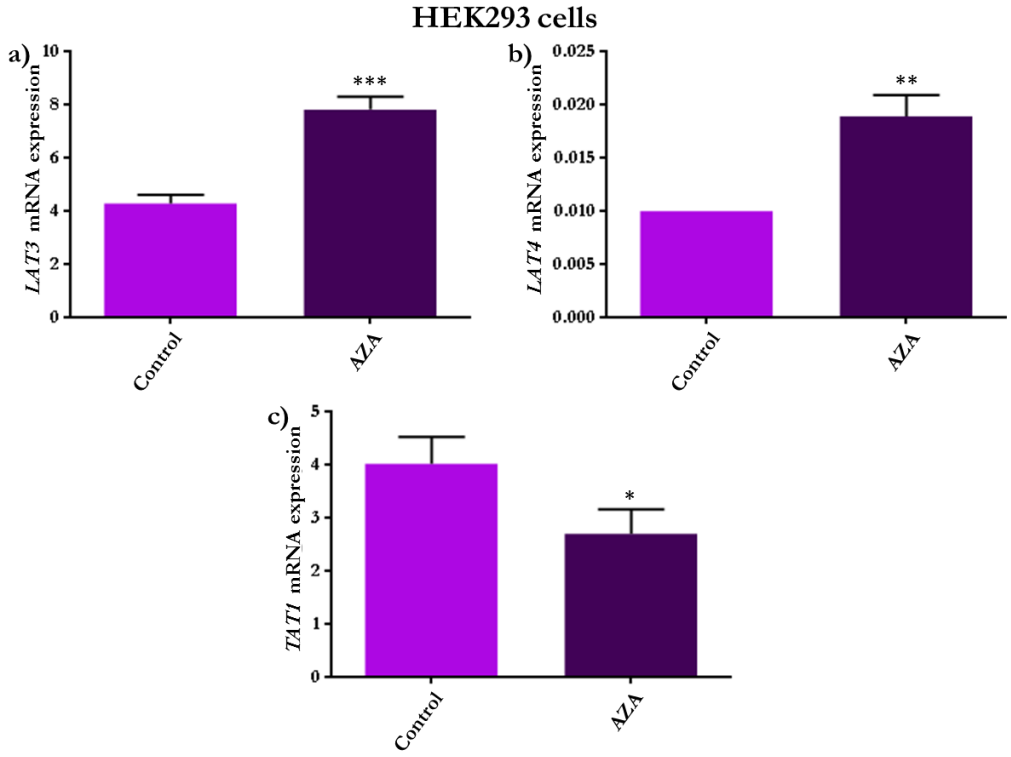

Figure 4.14: Relative mRNA expression of facilitated amino acid transporters in HEK293 cells in response to removal of methylation. a) *LAT3* mRNA expression was increased with AZA treatment. b) *LAT4* mRNA increased with removal of DNA methylation. c) *TAT1* mRNA was reduced with AZA treatment. Data presented as mean + SEM. n = 9 for both control and AZA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

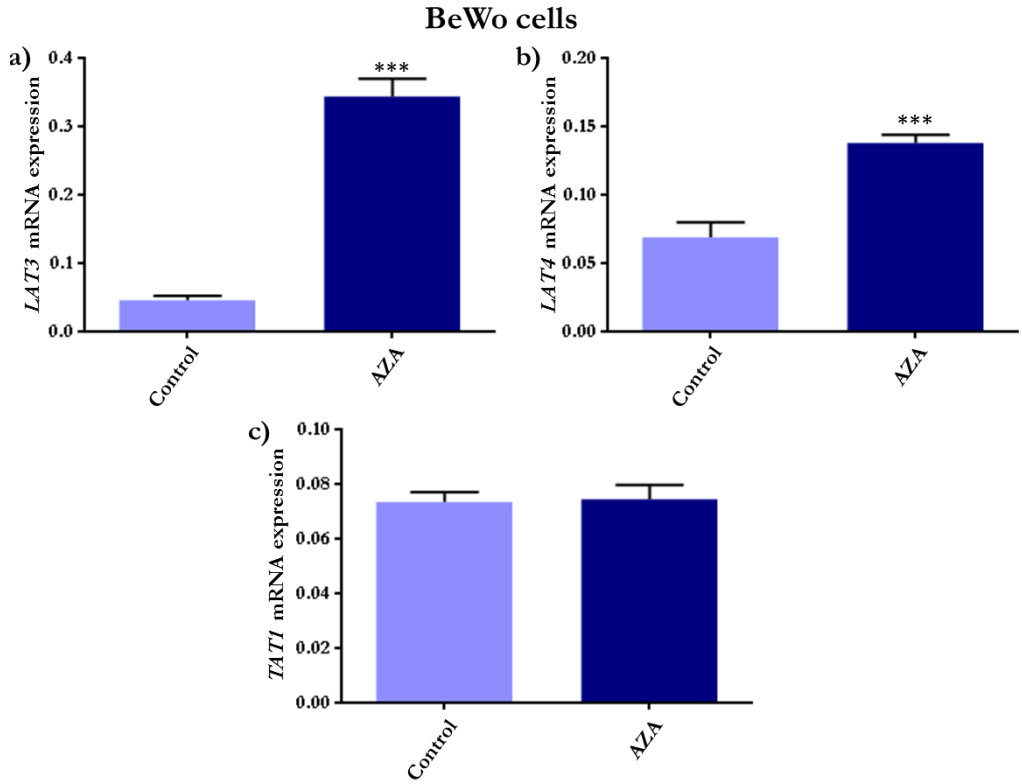

Figure 4.15: Relative mRNA expression of facilitated amino acid transporters in BeWo cells in response to removal of methylation. a) *LAT3* mRNA expression was increased with AZA treatment. b) *LAT4* mRNA increased with removal of DNA methylation. c) *TAT1* mRNA was not altered by removal of methylation. Data presented as mean + SEM. n = 9 for both control and AZA. *** $p < 0.001$.

Expression of vitamin D-related genes in response to removal of DNA methylation

In HEK293 cells, *megalin* mRNA expression was reduced with AZA treatment, whilst *RXRa* mRNA increased. *VDR* expression was not affected by the removal of methylation in HEK293 cells, and was not measured in BeWo cells. Both *megalin* and *RXRa* mRNA were unchanged in the BeWo cell line with inhibition of methylation (Figure 4.16 and Figure 4.17).

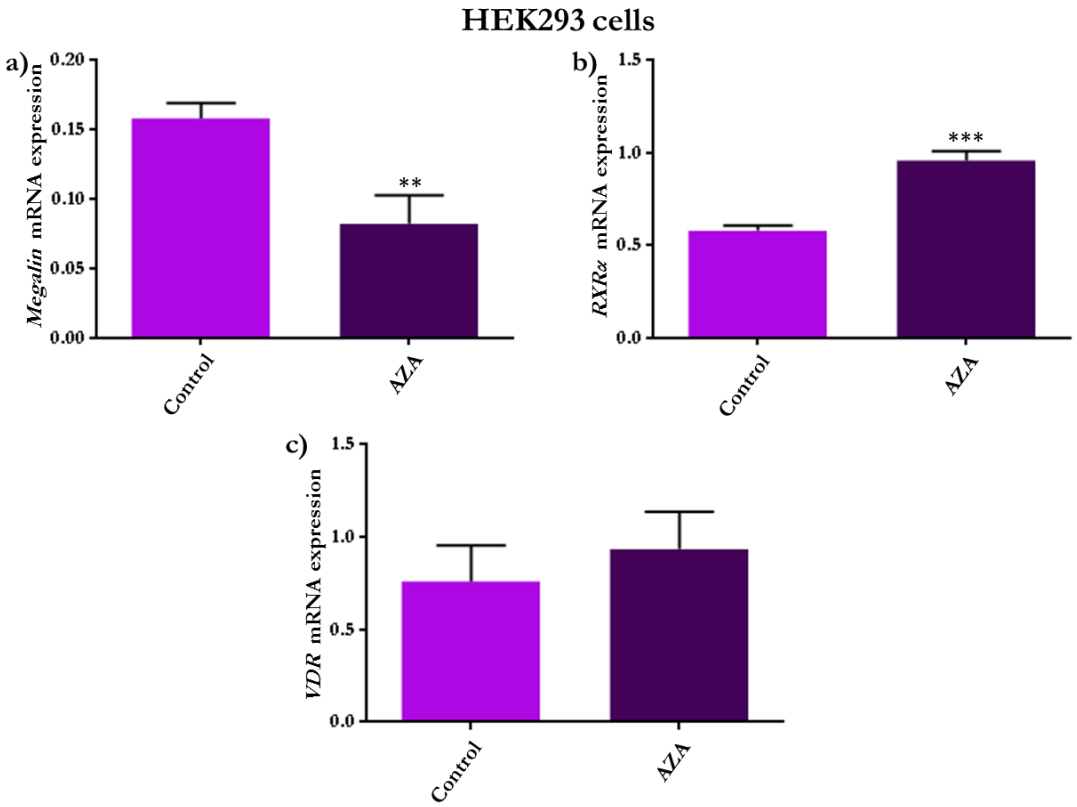

Figure 4.16: Relative mRNA expression of vitamin D-related genes in HEK293 cells in response to removal of methylation. a) *Megalin* mRNA decreased with AZA treatment. b) *RXRa* mRNA expression increased with inhibition of methylation. c) *VDR* mRNA was not affected by the removal of methylation. Data presented as mean + SEM. n = 9 for both control and AZA. ** $p < 0.01$, *** $p < 0.001$.

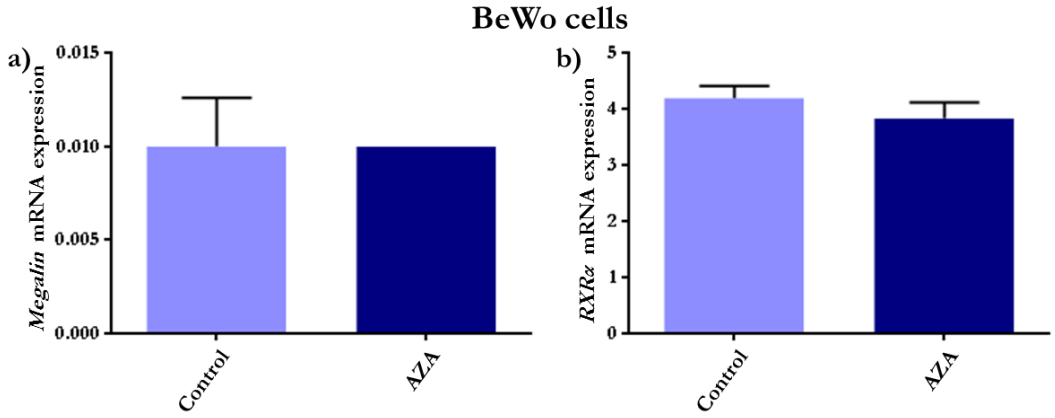

Figure 4.17: Relative mRNA expression of vitamin D-related genes in BeWo cells in response to removal of methylation. a) *Megalin* mRNA was unaffected by AZA treatment. b) *RXRa* mRNA expression was unchanged by inhibition of methylation. Data presented as mean + SEM. n = 9 for both control and AZA.

4.3.3. Effect of vitamin D on mRNA expression of nutrient transporters and vitamin D-related genes in HEK293 cells

As HEK293 cells were identified as expressing most of the components required for vitamin D transport and signalling, these were used to investigate the effect of vitamin D (25(OH)D and 1,25$(OH)_2$D) on nutrient transporter and vitamin D-related gene expression.

Effects of 25(OH)D on nutrient transporter and vitamin D-related gene expression in HEK293 cells

HEK293 cells were cultured with increasing concentrations of the inactive form of vitamin D; there was no alteration to mRNA expression of vitamin D-related or nutrient transport genes (Figure 4.18; Figure 4.19). Due to low *CYP24A1* expression in HEK293 cells the standard curve was not linear and mRNA expression was analysed using the 2^(-ddct) method. *LAT3* mRNA expression showed a trend ($p = 0.08$) for reduced expression in response to 100 nmol/l 25(OH)D compared to media control.

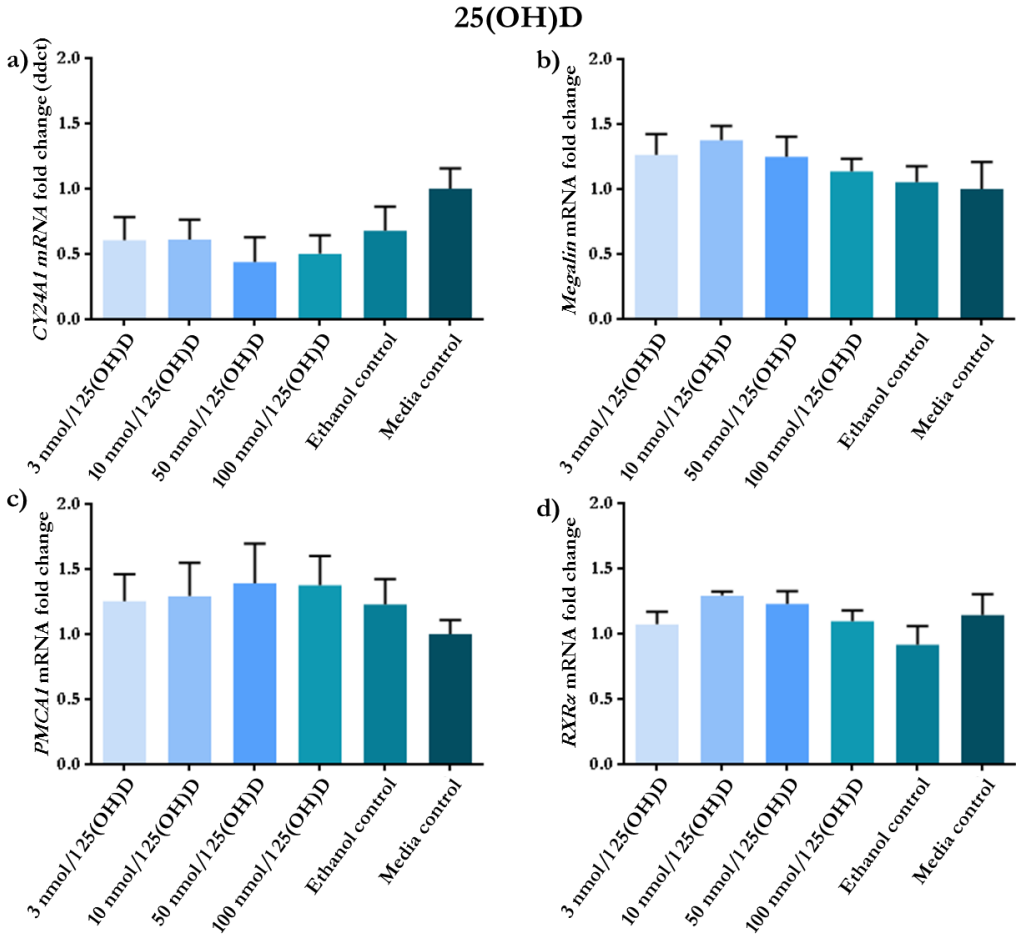

Figure 4.18: Relative mRNA expression of vitamin D-related and calcium transport genes in response to 25(OH)D. mRNA expression of a) CYP24A1, b) megalin, c) PMCA1 and d) RXRα were not affected by increasing concentrations of 25(OH)D. 3 nmol/l 25(OH)D n = 8, 10 nmol/l 25(OH)D n = 9, 50 nmol/l 25(OH)D n = 6, 100 nmol/l 25(OH)D n = 8, ethanol control n = 8, media control n = 8. Data presented as mean fold change + SEM.

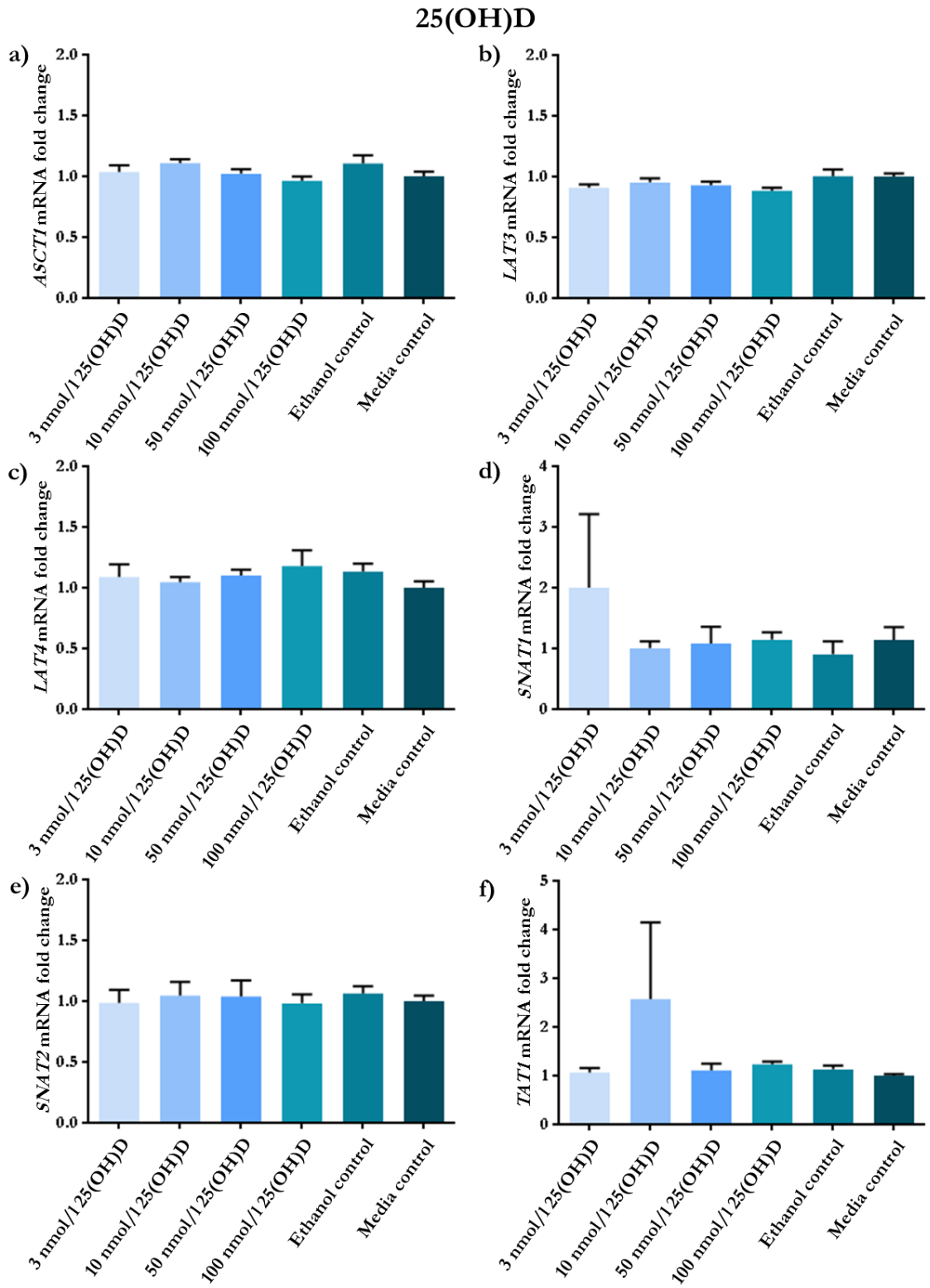

Figure 4.19: Relative mRNA expression of amino acid transporters in response to 25(OH)D. mRNA expression of a) *ASCT1*, b) *LAT3*, c) *LAT4*, d) *SNAT1*, e) *SNAT2* and f) *TAT1* was unaffected by 25(OH)D treatment. 3 nmol/l 25(OH)D n = 8, 10 nmol/l 25(OH)D n = 9, 50 nmol/l 25(OH)D n = 6, 100 nmol/l 25(OH)D n = 8, ethanol control n = 8, media control n = 8. Data presented as mean fold change + SEM.

Effects of 1,25(OH)$_2$D on nutrient transporter gene expression in HEK293 cells

Treatment of HEK293 cells with the active form of vitamin D also showed little impact on mRNA expression. Of the genes involved in vitamin D and calcium function that were tested, only *CYP24A1* showed increased expression at the two highest concentrations of 1,25(OH)$_2$D (Figure 4.20), while none of the amino acid transporters investigated showed alterations to mRNA expression (Figure 4.21).

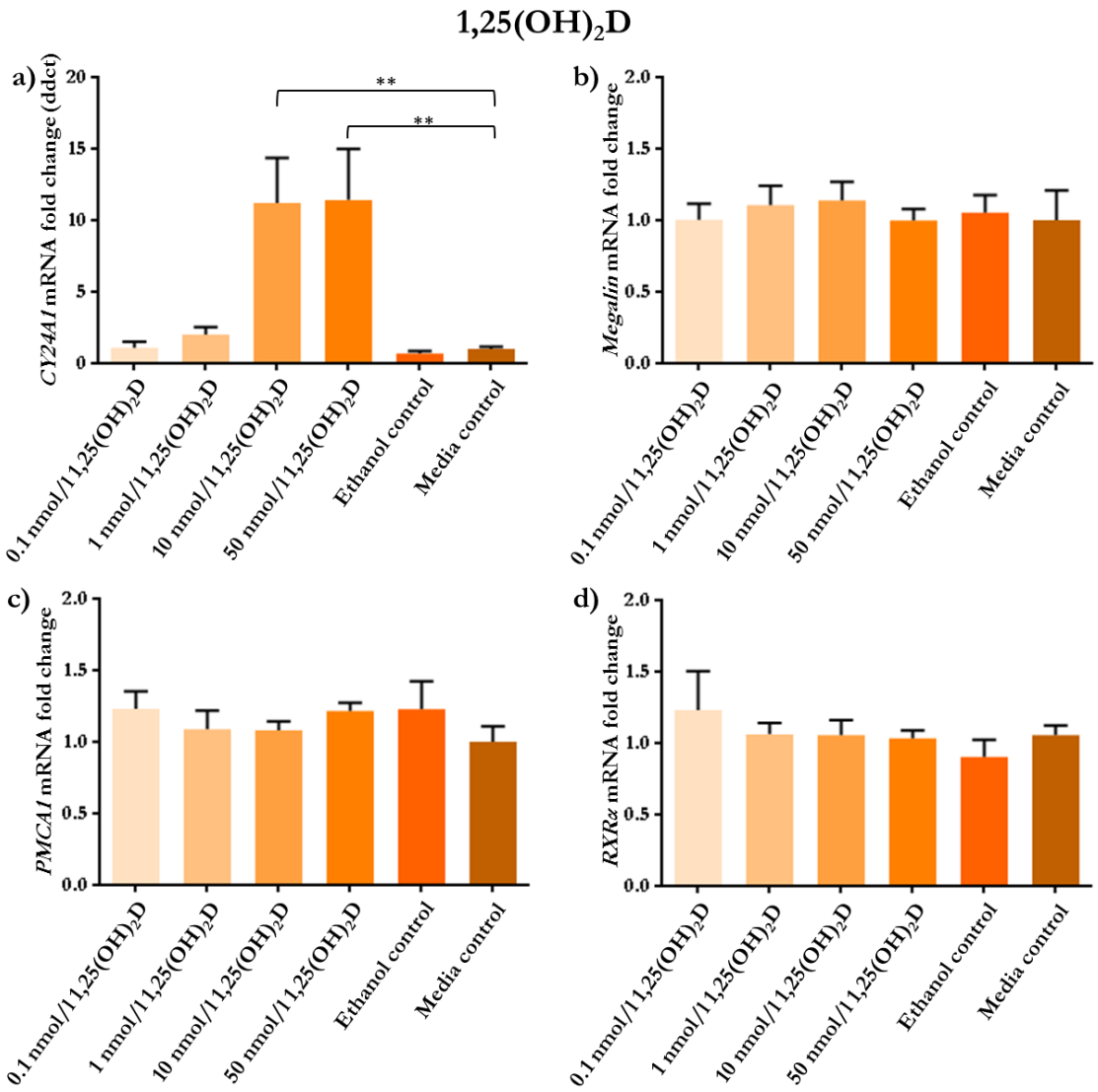

Figure 4.20: Relative mRNA expression of vitamin D and calcium genes in response to 1,25(OH)$_2$D. a) *CYP24A1* mRNA expression was significantly increased by 10 and 50 nmol/l 1,25(OH)$_2$D compared to media control. b) *Megalin* mRNA was not altered in response to 1,25(OH)$_2$D. c) *PMCA1* mRNA levels were not affected by 1,25(OH)$_2$D. d) *RXRa* mRNA levels were unaffected by 1,25(OH)$_2$D treatment. 0.1 nmol/l1,25(OH)$_2$D n = 8, 1 nmol/l 1,25(OH)$_2$D n = 9, 10 nmol/l 1,25(OH)$_2$D n = 7, 50 nmol/l 1,25(OH)$_2$D n = 8, ethanol control n = 8, media control n = 8. ** $p < 0.01$. Data presented as mean fold change + SEM.

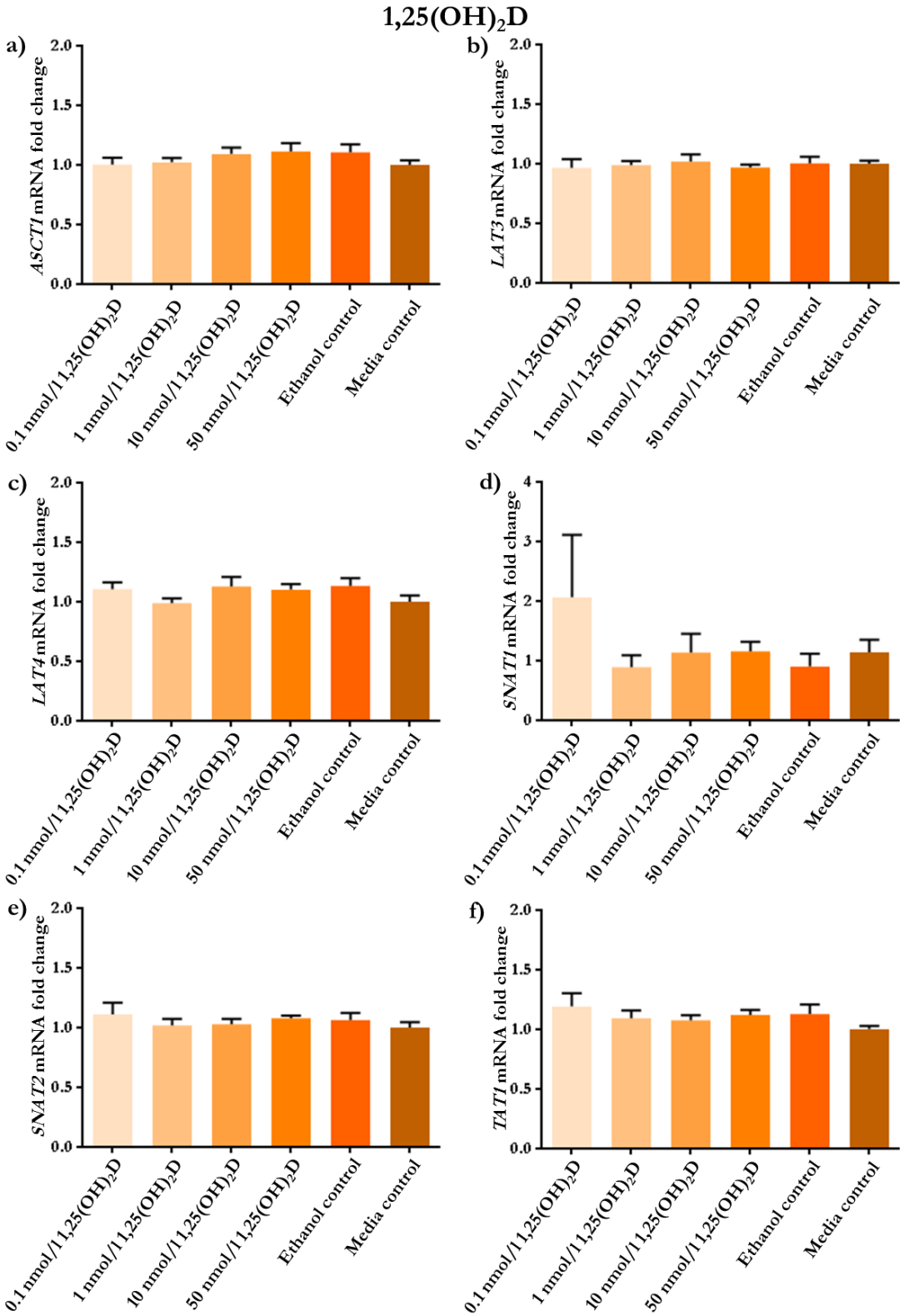

Figure 4.21: Relative mRNA expression of amino acid transporters in response to 1,25(OH)$_2$D. mRNA expression of a) *ASCT1*, b) *LAT3*, c) *LAT4*, d) *SNAT1*, e) *SNAT2* and f) *TAT1* was unaffected by 1,25(OH)$_2$D. 0.1 nmol/l 1,25(OH)$_2$D n = 8, 1 nmol/l 1,25(OH)$_2$D n = 9, 10 nmol/l 1,25(OH)$_2$D n = 7, 50 nmol/l 1,25(OH)$_2$D n = 8, ethanol control n = 8, media control n = 8. Data presented as mean fold change + SEM.

4.4. Discussion

The results presented in this chapter provide evidence for the use of the HEK293 cell line as a model for placental nutrient transport. However, culture of this cell line with vitamin D showed no effects on nutrient transporter expression. It is currently unclear whether this is a cell-specific effect in the HEK293 cell line or whether this also applies to human placenta. Differences in the response to vitamin D could arise due to the different functions of the placenta and kidney. Associations presented in Chapter 3 between placental expression of a vitamin D responsive gene and placental amino acid transporter expression suggests that vitamin D may affect nutrient transporter expression within the placenta. A model that more closely represents the human placenta is now required, such as human placental villous fragment culture, to explore this idea further.

4.4.1. Characterisation of BeWo and HEK293 cell lines as placental cell culture models

Housekeeping gene expression and stability

HKG expression levels and stability differed between the three cell types. Furthermore, mRNA expression levels of HKGs differed between the cell types with expression levels in BeWo cells typically higher than that in HEK293 and placenta. If genes expressed at a significantly higher level in BeWo cells were used for normalisation of target gene expression data this would skew qrt-PCR data and could result in BeWo cells appearing to have lower target gene expression than HEK293 or placenta. For this reason mRNA expression data were normalised to a single HKG, *UBC*, as this was the only HKG in which expression levels were not significantly different between the three cell types. The most stably expressed HKGs for placenta reported here differ from previously published data (Cleal *et al.*, 2009), which identified, *UBC*, *YWHAZ* and *TOP1* as the most stably expressed genes in placental tissue. However, this previous study investigated the expression of a wider range of HKGS and the extraction method as well as sex may influence the results. These results highlight that HKG stability can differ between cell types, and therefore should be assessed before comparison of mRNA expression between differing cell types.

The BeWo cell line as a potential placental cell model

Expression of vitamin D-related genes in BeWo cells did not completely match that of placenta. For example, the vitamin D metabolising enzymes *CYP2J2*, *CYP2R1* and *CYP24A1* were not detected in BeWo cells. Also the vitamin D transport genes *megalin* and *cubilin*, although present in placenta, were not detected in BeWo cells. *Cubilin* and *megalin* mRNA expression have previously been described in BeWo cells (Viola-Villegas *et al.*, 2009; Akour *et al.*, 2013), however for megalin the paper lacked the evidence to back up the claim. In addition, the response of BeWo cells to vitamin D may be limited, as although *RXRa* was detected in both placenta and BeWo, *VDR* expression was variable in the BeWo

samples tested and considerably lower than in placenta. Low expression of VDR at the mRNA and protein level in BeWo cells compared to placenta has previously been described as well as a blunted or non-existent VDRE-mediated response to vitamin D (Pospechova *et al.*, 2009).

The three facilitated amino acid transporters were expressed at significantly lower mRNA levels in BeWo cells compared to placenta; this was also evident at the protein level for TAT1. While BeWo cells have been used to study placental system A amino acid transport (Jones *et al.*, 2006), this expression data suggest that the BeWo cell line is not ideal for the study of placental facilitated amino acid transport. Transfection of the BeWo cell line with GFP-*TAT1* was attempted to increase expression levels in order to investigate regulation. However, despite many different methodologies, a transfection efficiency above 20% could not be achieved and this was not deemed high enough to study regulation of the transporter.

Altogether, the mRNA and protein expression data for facilitated amino acid transporters and vitamin D-related genes in BeWo cells has revealed that BeWo cells are lacking many components that are naturally expressed in placental tissue. A similar situation has been described in BeWo cells in relation to the renin-angiotensin system and the components that were expressed did not respond in the usual way to stimuli including cAMP and oestrogen (Wang *et al.*, 2013). This suggests that the cell line has undergone significant changes at both the molecular and physiological level, resulting in an altered function compared to the tissue that it supposedly represents. Although physiological responses of BeWo cells have not been investigated, the molecular components of the vitamin D pathway and of facilitated amino acid transporters have been characterised and indicate that expression at the molecular level is mismatched to that of placental tissue. The BeWo cell line may represent invasive trophoblast and therefore would not require nutrient transport systems. For this reason, the BeWo cell line will not be used as a model for the human placental syncytiotrophoblast.

Syncytialisation of BeWo cells was attempted to create a cell type similar to the placental syncytiotrophoblast. However, desmosome staining was unspecific and hCG measurement indicated that syncytialisation was not achieved as hCG production decreased with forskolin treatment. This could be due to increased apoptosis in response to forskolin as seen in BeWo and JEG-3 cells previously (Al-Nasiry *et al.*, 2006). Also non-placental cancer cells secrete hCG (Heyderman *et al.*, 1985) making it difficult to test for syncytium formation in a choriocarcinoma cell line. Indeed, wide variation (10-70%) in fusion rates of BeWo cells treated with forskolin has been reported (Wice *et al.*, 1990; Kudo *et al.*, 2003; Orendi *et al.*, 2010) bringing into question the reliability of the method. This line of investigation was not pursued further, due to the lack of robustness in measures of syncytium formation.

The HEK293 cell line as a potential placental cell model

mRNA expression of most of the vitamin D-related genes was detected in HEK293 cells, suggesting they may provide a good model to study the effects of vitamin D. Although expression of some genes was low or inconsistent, including *CYP24A1* and *CYP27B1*, expression of these has previously been demonstrated in HEK293 cells (Turunen *et al.*, 2007). The presence of *megalin* and *cubilin* suggests that vitamin D should be transported into the cell, while the presence of the metabolising enzymes suggests that the cell line should be able to activate and inactivate the various forms of vitamin D. Expression of *VDR* and *RXRa* imply that the cell line should be able to respond to vitamin D through VDR-mediated alterations to target gene expression. This is supported by work showing a classical increase in *CYP24A1* and decrease in *CYP27B1* mRNA when HEK293 cells were cultured with active vitamin D (Turunen *et al.*, 2007). In addition, mRNA expression of *LAT3* and *TAT1* was detected in HEK293 at levels similar to those in placenta. While mRNA expression of *LAT4* was significantly lower in HEK293 than placenta, protein expression of LAT4 was higher than that detected in placenta.

Overall, the HEK293 cell line appears a better model for the placental syncytiotrophoblast. Its expression of both vitamin D-related genes and amino acid transporters is more closely matched to that of placenta. Although, expression was not completely matched, the mismatch was far less than that observed between BeWo and placenta. Furthermore, the lack of some main components of the vitamin D signalling system in the BeWo cell line means that the cells responses to vitamin D are unlikely to be normal.

4.4.2. Effect of methylation on nutrient transporter mRNA expression in BeWo and HEK293 cells

Inhibition of DNA methylation in BeWo and HEK293 cells revealed cell- and gene-specific differences in mRNA expression. Firstly, HEK293 cells showed no mRNA expression of *EAAT4*, *EAAT5*, or b^{0+} mRNA, which matches placenta. Only *EAAT2* mRNA was not detected within BeWo cells. This provides further support for the choice of HEK293 cells for studies of placental function. Secondly, the study revealed that some genes are not affected by methylation within specific cell types. For example, *SNAT1* and *TAT1* were not affected by AZA treatment in BeWo cells, but mRNA expression increased or decreased, respectively, in HEK293 cells. This shows that methylation can result in tissue-specific gene expression changes. This has been reported in other studies where more than one cell line has been treated with AZA (Marik *et al.*, 2010; Habano *et al.*, 2011; Essa *et al.*, 2012; Hobaus *et al.*, 2013), and has also been shown in relation to placenta-specific methylation. For example, *CYP24A1* was shown to be highly methylated within placental cells compared to non-placental cells (Novakovic *et al.*, 2009).

Removal of methylation resulted in increased mRNA expression of over half of the genes tested in both HEK293 and BeWo cells, including the facilitated amino acid transporters *LAT3* and *LAT4* in both cell lines, and *EAAT2* and *RXRa* in the HEK293 cell line. This demonstrates that methylation may play a role in regulation of these genes. *LAT3* mRNA expression within the placenta has been positively associated with fetal growth (Cleal *et al.*, 2011), therefore methylation within the placenta affecting *LAT3* mRNA expression could impact upon fetal development. The same may be true for *EAAT2*, as studies have shown *EAAT2* is directly regulated by DNA methylation (Zschocke *et al.*, 2007). As we also observed an increase in *EAAT2* mRNA expression with the removal of methylation, this could mean that in our model *EAAT2* is also regulated by methylation. The use of AZA results in a general reduction in methylation of all genes within the cell, therefore it is unknown whether mRNA expression changes are a direct or indirect result of reduced methylation. Direct effects of reduced methylation include increased accessibility of the chromatin resulting in increased binding of transcription factors and co-activators (Ballestar and Wolfe, 2001). Alternatively, mRNA expression may be indirectly altered through methylation induced changes to activity of transcription factors, co-activators and co-repressors. For example, methylation of CREB-binding protein/p300 has been shown to abrogate binding of the co-factors to cAMP response element binding proteins (Xu *et al.*, 2001).

Not all genes that responded to AZA treatment showed increased mRNA expression. In HEK293 cells, *EAAT3*, *TAT1* and *megalin* showed reduced mRNA expression with AZA treatment, whilst in the BeWo cell line *EAAT3* and *EAAT4* mRNA was reduced. This reduction in expression may be due to methylation within negative response elements. nVDREs result in a down-regulation in mRNA expression upon *VDR* binding, such as is observed with *CYP27B1* (Takeyama and Kato, 2011). Removal of methylation in a negative response element would result in availability of the DNA for binding of transcriptional repressors. Alternatively, the genes may be negatively regulated by a factor that itself is methylated, and once de-methylated can act upon its target gene. Increased megalin protein expression has previously been associated with reduced methylation at CpGs within the *megalin* promoter region in JEG3 cells (Knutson *et al.*, 2000), whilst our study showed reduced *megalin* mRNA expression with reduced global DNA methylation in HEK293 but not BeWo cells. The differences observed could be due to the region methylated or cell-specific differences.

Only a few vitamin D-related genes were investigated, as characterisation of the methylation of the vitamin D enzymes, including *CYP24A1*, has previously been carried out (Novakovic *et al.*, 2009). Differing responses in *VDR* expression in response to AZA treatment have been reported. Some studies show no impact of AZA treatment on *VDR* expression (Habano *et al.*, 2011; Hobaus *et al.*, 2013), as we observed in HEK293 cells, while others report increased *VDR* expression (Pospechova *et al.*, 2009; Marik *et al.*, 2010; Essa *et al.*, 2012). *RXRa* mRNA expression in response to methylation

appears to be less extensively studied than *VDR*. In HEK293 cells *RXRa* mRNA expression increased with AZA treatment. However, other studies in colon cancer, BeWo and JEG3 cell lines reported no change in *RXRa* mRNA expression in response to AZA treatment (Pospechova *et al.*, 2009; Hobaus *et al.*, 2013). The contrasting outcomes between these studies and ours could be due to the different cell types used, or the differing concentrations of AZA administered to the cells. Pospechova *et al.* (2009) treated cells with 2.5 μmol/l of AZA, whilst we used 7 μmol/l. Methylation at specific sites within the *RXRa* gene promoter in cord tissue was negatively associated with percentage BMC in children at 4 years of age (Harvey *et al.*, 2014c). In addition, placentas from pre-eclamptic pregnancies showed increased methylation of *RXRa* which was associated with reduced protein expression of the nuclear receptor (Anderson *et al.*, 2015). These studies suggest that *RXRa* mRNA in the placenta may be modulated by methylation, and dysregulation of this may occur within pregnancy pathologies, which could have implications for later development of the offspring.

This study revealed differences between cell types in terms of the effects of DNA methylation on mRNA expression. Methylation levels in cancerous cells are altered in comparison to healthy tissue (Kulis and Esteller, 2010), which may influence the levels in BeWo cells as they originated from a choriocarcinoma. Therefore, for our placental cell model the HEK293 cell line is better suited as it less likely to show the aberrant methylation observed in cancer. The fact that we saw numerous differences in expression of genes required for normal placental function and fetal growth in response to methylation changes provides support for methylation as a mechanism of altering placental function. Epigenetic mechanisms are a candidate for the transmission of environmental cues from the mother to the placenta. The impact of the environment on placental methylation is supported by a link between methylation at a specific CpG within the *TAT1* promoter and measures of maternal fat mass prior to pregnancy (Chapter 3).

4.4.3. Effect of vitamin D on mRNA expression in HEK293 cells

Inactive 25(OH)D had no significant effect on expression of the genes investigated. mRNA expression of *CYP24A1*, a vitamin D responsive gene, was not altered suggesting 25(OH)D was not converted into the active $1,25(OH)_2D$ within these cells. This is supported by the fact that when HEK293 cells were cultured with 10 and 50 nmol/l $1,25(OH)_2D$ the classical increase in *CYP24A1* mRNA levels was observed, indicating that HEK293 cells can respond to vitamin D. This response at 10 nmol/l of $1,25(OH)_2D$ has previously been described (Turunen *et al.*, 2007). Although expression of CYP27B1 at the mRNA and protein level has previously been described in HEK293 cells (Alesutan *et al.*, 2013), the expressed protein may not be physiologically active. The current data would suggest that CYP27B1 may not be active in HEK293 cells.

Other than the classical up-regulation of *CYP24A1* with 1,25$(OH)_2$D, vitamin D did not affect mRNA expression of other vitamin D-related genes, however these are not included within the classical vitamin D negative feedback loop. 1,25$(OH)_2$D has previously been shown to increase mRNA expression of *VDR* in muscle cells (Pojednic *et al.*, 2015), suggesting that vitamin D genes not within the classical feedback loop can be regulated by vitamin D. Culture of HEK293 cells with various concentrations of 25(OH)D and 1,25$(OH)_2$D had no significant effects on amino acid transporter gene expression. This was expected for 25(OH)D considering the absence of an increase in *CYP24A1* expression, however *LAT3* mRNA expression in HEK293 cells treated with 100 nmol/l 25(OH)D showed a trend for a 10% reduction in expression compared to the media treated control, although this is unlikely to have a major impact on transport.

Even at the highest concentration of 1,25$(OH)_2$D there was no effect on *PMCA1* mRNA expression. Contrasting effects of 1,25$(OH)_2$D on *PMCA1* mRNA expression have been reported, including increased expression in intestinal explant culture (Balesaria *et al.*, 2009) and rat osteosarcoma cells (Glendenning *et al.*, 2001). The 1.8 fold increase in intestine is modest compared to the > 10 fold increase observed in our studies for *CYP24A1* mRNA expression. Other studies have reported no effect of 1,25$(OH)_2$D on *PMCA1* mRNA expression using cell types including CaCo-2 cells (Nakane *et al.*, 2007), cochlea epithelial cells (Yamauchi *et al.*, 2005; Yamauchi *et al.*, 2010) and human (Walters *et al.*, 1999) and rat duodenal biopsies (Brown *et al.*, 2002). Overall, the data presented here and in the available literature suggest that PMCA1 is not a vitamin D responsive calcium transporter. It would be interesting to investigate the response to vitamin D in a wider range of calcium transporters, as mRNA expression of other transporters, such as CaT1, has been reported to change upon exposure to active vitamin D (Walters *et al.*, 2007).

The lack of effect of active vitamin D on expression of the amino acid transporters tested is intriguing. Vitamin D has previously been shown to alter expression of nutrient transporters. Microarray analysis has revealed increased expression of transporters including choline transporter (*SLC44A2*), glucose-6-phosphate transporter (*SLC37A2*) and MCT7 (*SLC16A6*), which transports leucine, valine and isoleucine and belongs to the same family as *TAT1* (Ramagopalan *et al.*, 2010). Furthermore, 50 nmol/l 1,25$(OH)_2$D increased GLUT4 protein expression in 3T3L1 cells, which was physiologically reflected with increased glucose uptake (Manna and Jain, 2012). All the transporters investigated here showed associations with either 34 week 25(OH)D and/or DBP in placental samples from the SWS. Furthermore, *TAT1* expression in the SWS showed a positive trend for an association with *CYP24A1*, a potential biomarker for active vitamin D levels. The results here could mean that observations from the SWS placentas reflect indirect effects, possibly as a result of maternal nutrition or lifestyle. Alternatively, the results observed in the placenta may reflect placenta-specific interactions between the nutrient transporters and vitamin D, which may not be replicated in HEK293 cells. Different

responses in different tissues can occur as a result of varying expression of co-regulators. Vitamin D signalling involves numerous co-regulators, such as steroid receptor co-activators 1, 2 and 3, p300 and CREB-binding protein (Fetahu *et al.*, 2014), therefore an investigation into the expression of these in HEK293 and placenta would be interesting. One study showed synergistic effects on mRNA expression of a taurine transporter when a kidney cell line was cultured with a combination of $1,25(OH)_2D$ and retinoic acid (Chesney and Han, 2013), therefore it is possible that in the HEK293 cell line retinoic acid may also be required to initiate effects on mRNA expression mediated by the VDR/RXRα heterodimer. However, the above study also showed that treatment of a human breast cancer line in the same manner had no effect on mRNA expression of the transporter studied, suggesting that there are likely to be cell-specific responses to vitamin D and/or retinoic acid.

Maternal DBP levels in the SWS were associated with expression of some of the placental amino acid transporters. As discussed in chapter 3, this led us to think about the mechanism of vitamin D entry into the placenta. Cell culture media for these experiments was not supplemented with DBP, however the FBS added to the media contains albumin, and possibly DBP, although at unknown levels. Albumin functions in the same way as DBP for megalin- and/or cubilin-mediated endocytosis of vitamin D, although with a lower affinity. The observed increase in *CYP24A1* at 10 and 50 nmol/l $1,25(OH)_2D$ shows vitamin D did get into the cells, however, the level of transport is unknown. If the full amount of vitamin D added to the cell culture media was not entering the cell this may partly explain the lack of response in amino acid transporter mRNA expression. If only unbound vitamin D was being transported into the cells, the presence of albumin in the culture media could have resulted in vitamin D being sequestered outside the cell, resulting in transport into the cell only with the higher concentrations of vitamin D where presumably a larger percentage of the hormone was unbound. The higher binding affinity of albumin for 25(OH)D compared to $1,25(OH)_2D$ could explain why 25(OH)D had no impact on mRNA expression within HEK293 cells. This has previously been described in MCF-7 cells, *CYP24A1* expression was only induced upon exposure to vitamin D when the level of FBS in the cell culture media was reduced. This effect was not observed for $1,25(OH)_2D$ (Rowling *et al.*, 2006) and could explain why $1,25(OH)_2D$ but not 25(OH)D increased *CYP24A1* mRNA in HEK293 cells. In future, a measure of vitamin D uptake into the cells would be useful.

4.4.4. Limitations

There are several limitations to this study. Firstly, characterisation of the cell lines focussed on mRNA expression levels. This was decided on as the main focus of the study is to examine the effects of vitamin D which acts at the transcriptional level. However, this focus on mRNA expression has left questions about vitamin D metabolism within the chosen cell culture model unanswered. As discussed below, this could be pursued by analysis of CYP27B1 activity within the cell line. For the AZA studies,

a measure of cell viability following exposure would have been beneficial. It was noted that cells treated with AZA were less confluent at ~60% compared to ~80-90% when treated with control media. This anti-proliferative effect on cells treated with AZA has been previously described (Essa *et al.*, 2010; Marik *et al.*, 2010), although the concentration of AZA used for this study was within the previously described non-toxic range (Essa *et al.*, 2012). Equal amounts of RNA went into cDNA reactions and results were adjusted for HKGs, therefore differences in cell number should not have skewed data. However, a measure of cell viability would have shown that this effect was merely an anti-proliferative effect and not induction of apoptosis.

4.4.5. Future work

In future work, syncytialisation of the BeWo cell line could be pursued further, however more robust methods for evaluating cell fusion are required. Possible strategies to assess fusion could be to analyse expression of genes up-regulated during syncytialisation, such as *Spectrin Alpha, Non-Erythrocytic 1* and *Galactoside-binding soluble lectin 13*, by qrt-PCR (Orendi *et al.*, 2010). Alternatively, a method for assessing BeWo cell fusion has been developed using one set of BeWo cells expressing H2B-GFP in the nucleus, and one expressing Mit-DsRed2 in the cytoplasm. The number of fused cells can then be assessed by counting dual labelled cells using fluorescence microscopy or fluorescence-activated cell sorting (Kudo *et al.*, 2003). In addition, if syncytialisation of BeWo cells were pursued different strains of Bewo cells could be tested as syncytialisation rate is reported to differ depending on the strain of cells used (Orendi *et al.*, 2011).

Future work with the HEK293 cell line, could involve investigation into vitamin D metabolism within the cell line, such as investigating the activity of CYP27B1. This would be useful in light of the qrt-PCR data suggesting there was no transcriptional response to 25(OH)D. In addition, a greater number of calcium transporters could be investigated for changes to mRNA expression in response to vitamin D, as previous research has shown effects of vitamin D on calcium transporter expression.

Tissue-specific differences in the cellular response to vitamin D may occur, therefore although the HEK293 cell line was identified as the better model for the investigation, primary placental cells or placental villous fragment culture could provide alternative methods to investigate the effect of vitamin D on mRNA expression. It would be interesting to compare results using either of these methods to those in HEK293 to identify whether there are tissue-specific differences in vitamin D actions on nutrient transporter expression. Furthermore, if differences in the response to vitamin D were observed, the expression of vitamin D co-regulators could be compared in the cell types to see if variation in co-regulator expression was mediating altered cellular responses to vitamin D. In addition, exposure to the combination of $1,25(OH)_2D$ and retinoic acid could be explored as this was shown to

have a synergistic effect on expression of specific amino acid transporters in a kidney cell line (Chesney and Han, 2013).

It is possible that some genes, including *TAT1*, are highly methylated at VDREs. This would prevent vitamin D-mediated up-regulation in expression. It would be interesting to combine AZA and vitamin D treatment to see whether genes that did not respond to vitamin D in this study are able to respond upon removal of DNA methylation. As methylation levels have been shown to be altered in placental pathologies (Anderson *et al.*, 2015), this may provide useful insights into links between vitamin D, DNA methylation and placental mRNA expression in conditions such as pre-eclampsia. Moreover, synergistic effects of treatment with AZA and vitamin D have been described in some cancer cell lines (Marik *et al.*, 2010; Pan *et al.*, 2010; Essa *et al.*, 2012).

AZA treatment of the BeWo and HEK293 cell lines revealed specific changes in mRNA expression with the removal of methylation. This could be pursued further by investigating whether these are direct or indirect effects. Pyrosequencing of specific CpGs within the promoter regions of genes, such as *LAT3,* that were shown to be altered by AZA treatment could be used. However, this is a targeted approach, and only gives information about the selected CpGs. Alternatively, methylation arrays could be used to give a broader insight into genes with altered methylation status before focussing on specific CpGs. In addition, methylated and unmethylated reporter constructs for genes could be transfected into cell lines and reporter activity assessed. This would allow the impact of methylation on basal activity as well as activity in response to a stimulus, such as vitamin D, to be monitored. Furthermore, DNA methylation is not the only epigenetic mechanism which may affect placental function. Cells could be cultured with trichostatin A, a histone deacetylase inhibitor, to investigate the effect of histone acetylation on mRNA expression.

4.4.6. Conclusions

In conclusion, this study has identified the HEK293 cell line as the better model for the placental syncytiotrophoblast. The expression of numerous transporters was altered upon the removal of DNA methylation, implicating this as a key regulator of mRNA expression. However, mRNA expression of nutrient transporters within this cell line was not affected by vitamin D. It is currently unclear whether this is a cell-specific response of the HEK293 cell line, therefore an additional model, placental villous fragment culture, will be employed to investigate these responses within human placental tissue.

Chapter 5:
Placental uptake of vitamin D

5.1. Introduction

Many studies have described associations between maternal and umbilical cord 25(OH)D levels (Bouillon, 1981; Delvin, 1982; Hollis and Pittard, 1984; Markestad, 1984; Novakovic *et al.*, 2012) demonstrating that this inactive form is transported across the placenta. However, the evidence for 1,25$(OH)_2$D transport across the placenta is contradictory (Fleischman *et al.*, 1980; Delvin, 1982; Hollis and Pittard, 1984; Ishida *et al.*, 1988). Furthermore, the mechanism for uptake of vitamin D into the placenta is unclear.

5.1.1. The free hormone hypothesis

Initially, vitamin D was assumed to enter cells via passive diffusion as described by the free hormone hypothesis (Mendel, 1989). However, in recent years this hypothesis has come under scrutiny. As discussed in Chapter 1 section 1.5.3, both 25(OH)D and 1,25$(OH)_2$D bind the carrier proteins, DBP and albumin. These proteins show a high degree of homology in terms of amino acid sequence and protein structure (Cooke and David, 1985). Due to the large molar excess of albumin and DBP only a small fraction of vitamin D is free within the circulation. The free fraction of 25(OH)D is estimated to be 0.03%, while free 1,25$(OH)_2$D is thought to be slightly higher at 0.4%. While DBP is considered the main carrier protein for vitamin D, albumin is present within the circulation in much larger quantities, resulting in ~12% of 25(OH)D and ~14% of 1,25$(OH)_2$D being bound to albumin (Bikle *et al.*, 1985; Bikle *et al.*, 1986).

In Chapter 3, associations were presented between 25(OH)D and expression of placental nutrient transport genes. Unexpectedly, a greater number of associations were observed with expression of these genes and DBP levels. This could indicate an important role for DBP in mediating the effects of vitamin D within the placenta. In support for a key role of DBP, serum levels increase during pregnancy. This rise occurs alongside increased 1,25$(OH)_2$D levels. However, no rise in 25(OH)D occurs. Reports on the levels of free 1,25$(OH)_2$D during pregnancy are contradictory. Bikle *et al.* (1984) reported that the percentage of free 1,25$(OH)_2$D was reduced in pregnancy but, due to the higher levels of 1,25$(OH)_2$D, total free 1,25$(OH)_2$D is increased. However, other studies have reported that the concomitant increase in both 1,25$(OH)_2$D and DBP results in unchanged free 1,25$(OH)_2$D compared to non-pregnant controls (Van Hoof *et al.*, 2001). On the other hand, free 25(OH)D decreases during pregnancy (Bouillon, 1981). One explanation for this rise in DBP during pregnancy is that it prevents maternal catabolism of vitamin D and provides increased bound vitamin D for transport into the placenta, via a DBP-mediated mechanism.

One study examined vitamin D transport using the placental perfusion system. This study showed transfer of both 25(OH)D and $1,25(OH)_2D$, however huge variations in transport of 25(OH)D were observed between placentas. In some cases, 25(OH)D transport occurred at a greater rate than $1,25(OH)_2D$ transport, while in other placentas the reverse was observed. This study did investigate whether the presence of DBP affected 25(OH)D transport, and showed a reduction in transport in the presence of DBP. However, there are flaws to the methodology used to address this question. Firstly, the source of DBP used is described as male human serum, with no reference to the concentration of DBP present or to what other proteins may be present within this serum. Therefore, the molar concentration of DBP compared to vitamin D cannot be ascertained. Furthermore, results observed may reflect other components of the serum used. Secondly, the buffer used for all experiments contained albumin, therefore, this carrier protein may have bound to the vitamin D and the free transport observed may have been carrier protein-mediated (Ron *et al.*, 1984). Current literature support the transport of 25(OH)D across the placenta. Although $1,25(OH)_2D$ is not thought to cross the placenta (Shin *et al.*, 2010; Liu and Hewison, 2012) the data on associations between maternal and cord blood $1,25(OH)_2D$ levels are contradictory (Fleischman *et al.*, 1980; Delvin, 1982; Hollis and Pittard, 1984; Ishida *et al.*, 1988), therefore it is currently unknown whether $1,25(OH)_2D$ crosses the placenta.

It seems unlikely that vitamin D transport is dependent on the tiny fraction of unbound hormone, particularly in pregnancy, where reduced vitamin D transport to the fetus has been associated with reduced fetal growth, and defects in fetal and infant bone development (Javaid *et al.*, 2006; Harvey *et al.*, 2008; Bowyer *et al.*, 2009; Mahon *et al.*, 2010; Hart *et al.*, 2015). Reabsorption of vitamin D within the kidney is dependent on vitamin D binding to the carrier proteins, DBP and albumin (Nykjaer *et al.*, 1999; Nykjaer *et al.*, 2001), therefore it is possible that endocytosis of carrier protein bound vitamin D is important in the placental uptake of vitamin D.

5.1.2. Potential endocytic mechanisms

There are a range of endocytic mechanisms which could be responsible for vitamin D uptake into the placenta (Figure 5.1).

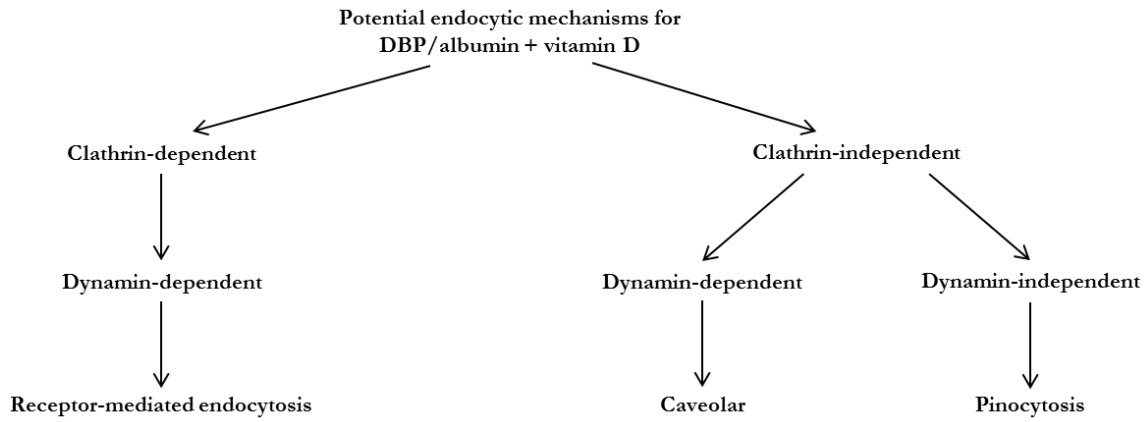

Figure 5.1: Potential mechanisms for DBP- and albumin-mediated uptake of vitamin D into the placenta. Modified from Mayor and Pagano (2007).

This study will specifically investigate the endocytic mechanisms of clathrin-dependent endocytosis, caveolae-dependent endocytosis and pinocytosis as well the role of megalin and cubilin in placental uptake of vitamin D. As described in Chapter 1.6.4 clathrin-dependent endocytosis involves clathrin coated pits (Figure 1.20; Doherty and McMahon, 2009), and is the mechanism for megalin- and cubilin-mediated uptake of albumin and DBP bound vitamin D within the kidney (Nykjaer *et al.*, 1999; Birn *et al.*, 2000; Zhai *et al.*, 2000; Nykjaer *et al.*, 2001). Cargo for clathrin-dependent endocytosis can be selected via the expression of specific membrane receptors, such as megalin and cubilin (see 5.1.3). Caveolae-dependent endocytosis functions in a similar manner to clathrin-dependent endocytosis but membrane invaginations are driven by the protein caveolin (Figure 1.20; Mayor and Pagano, 2007; Doherty and McMahon, 2009). However, caveolin proteins have not been identified within the placental syncytiotrophoblast (Lyden *et al.*, 2002; Linton *et al.*, 2003), so it seems unlikely that this endocytic mechanism is responsible for vitamin D uptake. Pinocytosis involves actin driven protrusions of the membrane, resulting in formation of the pinosome. The pinosome encapsulates external fluid and is internalised into the cell (Figure 1.20; Jones, 2007; Kerr and Teasdale, 2009). This investigation will use blockers of megalin, dynamin, clathrin-dependent endocytosis and pinocytosis to attempt to elucidate the mechanism of vitamin D entry into the human placenta.

5.1.3. Receptor-mediated endocytosis via megalin and cubilin

Megalin and cubilin mediate endocytosis of albumin and DBP bound vitamin D in renal proximal tubule cells. These endocytic receptors bind DBP or albumin when bound to 25(OH)D or $1,25(OH)_2D$ and mediate their uptake into the cell (Nykjaer *et al.*, 1999; Nykjaer *et al.*, 2001). Expression of both megalin mRNA and protein and cubilin protein has been demonstrated within the human placenta (Sahali *et al.*, 1992; Lundgren *et al.*, 1997), raising the possibility of this mechanism for vitamin D transport from the maternal circulation into the placenta (Figure 5.2).

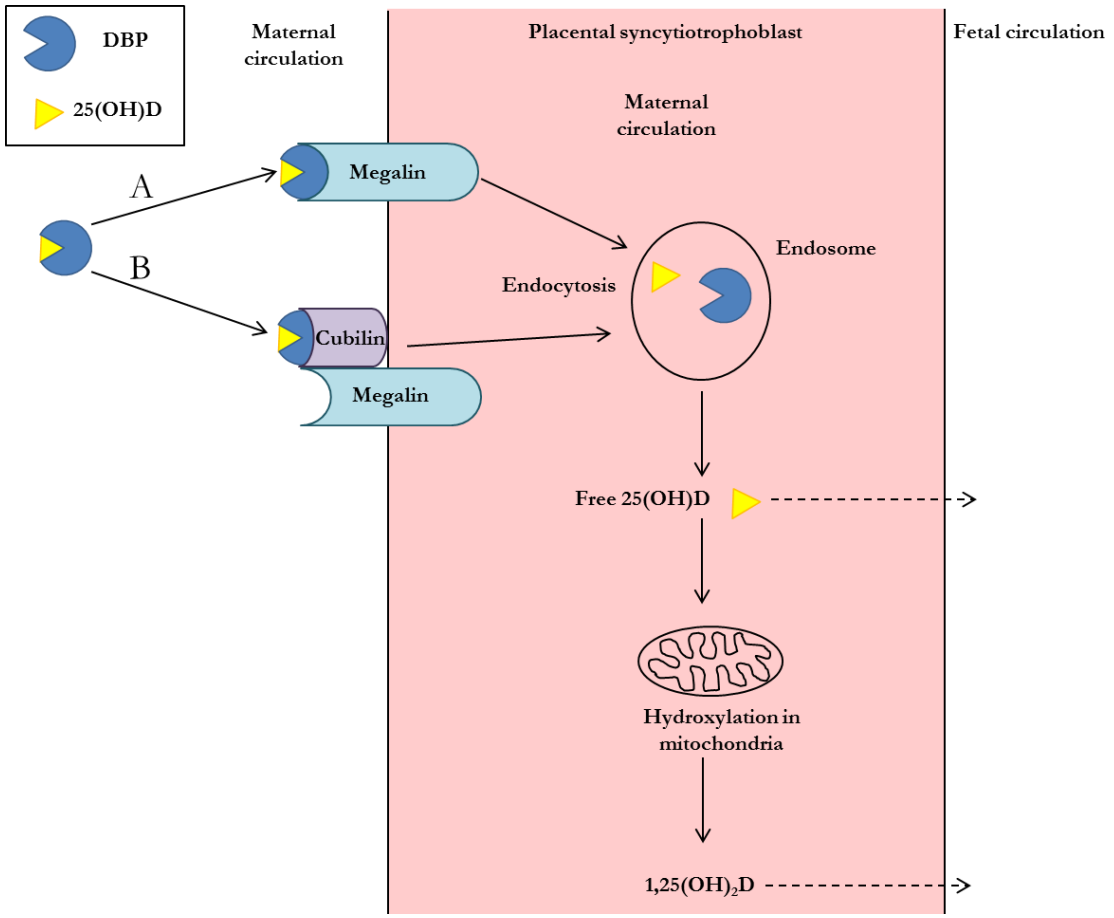

Figure 5.2: Potential mechanisms of cubilin and/or megalin receptor-mediated endocytosis into the placenta. 25(OH)D-DBP complexes could enter the placenta via binding to megalin or cubilin. A. indicates 25(OH)D-DBP entry via binding to megalin and subsequent internalisation of the megalin-ligand complex. B. indicates internalisation of 25(OH)D-DBP via binding to cubilin. Cubilin requires interaction with megalin to internalise cubilin-ligand complexes. Following receptor-mediated endocytosis via either mechanism, the 25(OH)D would dissociate from DBP within the endosome, leaving 25(OH)D free. The free 25(OH)D can then be hydroxylated to form $1,25(OH)_2D$ within the mitochondria. Dashed lines indicate potential transport of vitamin D into the fetal circulation. This diagram illustrates DBP-mediated entry of vitamin D, but albumin-mediated entry could also occur via the same process.

Megalin is a 600 kDa transmembrane protein of the low-density lipoprotein receptor family and binds a number of ligands, including DBP, albumin and low-density lipoprotein (Verroust and Christensen, 2002). Upon ligand binding, the megalin-ligand complex is internalised into the cell. While megalin can function independently, cubilin cannot. Cubilin is a 400 kDa peripheral membrane protein, lacking transmembrane and intracellular domains. Ligands of cubilin include, DBP, albumin (Birn *et al.*, 2000) and apolipoprotein A-I (Verroust and Christensen, 2002). Once bound to a ligand, cubilin associates with megalin, and megalin mediates the internalisation of this dual receptor ligand complex. Within the kidney, cubilin is likely to be the major ligand binding partner for DBP as removal of both megalin and cubilin had little additional effect on loss of DBP in the urine compared to removal of cubilin alone (Nykjaer *et al.*, 2001). A similar story has been described for cubilin binding to albumin within the kidney (Birn *et al.*, 2000). This dual receptor function could also be important for vitamin D uptake within the placenta (Figure 5.2B), alternatively, megalin may act alone to mediate placental uptake of vitamin D (Figure 5.2A).

Albumin uptake in placental explants has been shown to be reduced by inhibition of clathrin-dependent endocytosis, but not through specific inhibition of megalin-mediated transport (Lambot *et al.*, 2006). This could indicate alternative entry systems for DBP and albumin into the placenta, or may show that both DBP and albumin are internalised via a non-megalin-mediated mechanism. In support of the presence of alternative uptake mechanisms, DBP uptake has been described in cells which do not express megalin or cubilin (Chun *et al.*, 2010).

5.1.4. Aims

The aim of this study is to investigate vitamin D entry into the placenta. Specifically, the aims of this investigation are:

1. To study whether both 25(OH)D and 1,25$(OH)_2$D are transported into the placenta.
2. To investigate the role of DBP and albumin in mediating entry of both vitamin D metabolites into the placenta.
3. To investigate the mechanism for 25(OH)D and 1,25$(OH)_2$D entry into the placenta through the use of blockers of specific endocytic routes.
4. To investigate whether 25(OH)D and 1,25$(OH)_2$D alone or in combination with albumin or DBP causes changes to expression of genes involved in amino acid, calcium and vitamin D transport and vitamin D metabolism.

5.2. Methods

Placental villous fragment culture was used to investigate the uptake of 25(OH)D and 1,25$(OH)_2$D into the human placenta. Uptake was studied through two methods; visualisation of FITC-albumin and mRNA expression of the vitamin D responsive gene *CYP24A1*. FITC-albumin was used as it has a high homology to DBP, and can bind and transport both 25(OH)D and 1,25$(OH)_2$D. The mechanism of uptake of 25(OH)D and 1,25$(OH)_2$D was investigated using blockers for specific forms of endocytosis. Expression levels of specific vitamin D-related and nutrient transport genes were also studied to investigate placental gene expression changes in response to vitamin D.

5.2.1. Placenta collection

Full term human placentas were collected within 30 min of delivery from MAVIDOS or by Dr E.Lofthouse from uncharacterised pregnancies, of which there are no data on the women or infants as there are for SWS and MAVIDOS. These studies were conducted according to the guidelines in the Declaration of Helsinki, and the Southampton and South West Hampshire Research Ethics Committee approved all procedures. Written informed consent was obtained from all participating women.

5.2.2. Placental villous fragment culture

Placental villous fragments (Figure 5.3) of ~10 mg were dissected from the maternal facing side of the placenta and any maternal decidual tissue was removed (section 2.4.2). Placental villous fragments were stored in Tyrodes buffer at RT whilst the remaining fragments were dissected. Placental villous fragments were exposed to 25(OH)D alone or with albumin, or 1,25$(OH)_2$D alone, with DBP or albumin to investigate the effect of carrier proteins on vitamin D uptake into the placenta. The mechanism of vitamin D uptake was investigated through culturing placental villous fragments with each type of vitamin D alongside carrier proteins combined with exposure to inhibitors of specific endocytic processes.

Figure 5.3: A placental villous fragment in Tyrodes buffer.

Villous fragments that were to be exposed to endocytic blockers for the duration of the experiment were incubated in a pre-incubation buffer containing the specific blocker for 30 min at 37ºC. Villous fragments that were not to be exposed to endocytic blockers for the experiment were incubated in Tyrodes at 37ºC for the pre-incubation period. The function of each of the endocytic blockers used is shown in Table 5.1. Placental villous fragments were then transferred to their experimental buffers for the duration of the experiment.

Table 5.1: Functions of endocytic blockers used for placental villous fragment studies.

Endocytic blocker	Function
Amiloride hydrochloride hydrate	Inhibits pinocytosis (West *et al.*, 1989)
Chlorpromazine hydrochloride	Inhibits clathrin-mediated endocytosis (Wang *et al.*, 1993)
Dynasore	Inhibits dynamin-dependent endocytosis (Macia *et al.*, 2006)
Gentamicin	Inhibitor of megalin (Dagil *et al.*, 2013)

Experiments for microscopy

Following pre-incubation with normal Tyrodes buffer at RT, placental villous fragments were incubated with 150 nmol/l FITC-albumin in Tyrodes at 4ºC and 37ºC. One villous fragment was incubated per 1.5 ml eppendorf in 500 μl of buffer. Experiments had a total duration of 60 min with samples taken at 5, 15, 30 and 60 min. Control fragments were also incubated with 1.43 μmol/l FITC-dextran at 4ºC and 37ºC for 60 min. FITC-dextran was used as a control as it has been shown not to be transported into human placenta during placental perfusion experiments (Balakrishnan *et al.*, 2010; Balakrishnan *et al.*, 2011). To investigate the role of endocytosis in uptake of FITC-albumin, placental villous fragments were pre-incubated with 5 mmol/l amiloride hydrochloride hydrate or 80 μmol/l dynasore in Tyrodes buffer. Villous fragments were then incubated with 150 nmol/l FITC-albumin plus the respective endocytic blocker at 37ºC for 5 and 15 min for amiloride and 60 min for dynasore. At each timepoint buffer was removed and villous fragments were fixed in 4% PFA and stored at 4ºC. The day after experimentation, placental fragments were transferred from PFA to 0.1% sodium azide and stored at 4ºC until lectin staining. Numbers for microscopy data are n = 5 for FITC-albumin uptake at 4ºC for 5, 15, 30 and 60 min, and at 37ºC for 5, 15 and 30 min. n = 6 for FITC-albumin uptake at 37ºC for 60 min. FITC-dextran data is n = 4 at both temperatures. FITC-albumin uptake with dynasore is n = 1. FITC-albumin uptake with amiloride is n = 3 at both time points.

To ensure the integrity of placental villous fragments for the longer duration RNA experiments, placental fragments were incubated at 37ºC for 8 h in Tyrodes buffer, before fixing in 4% PFA.

Experiments for RNA

For RNA studies, placental villous fragments were pre-incubated with either 80 μmol/l dynasore, 5 mmol/l amiloride hydrochloride hydrate, 100 μmol/l gentamicin, 1.4 mmol/l chlorpromazine hydrochloride or control Tyrodes for 30 min at 37ºC. Following pre-incubation, placental villous

fragments were then transferred to experimental buffers and incubated at 37ºC. Conditions were conducted with three placental fragments in a 2 ml eppendorf with 500 µl buffer. All experimental conditions for RNA are displayed in Table 5.2. The response to vitamin D was initially measured over a 12 h time period with samples taken at 2, 4, 6, 8 and 12 h (n = 2). This was carried out with 100 nmol/l 25(OH)D, 100 nmol/l 25(OH)D + 0.7 mmol/l BSA, 50 nmol/l $1,25(OH)_2D$, 50 nmol/l $1,25(OH)_2D$ + 0.7 mmol/l BSA, and ethanol + 0.7 mmol/l BSA (vehicle control). All other RNA experiments were carried out for 8 h, as at this time placental fragments were shown to respond adequately to vitamin D. By 12 h HKG expression showed a decline indicating that the function of the placental fragment may have been deteriorating at the later time point. At the end of the experiment, buffer was removed and villous fragments were snap frozen on dry ice and stored at -80ºC.

Table 5.2: Experimental conditions for 8 h RNA studies with placental villous fragments.

Vitamin D / solvent	Carrier protein / protein control	Endocytic blocker	n	Figure
100 nmol/l 25(OH)D	-	-	4	5.4a
100 nmol/l 25(OH)D	0.7 mmol/l BSA	-	4	5.4a
20 µmol/l 25(OH)D	-	-	6	5.6a, 5.10
20 µmol/l 25(OH)D	0.7 mmol/l BSA	-	6	5.6a, 5.10
20 µmol/l 25(OH)D	0.7 mmol/l BSA	5 mmol/l amiloride hydrochloride hydrate	1	5.10
50 nmol/l $1,25(OH)_2D$	-	-	11	5.4b, 5.6b, 5.11, 5.12
50 nmol/l $1,25(OH)_2D$	0.7 mmol/l BSA	-	11	5.4b, 5.6b, 5.12
50 nmol/l $1,25(OH)_2D$	0.7 mmol/l BSA	5 mmol/l amiloride hydrochloride hydrate	5	5.12
50 nmol/l $1,25(OH)_2D$	0.7 mmol/l BSA	100 µmol/l gentamicin	5	5.12
50 nmol/l $1,25(OH)_2D$	0.7 mmol/l BSA	80 µmol/l dynasore	5	5.12
50 nmol/l $1,25(OH)_2D$	0.7 mmol/l BSA	1.4 mmol/l chlorpromazine hydrochloride	5	
50 nmol/l $1,25(OH)_2D$	5 µmol/l DBP	-	6	5.6b, 5.11
50 nmol/l $1,25(OH)_2D$	5 µmol/l DBP	80 µmol/l dynasore	4	5.11
50 nmol/l $1,25(OH)_2D$	Gelatin (to match BSA)	-	5	5.6b
50 nmol/l $1,25(OH)_2D$	1 X amino acid mix	-	5	5.6b
Ethanol	-	-	6	5.6, 5.10
Ethanol	0.7 mmol/l BSA	-	11	5.4, 5.6, 5.10, 5.11, 5.12
Ethanol + DMSO	0.7 mmol/l BSA	-	5	5.11, 5.12
Ethanol	0.7 mmol/l BSA	5 mmol/l amiloride hydrochloride hydrate	6	5.10, 5.12
Ethanol	0.7 mmol/l BSA	100 µmol/l gentamicin	5	5.12
Ethanol	0.7 mmol/l BSA	80 µmol/l dynasore	5	5.11, 5.12
Ethanol	0.7 mmol/l BSA	1.4 mmol/l chlorpromazine hydrochloride	5	

Sample numbers for each condition are displayed. Chlorpromazine hydrochloride resulted in reduced RNA yield and poor quality RNA so these samples were not analysed further.

5.2.3. Lectin staining

Placental villous fragment samples were stained with lectins to highlight placental structures as described in General methods 2.4.3. Briefly, samples stored in 0.1% sodium azide were washed in PBS and permeabilised for 2 hr in 1% Triton X 100. This was followed by further PBS washes and overnight incubation in lectin stains (Table 5.3). Fragment samples that were used to investigate uptake of FITC labelled proteins were stained with biotin-DSL and rhodamine-PSA. Samples that were used to ensure the integrity of the villous fragments after 8 h of incubation were stained with FITC-AAL, biotin-DSL and rhodamine-PSA.

Table 5.3: Lectin stains used for placental villous fragment staining.

Lectin	Conjugated to	Dilution used	Placental structure stained
AAL	FITC	1:100	Capillaries
DSL	Biotin	1:200	MVM
PSA	Rhodamine	1:500	Villous stroma

The following day samples were washed in PBS and incubated with Streptavidin 680LT (1:500 in PBS) for 2 hrs at RT on a shaker, to allow visualisation of the biotinylated lectin. Villous fragments were washed in PBS and incubated with DAPI (2 µl 1 mg/ml DAPI in 1 ml PBS) for 15 min, for visualisation of the nuclei. Following PBS washes, villous fragments were then cleared through a series of TDE concentrations, before being stored in 97% TDE at 4ºC. Placental villous fragments were visualised on the SP5 fluorescent confocal microscope. Images were obtained through a series of z-sections through the placental fragment. 6 sections were taken for each image at 10 µm intervals beginning at the tip of the villi, and a minimum of 5 images were taken for each placental villous fragment. A minimum of 2 images were taken for each FITC-dextran placental fragment. For more detailed images, a greater number of z-sections were taken at smaller intervals to create z-stacks.

5.2.4. mRNA expression

RNA was extracted using the miRNeasy kit as described in General methods 2.5.2. RNA quantity and purity were assessed through NanoDrop™ measurements and agarose gel electrophoresis (section 2.5.3). 200 ng of RNA was reverse transcribed using the Precision nanoScript™2 or the Promega protocol as described in section 2.5.4. NECs were synthesised at the same time as cDNA.

qrt-PCR was carried out to determine expression levels of genes of interest as described in section 2.5.8 using Roche UPL and Primer Design Perfect Probe assays. Primers are displayed in Table 5.4. For each qrt-PCR assay, samples (4 ng) were run alongside a standard curve, CV controls, NECs and NTCs in triplicate (Table 2.6). UPL qrt-PCR cycling conditions were 95°C for 10 min; 45

cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 s; 50°C for 30 s, with data collection at the 72°C step. Perfect Probe HKG qrt-PCR cycling conditions were 95°C for 10 min; 50 cycles of 95°C for 10 s, 50°C for 30 s and 72°C for 15 s; 50°C for 30 s, with data collection at the 50°C step. Cp values were determined by the second derivative method and were converted to DNA concentration (ng/5 μl) using a standard curve (see General methods 2.5.8). Two HKGS, *TOP1* and *YWHAZ*, were measured in the samples. However, *TOP1* was shown to be altered with vitamin D treatment, therefore only *YHWAZ* was used for data normalisation.

mRNA expression of specific vitamin D-related and nutrient transport genes were measured. *CYP24A1* mRNA expression was measured as a proxy for the response to vitamin D, as mRNA expression of this gene increases in response to vitamin D. The mRNA expression of the amino acid transporters *LAT3* and *TAT1* were investigated as *LAT3* was associated with maternal 25(OH)D and DBP at 34 weeks gestation in SWS placentas (Chapter 3), while *TAT1* was associated with DBP. The expression of an amino acid transporter that did not associate with 25(OH)D in the SWS placentas, *ASCT2*, was also explored. In addition, expression of the calcium transporter, *PMCA1* was measured. This gene was not associated with maternal vitamin D or DBP in SWS placentas, but previous links between vitamin D and *PMCA1* mRNA expression have been shown (Glendenning et al., 2000, 2001; Walters et al., 2007; Balesaria et al., 2009). Expression of the vitamin D-related genes, *cubilin*, *megalin*, *legumain* and *CYP27B1* were also measured.

Table 5.4: Primers and probes used for qrt-PCR on placental villous fragments.

Gene	Accession number	Primer sequence (5'-3')	UPL number	Amplicon (bp)
Vitamin D genes				
Cubilin	NM_001081.3	F: GGACAATGTCAGAATAGCTTCGT R: CAGTGGCTAGCAGGGCTTT	10	8624 – 8696
CYP24A1	NM_001128915.1, NM_000782.4	F: GAAAGAATTGTATGCTGCTGTCA R: CACATTAGACTGTTTGCTGTCGT	78	1337 – 1408 1337 – 1408
CYP27B1	NM_000785.3	F: CTTGCGGACTGCTCACTG R: CGCAGACTACGTTGTTCAGG	63	520 – 642
Legumain	NM_001008530.2, NM_005606.6	F: GGAAACTGATGAACACCAATGA R: GGAGACGATCTTACGCACTGA	25	1311 – 1423 1193 – 1305
Megalin	NM_004525.2	F: TTGTTTTGATGCCTCTGATGA R: AGCTAGGCATGTTCGCTCAG	34	8877 – 8937
Amino acid transporter genes				
ASCT2	NM_001145144.1, NM_001145145.1, NM_005628.2	F: GAGGAATATCACCGGAACCA R: AGGATGTTCATCCCCTCCA	43	114 – 179 304 – 369 1250 – 1315
LAT3	NM_001198810.1, NM_003627.5	F: GCCCTCATGATTGGCTCTTA R: CCGGCATCGTAGATCAGC	29	623 – 642 794 – 864
TAT1	NM_018593.4	F: GGTGTGAAGAAGGTTTATCTACAGG R: AGGGCCCCAAAGATGCTA	6	1238 – 1329
Calcium transporter genes				
PMCA1	NM_001001323.1, NM_001682.2	F: CCATAGTATCATTGGGCCTTTC R: CTTCTTCCTCCCCAACAGAA	75	534 – 620 534 – 620

F = forward primer, R = reverse primer.

RNA sequencing was performed on placental villous fragments exposed to 20 μmol/l 25(OH)D, 20 μmol/l 25(OH)D + 0.7 mmol/l BSA, and ethanol + 0.7 mmol/l BSA control (n = 4 for each condition). 450 ng of total RNA was prepared at a dilution of 30 ng/μl and paired-end RNA sequencing was carried out by Expression Analysis Inc (Durham, North Carolina, USA) as described in section 2.5.9. Genes were identified from the sequencing information by aligning raw sequencing data against the human transcriptome.

5.2.5. Data analysis

All data were analysed in SPSS and graphs were created in GraphPad Prism 6. Unless otherwise stated, data are presented as mean + SEM. $p < 0.05$ was deemed statistically significant.

Analysis of FITC-protein uptake

The average FITC-protein uptake for each placental fragment was calculated using ImageJ as described in Section 2.8.3. Data were then tested for normal distribution and log transformed or square rooted if non-normally distributed. Equal variance was established using the Levene statistic and p values were selected from outputs accordingly. A two-way ANOVA was used to explore the effects of temperature and time on FITC-albumin uptake. T-tests were used to investigate differences in FITC-albumin uptake compared to FITC-dextran, and to examine the effects of endocytic blockers on FITC-albumin uptake.

Analysis of mRNA expression measured by qrt-PCR

Target gene expression was determined through the use of the standard curve as described in section 2.5.8, and then normalised to HKG expression. The standard curve for *ASCT2* failed so mRNA expression levels were calculated using $2^{-\Delta\Delta cp}$ as described in section 2.5.8. Data were normalised to *YHWAZ* expression, and are expressed as mean fold change relative to ethanol + albumin control + SEM. Data were tested for normal distribution and log transformed or square rooted if non-normally distributed. Equal variance within the data sets was analysed using the Levene statistic. A two-way ANOVA was used to investigate the effect of time and vitamin D treatment on *CYP24A1* fold change in relative mRNA expression with a Least Significant Difference test for pairwise comparisons. All other qrt-PCR data were analysed with one-way ANOVA and Tukey's post hoc test to identify which groups differed significantly. Where the Levene statistic showed unequal variance of data, the Games-Howell post hoc test was used. For data that was still not normally distributed following transformation, a Kruskal Wallis test was used to compare groups.

Analysis of mRNA expression measured by RNA sequencing

RNA sequencing data were analysed by three two-way comparisons using t-tests. 20 μmol/l 25(OH)D was compared to ethanol + albumin control, 20 μmol/l 25(OH)D + 0.7 mmol/l BSA was compared to ethanol + albumin control and 20 μmol/l 25(OH)D + 0.7 mmol/l BSA was compared to 20 μmol/l 25(OH)D. Data are presented as fold change relative to control. For the 20 μmol/l 25(OH)D + 0.7 mmol/l BSA compared to 20 μmol/l 25(OH)D analysis data are presented as fold change relative to 20 μmol/l 25(OH)D. A fold change of 2 was set for the threshold above which a gene was classed as up-regulated, while a threshold of 0.5 was set as the fold change for down-regulation of gene expression. RNA sequencing data were specifically searched for genes that have been focussed on in this thesis. Vitamin D-related genes searched for were *cubilin*, *CYP2J2*, *CYP24A1*, *CYP27A1*, *CYP27B1*, *legumain*, *megalin*, *RXRa* and *VDR*. Amino acid transporter genes searched for were *4F2hc*, *ASCT1*, *ASCT2*, *EAAT2*, *EAAT3*, *LAT1*, *LAT2*, *LAT3*, *LAT4*, *SNAT1*, *SNAT2*, *SNAT4*, *TAT1*, *xCT*, *y⁺LAT1* and *y⁺LAT2*. Calcium transport genes searched for were *calbindin D-9K*, *calbindin D-28K*, *CaT1*, *CaT2*, *NCX1*, *NCX2*, *NCX3*, *PMCA1* and *PMCA4*. Other genes searched for were *DNMT1*, *DNMT3b*, *LPL*, *PTH1R*, *PTHrP* and *TXNIP*.

5.3. Results

5.3.1. Vitamin D and albumin uptake into placental villous fragments

The effect of albumin on vitamin D uptake into placental villous fragments was investigated. Initially, a timecourse was carried out over a period of 12 h and uptake of vitamin D was assessed through mRNA expression of the vitamin D responsive gene, *CYP24A1*. 25(OH)D and 1,25$(OH)_2$D were investigated in the presence and absence of albumin, and controls were incubated with ethanol and albumin. This revealed a significant effect of 25(OH)D with albumin (Figure 5.4a) as well as 1,25$(OH)_2$D and 1,25$(OH)_2$D with albumin on *CYP24A1* mRNA expression (Figure 5.4b). The presence of albumin resulted in a further increase in mRNA expression compared to 1,25$(OH)_2$D alone (Figure 5.4b).

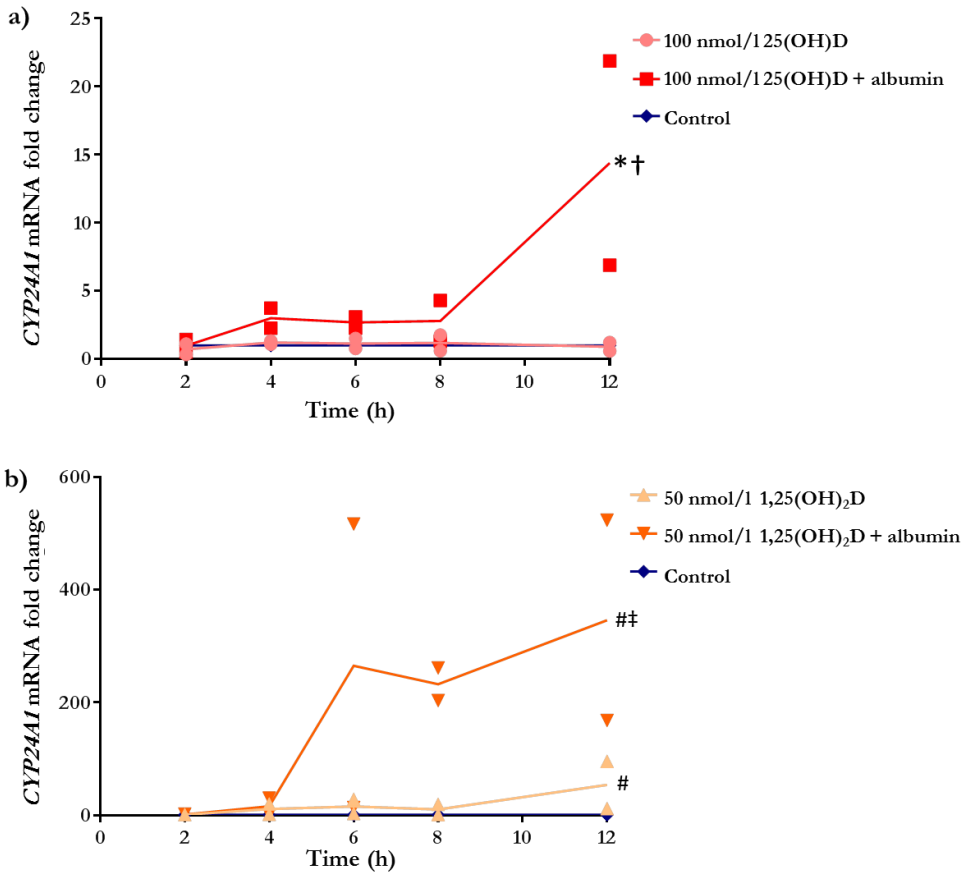

Figure 5.4: Timecourse of *CYP24A1* relative mRNA fold change in response to 100 nmol/l 25(OH)D and 50 nmol/l 1,25(OH)2D alone and in the presence of albumin. a) *CYP24A1* mRNA fold change in response to 25(OH)D. b) *CYP24A1* fold change in response to 1,25$(OH)_2$D. Two-way ANOVA revealed a significant effect of both time and vitamin D treatment on *CYP24A1* mRNA expression ($p < 0.001$). Post hoc analysis revealed a significantly higher fold change in mRNA expression in placental fragments exposed to 100 nmol/l 25(OH)D + albumin compared to control (* $p < 0.05$) and 25(OH)D († $p < 0.05$) treated fragments. There was also a significantly higher fold change in mRNA expression in placental fragments exposed to 50 nmol/l 1,25$(OH)_2$D and 1,25$(OH)_2$D with albumin compared to control (# $p < 0.001$), and in fragments treated with 1,25$(OH)_2$D with albumin compared to 1,25$(OH)_2$D alone (‡ $p < 0.001$). All conditions n = 2. Data are plotted as single data points, with lines showing average of the two points.

The effect of albumin on vitamin D uptake into the placenta was studied further with experiments taking place over an 8 h period. The 8 h time point was decided upon as this was enough time to induce changes to mRNA expression, as supported by Figure 5.4. Furthermore, after 12 h HKG expression was slightly lower in all placental fragments, suggesting function may have been in decline at this later time. The morphology of placental villous fragments following 8 h in control buffer was studied using fluorescent lectin stains, to ensure the gross morphology of the fragment was unaffected by this time in culture (Figure 5.5). As seen below, there were no obvious signs of deterioration or morphological changes within the placental villous fragments following 8 h in culture.

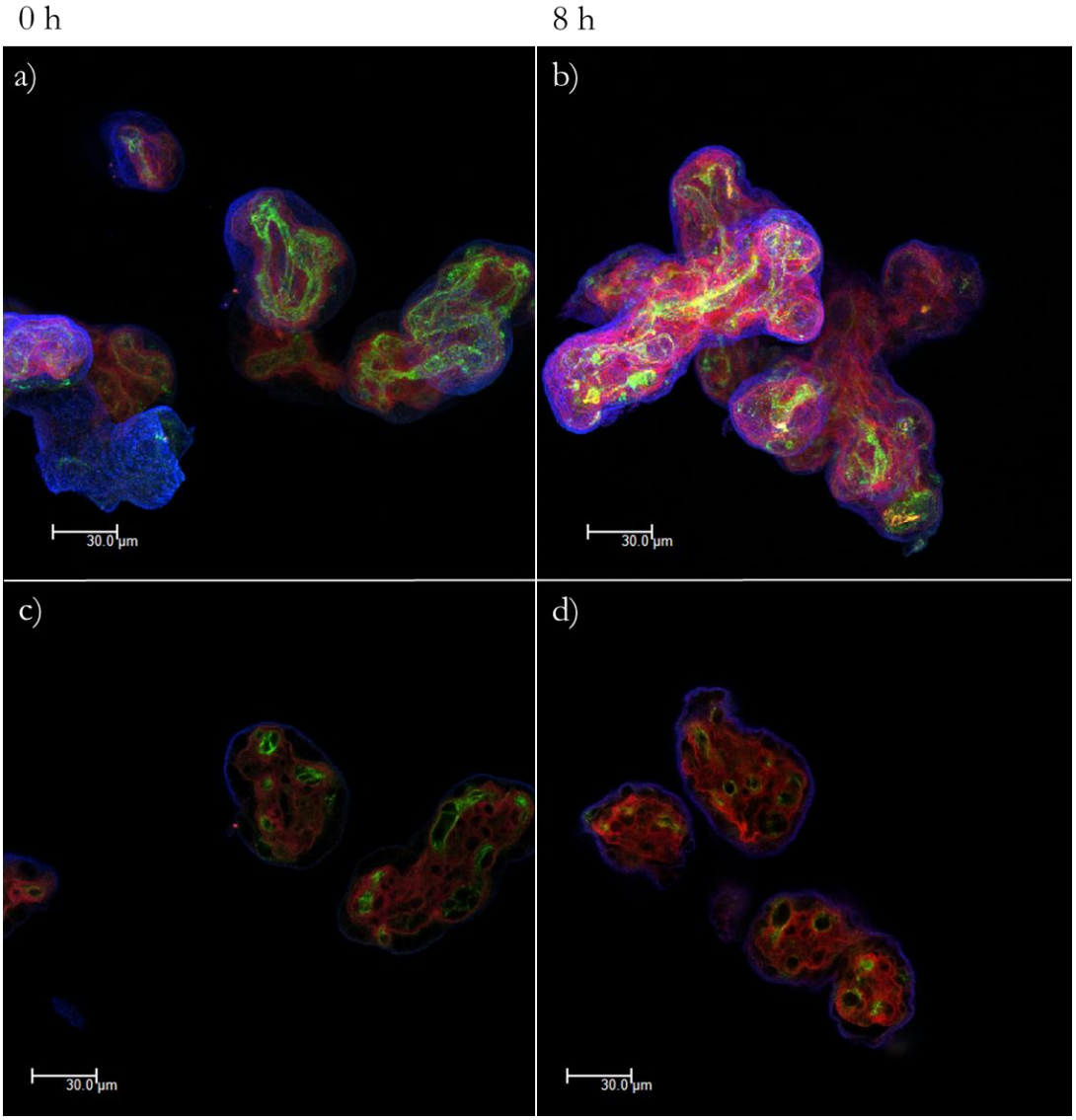

Figure 5.5: Placental villous fragment gross morphology was not affected by 8 h in culture. a) Maximum projection of a placental villous fragment fixed at 0 h. b) Maximum projection of a placental villous fragment fixed following 8 h incubation. c) A slice through a placental fragment fixed at 0 h. d) A slice through a placental fragment fixed after 8 h. Red indicates the villous stroma stained by rhodamine-PSA, blue indicates the MVM stained by biotin-DSL, green indicates FITC-AAL staining of capillaries.

The uptake of 25(OH)D and 1,25$(OH)_2$D was investigated further in the placental villous fragments. The concentration of 25(OH)D was increased to 20 μmol/l to investigate whether there was an effect of inactive vitamin D at higher doses. This showed that 25(OH)D alone caused an increase in *CYP24A1* mRNA expression, however the presence of albumin resulted in a much larger increase in mRNA expression, with an average 49 fold change (Figure 5.6a). A more detailed investigation was carried out into the uptake of 1,25$(OH)_2$D. Alongside the effects of albumin, the effect of DBP was also investigated, as were the effects of some additional controls; a gelatin control, to investigate the effect of the presence of a non-vitamin D binding protein, and an amino acid mix corresponding to the levels in the maternal circulation. *CYP24A1* showed increased mRNA expression in response to 1,25$(OH)_2$D alone, with albumin and with DBP. However there was no further significant increase with the presence of albumin or DBP (Figure 5.6b).

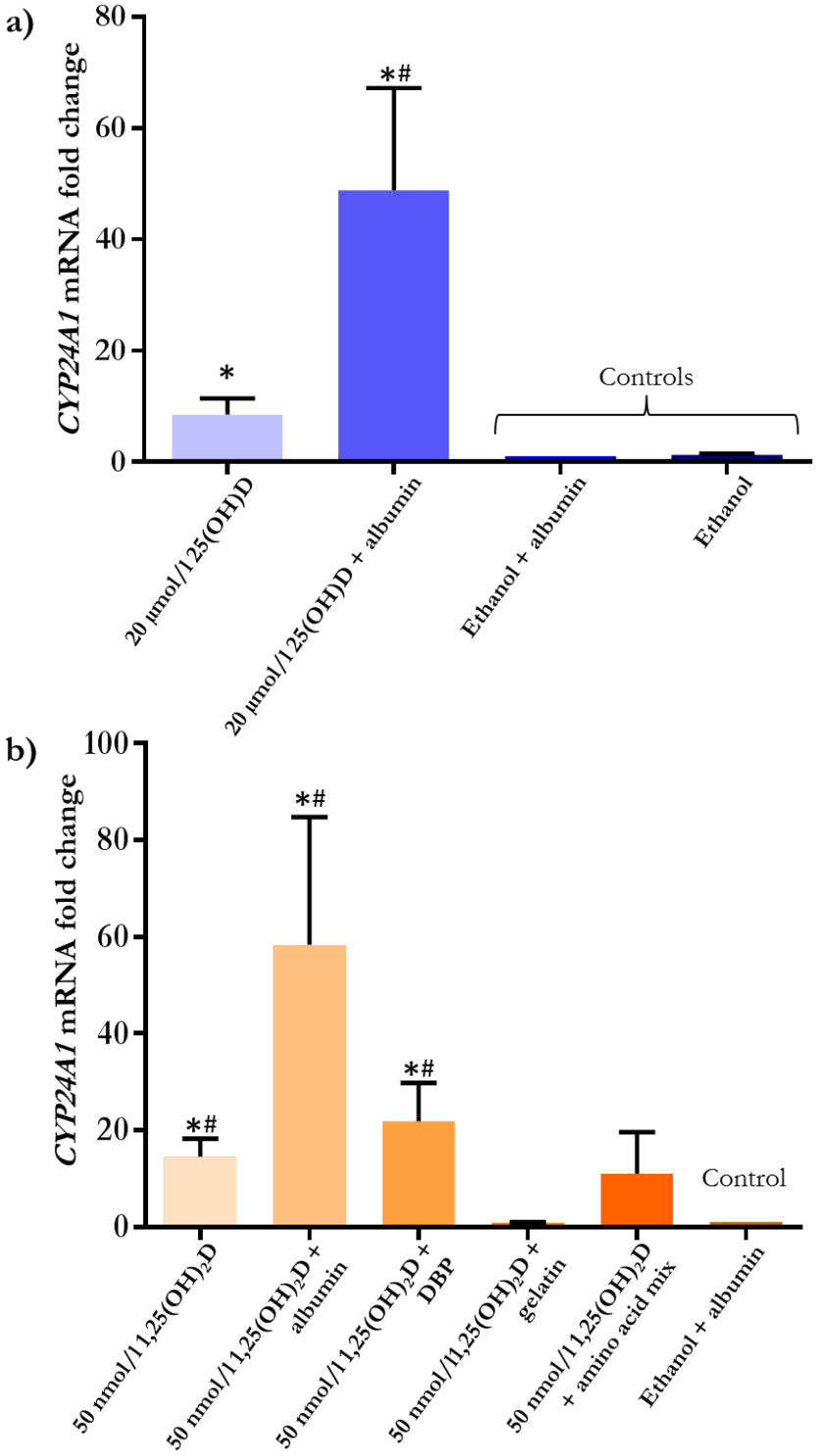

Figure 5.6: The effect of albumin and/or DBP with 25(OH)D and 1,25(OH)$_2$D on relative mRNA expression of *CYP24A1* in placental villous fragments. a) *CYP24A1* relative mRNA expression increased with 25(OH)D + albumin compared to controls (ethanol + albumin, and ethanol alone) * $p < 0.05$, and compared to 25(OH)D alone # $p < 0.01$. b) *CYP24A1* relative mRNA expression was not significantly increased further by the presence of albumin or DBP. *CYP24A1* expression increased with 1,25(OH)$_2$D ($p < 0.01$), 1,25(OH)$_2$D + albumin ($p < 0.001$) and 1,25(OH)$_2$D + DBP ($p < 0.01$) compared to 1,25(OH)$_2$D + gelatin* and ethanol + albumin#. Data are presented as mean fold change relative to ethanol + albumin control + SEM. Samples numbers are displayed in Table 5.2.

As albumin may facilitate entry of 25(OH)D into the placenta. FITC-albumin uptake into placental villous fragments was measured over a time period of 60 min at 4ºC and 37ºC (Figure 5.7). This revealed a significant increase in FITC-albumin uptake at the higher temperature, indicative of the involvement of a specific mechanism of uptake. The effect of time on uptake was not significant, although there was a trend for increased uptake with time. Some placental fragments were classed as non-responders, as they showed a consistently low FITC-albumin uptake at 37ºC. For purity of data these were included in the analysis, but when analysed without the non-responders, the effect of time became significant ($p = 0.02$), and the effect of temperature became even more significant ($p < 0.001$). As a control measure, FITC-dextran uptake was also studied (Figure 5.7b), the uptake of this protein, which has a similar molecular weight to albumin, was negligible at both temperatures. Representative images of FITC-albumin uptake at 5, 15, 30 and 60 min and of FITC-dextran are shown in Figure 5.8.

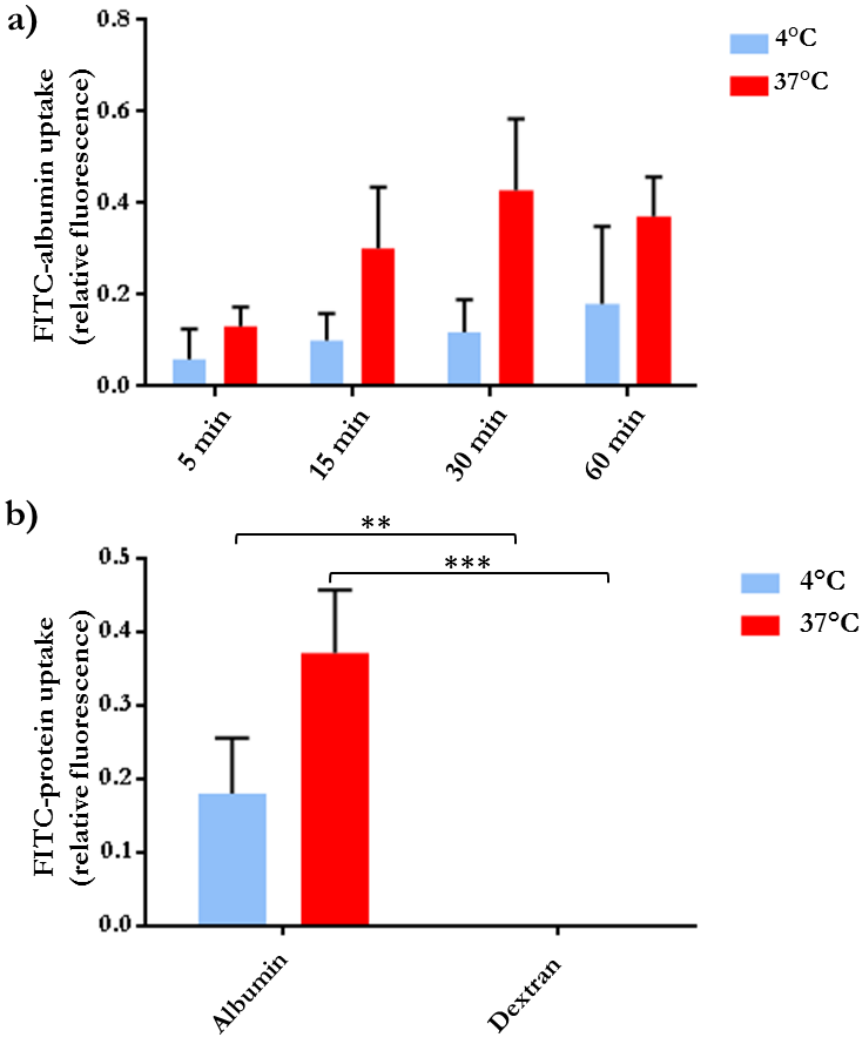

Figure 5.7: FITC-albumin and FITC-dextran uptake in placental villous fragments. a) Timecourse of FITC-albumin uptake at 4°C and 37°C. Two-way ANOVA revealed a significant effect of temperature on FITC-albumin uptake $p = 0.004$, and a trend for an effect of time $p = 0.08$. $n = 5$ at each point, except 60 min at 37°C which has $n = 6$. b) FITC-dextran uptake was not observed in placental villous fragments. Significantly higher levels of FITC-albumin compared to FITC-dextran uptake were observed at 60 min at both temperatures investigated. $n = 5$ FITC-albumin at 4°C, $n = 6$ FITC-albumin at 37°C, and $n = 4$ FITC-dextran at both temperatures. Data are presented as mean FITC-protein uptake + SEM. ** $p < 0.01$, *** $p < 0.001$.

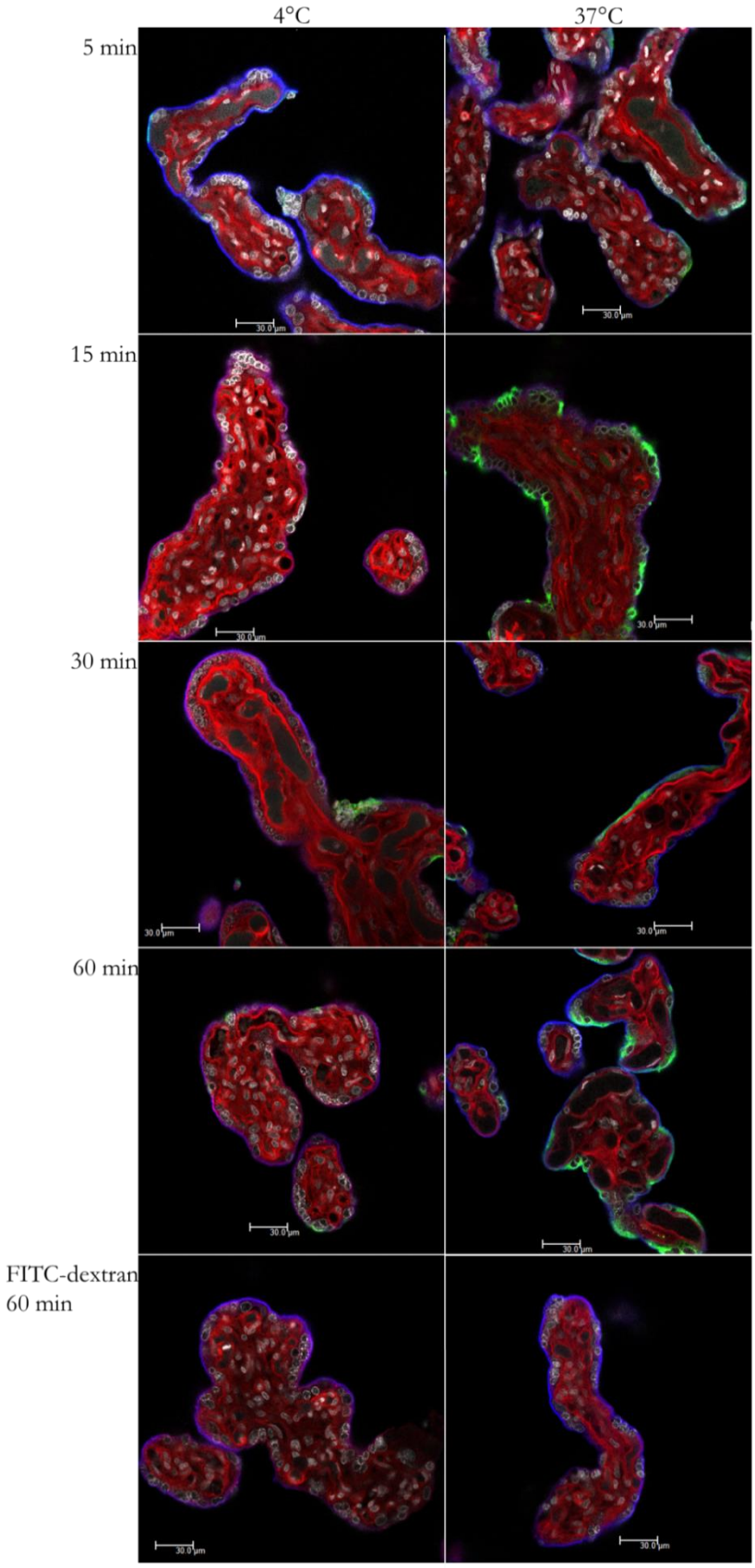

Figure 5.8: Representative images of FITC-albumin and FITC-dextran uptake over a 60 min timecourse at 4°C and 37°C. Confocal fluorescence microscopy images of FITC-albumin and FITC-dextran. Green indicates FITC-protein, red indicates the villous stroma stained by rhodamine-PSA, blue indicates the MVM stained by biotin-DSL, and grey indicates DAPI stained nuclei.

Further images of FITC-albumin uptake into the placental villous fragments are displayed in Figure 5.9. The top panel shows a lower magnification image, which highlights the uneven distribution of FITC-albumin uptake into the villous fragments. The bottom panel shows a higher magnification image, and this shows FITC-albumin in punctate dots within the stroma, which could correspond to vesicular uptake.

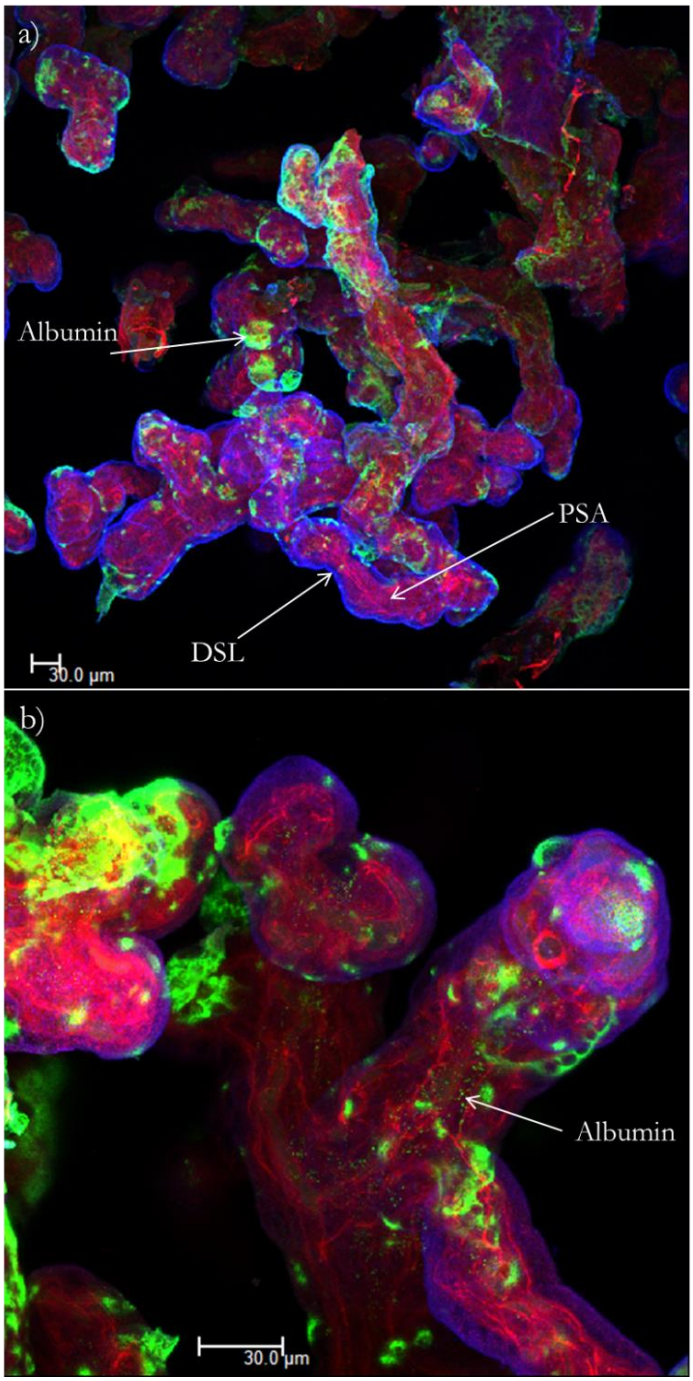

Figure 5.9: Low and high magnification maximum projections of FITC-albumin uptake in placental villous fragments. a) Maximum projection of FITC-albumin uptake at x 20 magnification. The projection was created from 78 slices at a distance of 1.5 µm. b) Maximum projection of FITC-albumin uptake at x 64 magnification. The projection was created from 105 slices at a distance of 1.5 µm. Green indicates FITC-albumin, red indicates the villous stroma stained by rhodamine-PSA and blue indicates the MVM stained by biotin-DSL. DAPI stained nuclei were omitted from the projection for clarity.

5.3.2. Investigation of endocytic uptake mechanism

The effect of endocytic blockers on vitamin D uptake was analysed through the expression of *CYP24A1*. The impact of amiloride on the uptake of 25(OH)D was investigated. Unfortunately, statistical analysis could not be conducted on these data due to low sample numbers, but a marked decrease in the induction of *CYP24A1* mRNA expression was observed when placental villous fragments were cultured with 5 mmol/l amiloride alongside 25(OH)D and albumin (Figure 5.10).

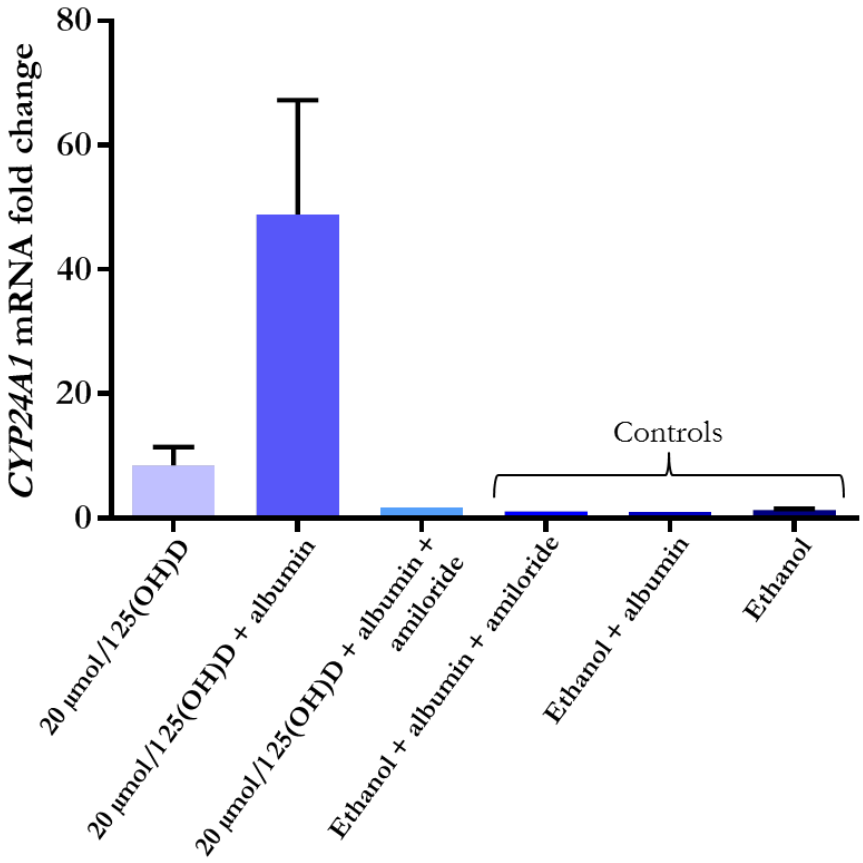

Figure 5.10: The effect of amiloride and 25(OH)D on relative mRNA expression of *CYP24A1*. Amiloride resulted in decreased *CYP24A1* relative mRNA expression, however due to low numbers statistical analysis could not be carried out. Sample numbers are displayed in Table 5.2. Data are presented as mean fold change relative to ethanol + albumin control + SEM.

The uptake of $1,25(OH)_2D$ with DBP and albumin was also investigated alongside endocytic blockers. We attempted to inhibit DBP-mediated uptake with dynasore. The response to dynasore was variable, and overall showed no significant effect on DBP-mediated uptake (Figure 5.11). Albumin-mediated uptake of $1,25(OH)_2D$ was investigated using a wider range of endocytic blockers; amiloride, chlorpromazine hydrochloride, dynasore and gentamicin. Chlorpromazine hydrochloride resulted in a substantially reduced RNA yield and poor RNA quality when samples were run on agarose gels, so samples were not analysed further. The use of gentamicin, a competitive inhibitor for megalin, did not significantly reduce *CYP24A1* mRNA expression. As seen with DBP, results on albumin-mediated

uptake with dynasore were varied and did not differ significantly from controls or 1,25(OH)$_2$D-treated villous fragments. Treatment with amiloride, resulted in a significant reduction in *CYP24A1* mRNA expression, compared to 1,25(OH)$_2$D alone and 1,25(OH)$_2$D with albumin, indicating a possible reduction in 1,25(OH)$_2$D uptake (Figure 5.12).

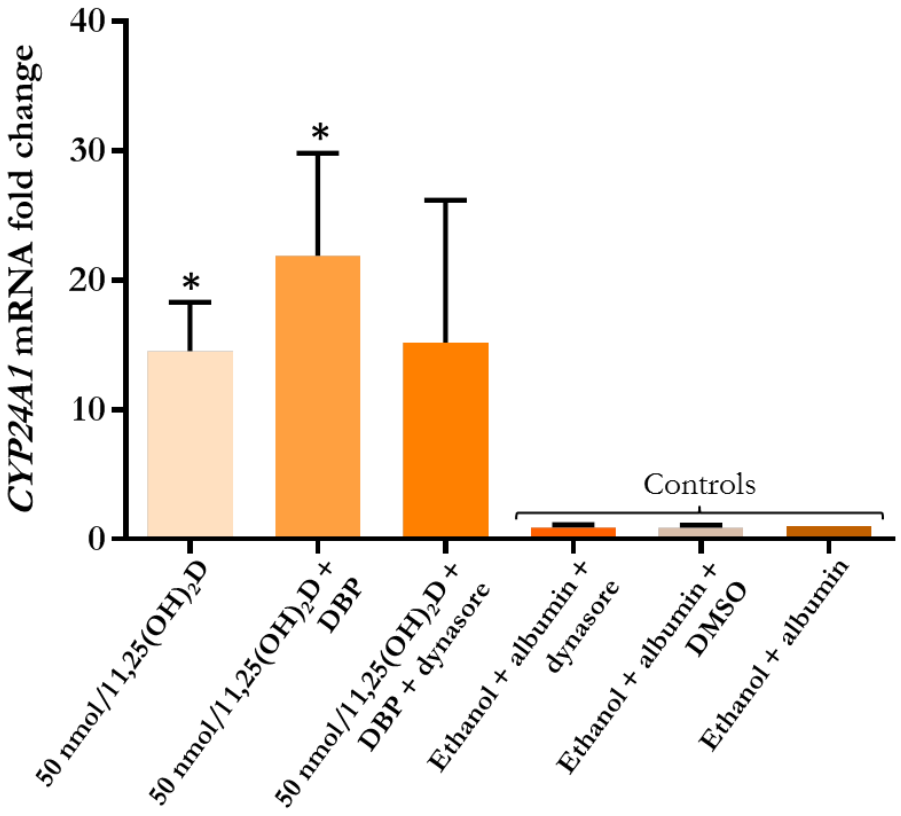

Figure 5.11: The effect of dynasore and 1,25(OH)$_2$D on relative mRNA expression of *CYP24A1* in placental villous fragments. 80 μmol/l dynasore did not significantly affect *CYP24A1* relative mRNA expression. *CYP24A1* mRNA expression was significantly increased by 50 nmol/l 1,25(OH)$_2$D and 50 nmol/l 1,25(OH)$_2$D + DBP (* $p < 0.01$) compared to controls; ethanol + albumin + dynasore, ethanol + albumin + DMSO and ethanol + albumin ($p = 0.001$ for 50 nmol/l 1,25(OH)$_2$D vs ethanol + albumin + DMSO). Sample numbers are displayed in Table 5.2. Data are presented as mean fold change relative to ethanol + albumin control + SEM.

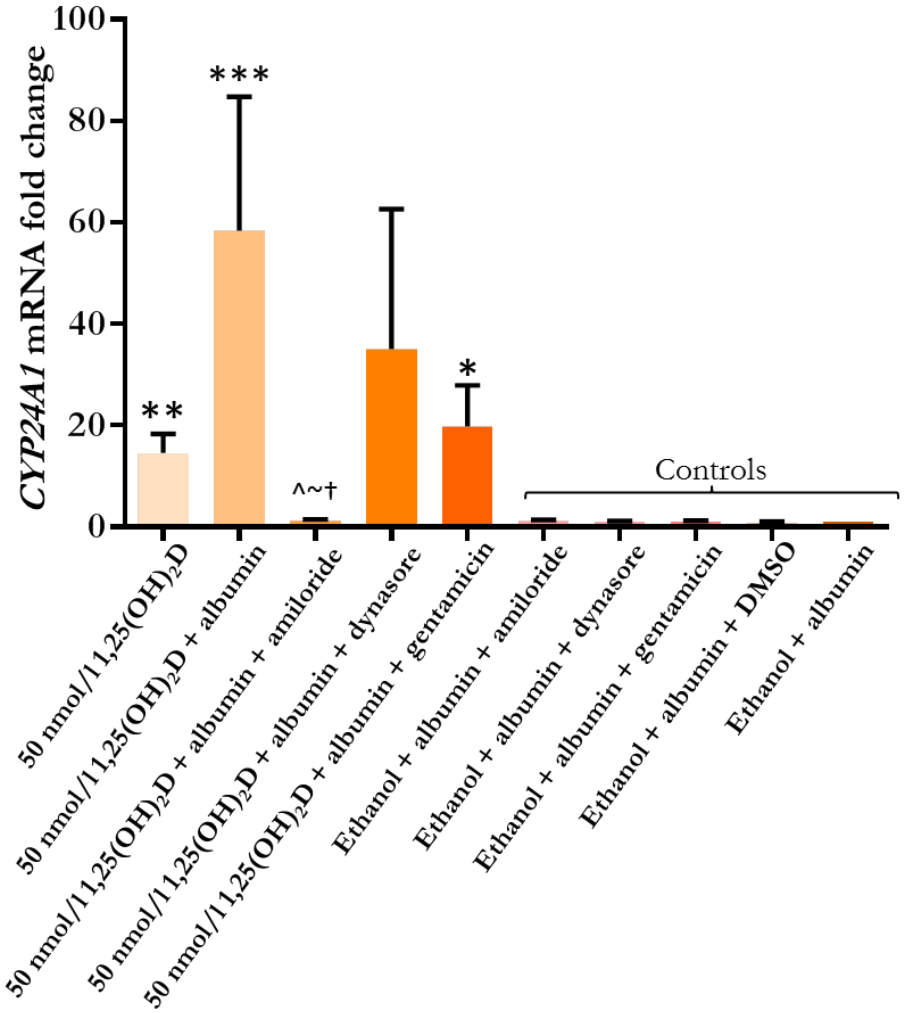

Figure 5.12: The effect of endocytic blockers and 1,25(OH)$_2$D on relative mRNA expression of *CYP24A1* in placental villous fragments. 5 mmol/l amiloride significantly reduced relative *CYP24A1* mRNA expression. *CYP24A1* relative mRNA expression was increased compared to controls with 1,25(OH)$_2$D ** $p < 0.01$, with 1,25(OH)$_2$D + albumin *** $p < 0.001$ and 1,25(OH)$_2$D + albumin + gentamicin * $p < 0.05$. The 1,25(OH)$_2$D-mediated up-regulation of *CYP24A1* mRNA expression was significantly reduced with addition of amiloride compared to 1,25(OH)$_2$D ^ $p < 0.01$, 1,25(OH)$_2$D + albumin ~ $p < 0.001$, and 1,25(OH)$_2$D + albumin + gentamicin † $p < 0.05$. Exposure to gentamicin had no effect on 1,25(OH)$_2$D-mediated up-regulation of *CYP24A1* expression. Exposure to dynasore resulted in highly variable results, which did not differ from controls or fragments treated with vitamin D. Sample numbers are displayed in Table 5.2. Data are presented as mean fold change relative to ethanol + albumin control + SEM.

The mechanism of vitamin D uptake was investigated further using confocal microscopy. The uptake of FITC-albumin was explored in the presence of dynasore, a blocker of dynamin-dependent endocytosis, and amiloride, a pinocytosis inhibitor. Exposure to 80 μmol/l dynasore did not appear to inhibit uptake of FITC-albumin. There may be an increase in FITC-albumin uptake in response to dynasore (Figure 5.13), however, due to low sample numbers statistical analysis could not be conducted.

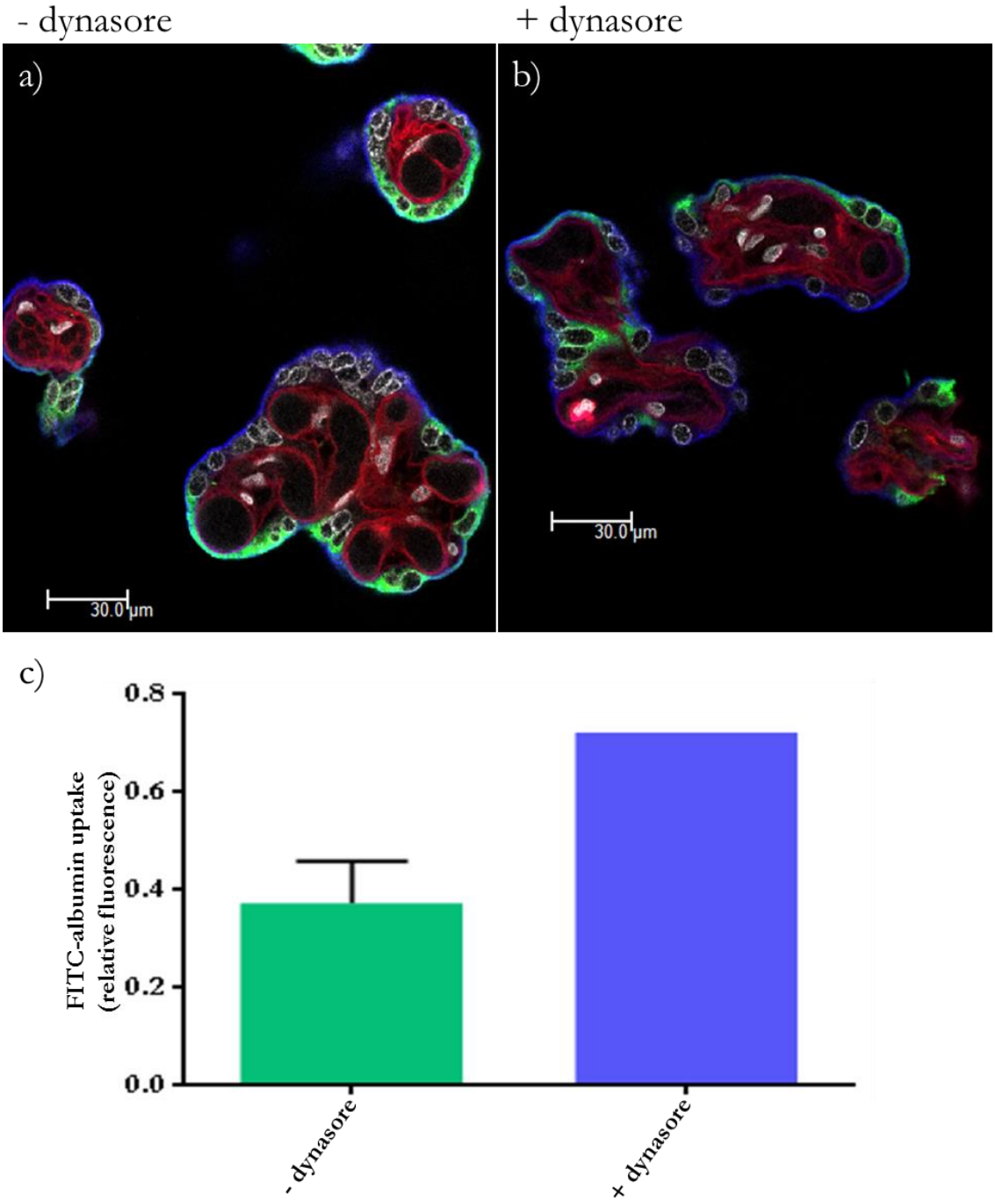

Figure 5.13: Effect of dynasore, a blocker of dynamin-mediated endocytosis, on FITC-albumin uptake in placental villous fragments. a) Representative image of FITC-albumin uptake in control buffer. b) Representative image of FITC-albumin uptake in the presence of 80 µmol/l dynasore. Green indicates FITC-albumin, red indicates the villous stroma stained by rhodamine-PSA, blue indicates the MVM stained by biotin-DSL and grey indicates DAPI stained nuclei. c) FITC-albumin uptake did not appear to be affected by treatment with dynasore. There may be a slight increase with dynasore treatment but due to the low numbers statistical analysis could not be carried out on these data. n = 6 without dynasore, n = 1 with dynasore. Data are presented as mean + SEM.

Exposure of placental villous fragments to amiloride resulted in a significant down-regulation in FITC-albumin uptake following 5 min incubation with FITC-albumin (Figure 5.14). The effect at 15 min was not statistically significant ($p = 0.12$). However, when non-responders were removed the effect at 15 min also became significant ($p = 0.01$; Figure 5.14).

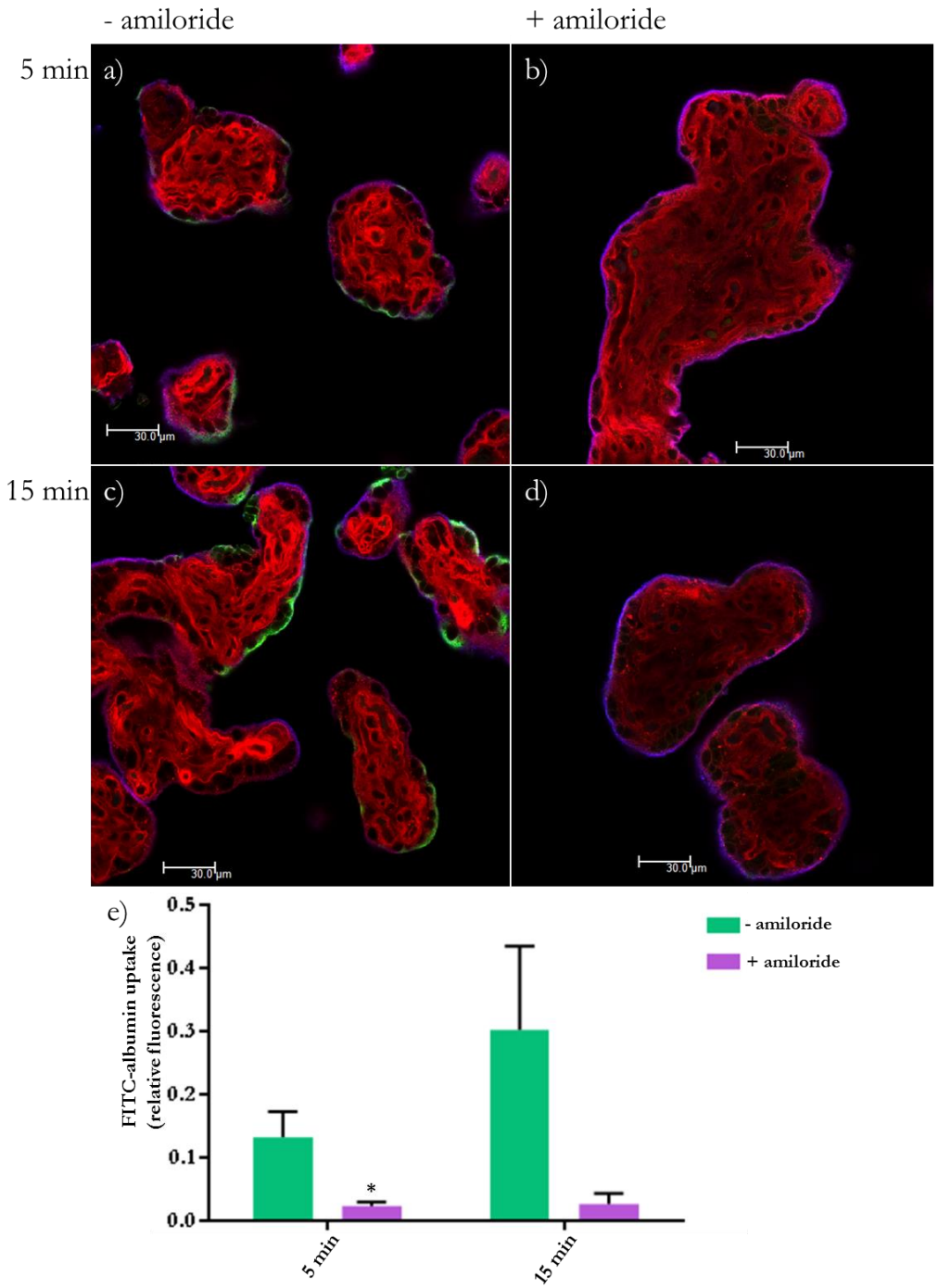

Figure 5.14: Effect of amiloride, an inhibitor of pinocytosis, on FITC-albumin uptake in placental villous fragments. a-d) Representative images of FITC-albumin uptake in placental fragments at 5 and 15 min with and without 5 mmol/l amiloride. Green indicates FITC-albumin, red indicates the villous stroma stained by rhodamine-PSA and blue indicates the MVM stained by biotin-DSL. e) FITC-albumin uptake was reduced with exposure to amiloride. At 5 min, FITC-albumin uptake was significantly reduced upon exposure to amiloride. At 15 min, there was no significant effect of amiloride exposure. n = 5 and 3 for FITC-albumin without and with amiloride, respectively. Data are presented as mean + SEM. * $p < 0.05$.

5.3.3. Effect of vitamin D and albumin or DBP on nutrient transporter expression in placental villous fragments

The effect of vitamin D on the expression of a small number of placental nutrient transporters was investigated. The impact of 1,25(OH)$_2$D alone and in combination with albumin or DBP on expression of vitamin D-related genes was explored. *Cubilin* showed a trend ($p = 0.07$) for reduced mRNA expression in response to 1,25(OH)$_2$D with DBP, in comparison to the ethanol + albumin control (Figure 5.15a). However, no changes in mRNA expression in response to 1,25(OH)$_2$D with or without carrier protein, were observed for *CYP27B1*, *legumain* and *megalin* (Figure 5.15b-d).

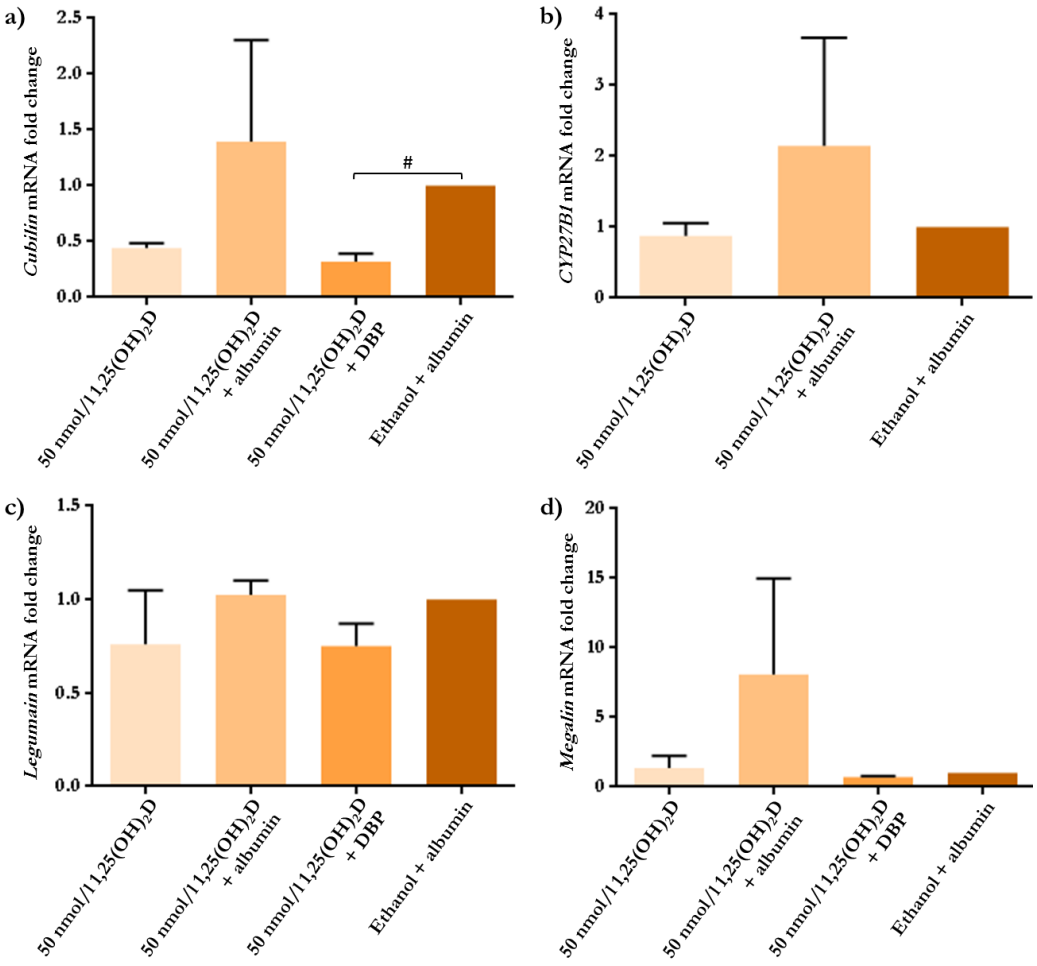

Figure 5.15: The effect of 1,25(OH)$_2$D exposure in the presence and absence of carrier proteins on relative mRNA expression of genes involved in vitamin D function in placental villous fragments. a) *Cubilin* showed a trend for reduced expression when placental fragments were cultured with 1,25(OH)$_2$D + DBP. n = 4 for 50 nmol/l 1,25(OH)$_2$D, 50 nmol/l 1,25(OH)$_2$D + albumin and ethanol + albumin. n = 2 for 1,25(OH)$_2$D + DBP. b) *CYP27B1* expression was not affected by culture with 1,25(OH)$_2$D alone or with DBP or albumin. n = 2 for all conditions. c) *Legumain* was unaffected by 1,25(OH)$_2$D alone or with DBP or albumin. n = 2 for all conditions. d) mRNA expression of *megalin* showed no significant changes in response to 1,25(OH)$_2$D or 1,25(OH)$_2$D with binding proteins. n = 4 for 50 nmol/l 1,25(OH)$_2$D, 50 nmol/l 1,25(OH)$_2$D + albumin and ethanol + albumin. n = 2 for 1,25(OH)$_2$D + DBP. Data are presented as mean fold change relative to ethanol + albumin control + SEM. $^{\#}$ $p < 0.1$.

Expression of placental amino acid and calcium transporters was also explored in response to both 25(OH)D and 1,25(OH)$_2$D. *LAT3* mRNA expression showed increased expression with 20 µmol/l 25(OH)D compared to 20 µmol/l 25(OH)D + albumin (trend, p = 0.07) and ethanol + albumin (Figure 5.16a). However, results did not differ significantly from the ethanol control, so it cannot be ruled out that effects are due to the ethanol used to dissolve the 25(OH)D in, rather than the 25(OH)D itself. *TAT1* mRNA expression showed a trend (p = 0.06) for altered expression, as only a trend was observed post-hoc tests could not be conducted to identify where the trend occurred, however, the results show a similar pattern to *LAT3* results with reduced expression in both conditions containing albumin (Figure 5.16b). *PMCA1* mRNA expression did not differ upon exposure to 20 µmol/l 25(OH)D alone or with albumin (Figure 5.16c).

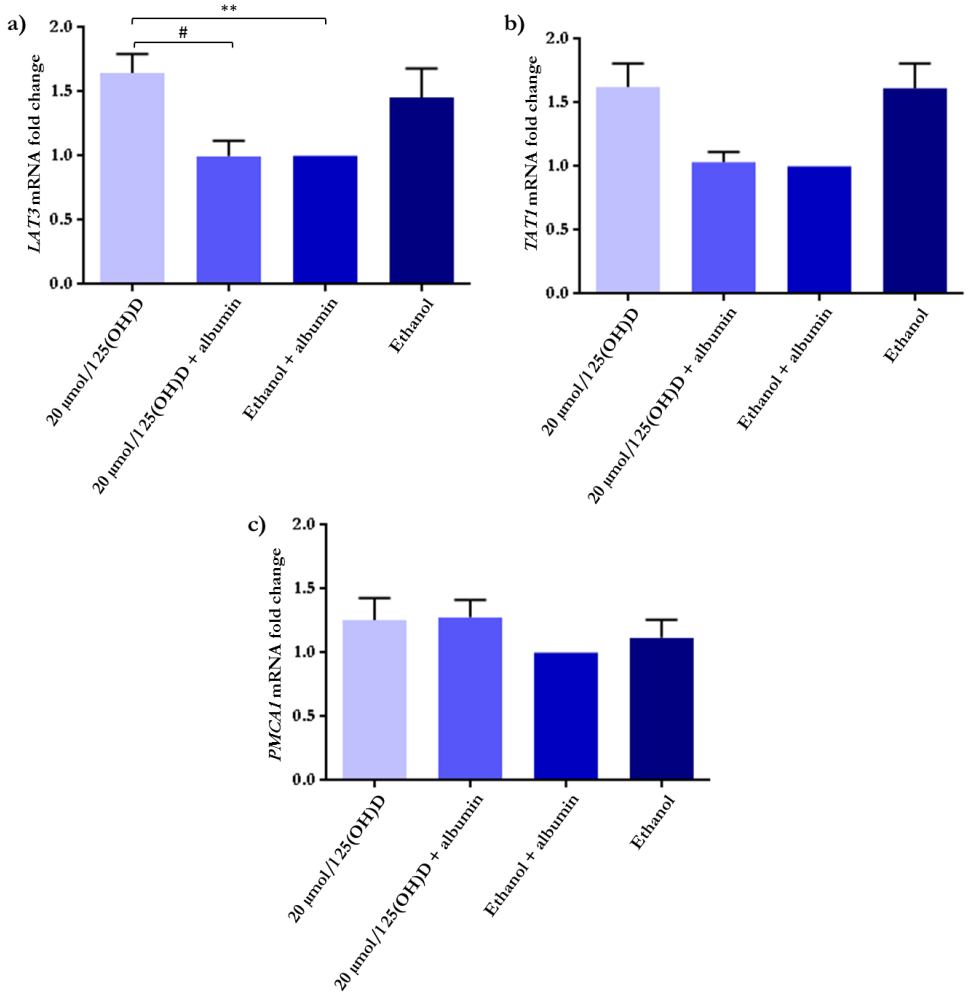

Figure 5.16: The effect of 25(OH)D exposure in the presence and absence of albumin on relative mRNA expression of amino acid and calcium transport genes in placental villous fragments. a) *LAT3* mRNA expression was significantly increased by 20 µmol/l 25(OH)D in comparison to ethanol + albumin, and 20 µmol/l 25(OH)D + albumin (trend). n = 5 for 20 µmol/l 25(OH)D, 20 µmol/l 25(OH)D + albumin and ethanol. n = 11 for ethanol + albumin. b) *TAT1* mRNA expression showed a trend for altered expression. n = 5 for 20 µmol/l 25(OH)D, 20 µmol/l 25(OH)D + albumin and ethanol. n = 8 for ethanol + albumin. c) *PMCA1* mRNA expression was unaffected by exposure to 20 µmol/l 25(OH)D and/or albumin. n = 5 for 20 µmol/l 25(OH)D, 20 µmol/l 25(OH)D + albumin and ethanol. n = 10 for ethanol + albumin. Data are presented as mean fold change relative to ethanol + albumin control + SEM. $^{\#}$ $p < 0.1$, ** $p < 0.01$.

Expression of the amino acid and calcium transporter genes was also investigated in response to $1,25(OH)_2D$. *ASCT2* and *LAT3* showed no response to $1,25(OH)_2D$ alone or with albumin or DBP (Figure 5.17a-b). *TAT1* mRNA expression showed significant differences in expression with $1,25(OH)_2D$, albumin and DBP. Specifically, 50 nmol/l $1,25(OH)_2D$ resulted in increased *TAT1* expression in comparison to ethanol + albumin and $1,25(OH)_2D$ + albumin. While expression was significantly higher with $1,25(OH)_2D$ + DBP compared to $1,25(OH)_2D$ + albumin (Figure 5.17c). *PMCA1* mRNA levels also showed changes in response to $1,25(OH)_2D$ + albumin; mRNA expression was increased with $1,25(OH)_2D$ + albumin compared to $1,25(OH)_2D$ alone and $1,25(OH)_2D$ + DBP. However, levels did not differ significantly from ethanol + albumin control (Figure 5.17d).

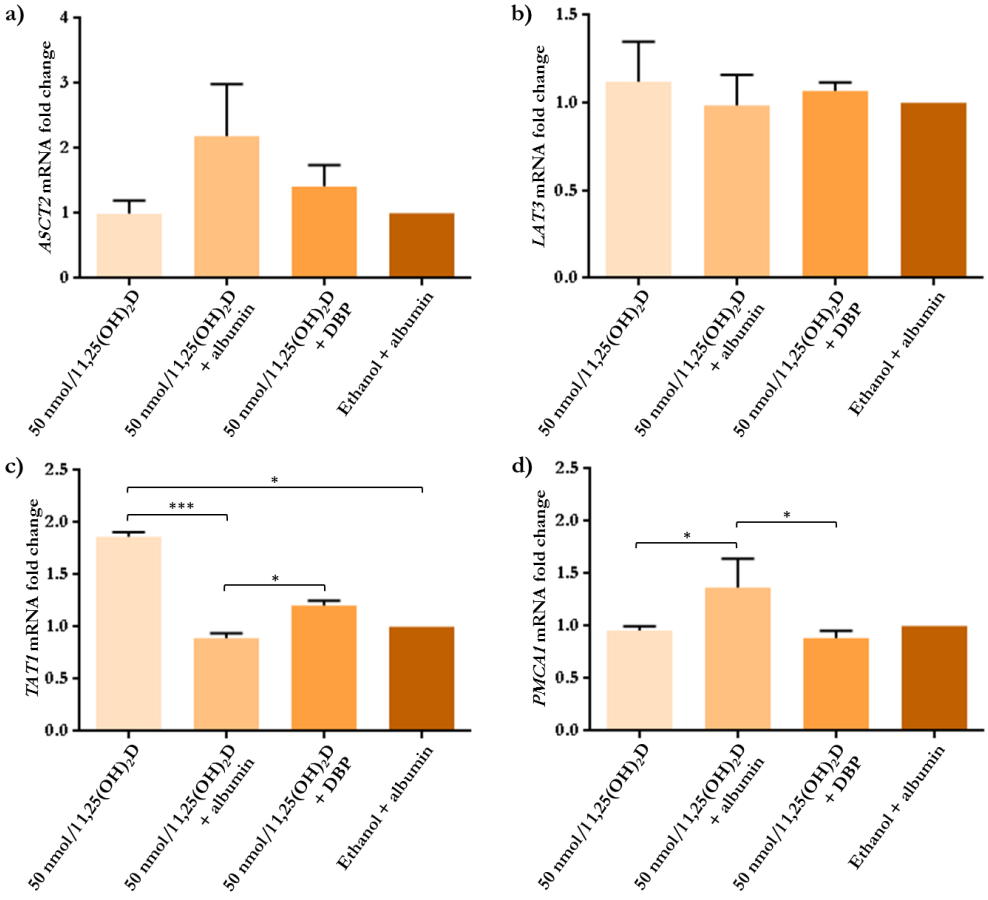

Figure 5.17: The effect of $1,25(OH)_2D$ exposure in the presence and absence of albumin and DBP on relative mRNA expression of amino acid and calcium transport genes in placental villous fragments. a) Expression of *ASCT2* was not affected by $1,25(OH)_2D$, albumin or DBP. n = 4 for 50 nmol/l $1,25(OH)_2D$, 50 nmol/l $1,25(OH)_2D$ + albumin, and ethanol + albumin. n = 2 for $1,25(OH)_2D$ + DBP. b) $1,25(OH)_2D$ showed no impact on *LAT3* mRNA levels. n = 7 for 50 nmol/l $1,25(OH)_2D$ and 50 nmol/l $1,25(OH)_2D$ + albumin, n = 4 for $1,25(OH)_2D$ + DBP and ethanol + albumin. c) *TAT1* mRNA expression was significantly increased by 50 nmol/l $1,25(OH)_2D$ alone compared to 50 nmol/l $1,25(OH)_2D$ + albumin, and ethanol + albumin. Expression levels were also significantly increased by 50 nmol/l $1,25(OH)_2D$ + DBP compared to 50 nmol/l $1,25(OH)_2D$ + albumin. n = 5 for 50 nmol/l $1,25(OH)_2D$ and 50 nmol/l $1,25(OH)_2D$ + albumin, n = 4 for 50 nmol/l $1,25(OH)_2D$ + DBP and 8 for ethanol + albumin. d) 50 nmol/l $1,25(OH)_2D$ + albumin resulted in a significant increase in *PMCA1* expression compared to 50 nmol/l $1,25(OH)_2D$ and 50 nmol/l $1,25(OH)_2D$ + DBP. n = 7 for 50 nmol/l $1,25(OH)_2D$ and 50 nmol/l $1,25(OH)_2D$ + albumin. n = 4 for 50 nmol/l $1,25(OH)_2D$ + DBP, and n = 10 for ethanol + albumin. Data are presented as mean fold change relative to ethanol + albumin control + SEM. * $p < 0.05$, *** $p < 0.001$.

RNA sequencing: The effect of exposure to 25(OH)D alone and with albumin was investigated in more detail by RNA sequencing. Genes of interest for this thesis were searched for within the RNA sequencing data set. Exposure of placental villous fragments to 20 μmol/l 25(OH)D resulted in a significant up-regulation of *CYP24A1* and *TXNIP*, and a significant down-regulation of *xCT*, *CYP27B1* and *CaT1* compared to control treated placental fragments (Table 5.5). In contrast, exposure to 20 μmol/l 25(OH)D with albumin resulted in a significant up-regulation in mRNA levels of *CYP24A1* and *NCX2* compared to control treated placental villous fragments. None of the genes tested showed a significant down-regulation in response to treatment with 20 μmol/l 25(OH)D with albumin compared to control treated fragments (Table 5.5). When 20 μmol/l 25(OH)D with albumin exposure was compared with 20 μmol/l 25(OH)D alone, the addition of albumin resulted in increased mRNA expression of *xCT*, *CYP24A1*, *CaT1* and *CYP27B1*, and significant down-regulation of *TXNIP* (Table 5.5).

Table 5.5: RNA sequencing data showing genes up- or down-regulated in response to 20 μmol/l 25(OH)D alone or with albumin in placental villous fragments.

20 μmol/l 25(OH)D vs control			20 μmol/l 25(OH)D + 0.7 mmol/l albumin vs control			20 μmol/l 25(OH)D + 0.7 mmol/l albumin vs 20 μmol/l 25(OH)D		
Gene	**Fold change**	**p**	**Gene**	**Fold change**	**p**	**Gene**	**Fold change**	**p**
Up-regulated genes			**Up-regulated genes**			**Up-regulated genes**		
CYP24A1	**3.7**	**0.04**	***CYP24A1***	**18.3**	**< 0.001**	***xCT***	**8.4**	**<0.001**
TXNIP	**2.8**	**< 0.001**	***NCX2***	**4.3**	**< 0.001**	***CYP24A1***	**4.9**	**0.002**
NCX2	1.9	0.05	*xCT*	2.0	0.06	***CaT1***	**3.7**	**0.002**
CYP27A1	**1.8**	**0.03**	*Megalin*	1.8	0.11	*NCX3*	3.2	0.12
4F2hc	**1.8**	**0.03**	*Calbindin D28K*	1.8	0.54	***CYP27B1***	**3.0**	**0.002**
Cubilin	1.8	0.10	*SNAT4*	1.5	0.14	*CaT2*	2.8	0.34
RXRα	**1.7**	**< 0.001**	***VDR***	**1.5**	**< 0.05**	*NCX2*	2.3	0.06
ASCT1	**1.4**	**0.02**	***4F2hc***	**1.4**	**0.04**	*SNAT1*	2.1	0.08
PTH1R	1.4	0.09	***RXRα***	**1.4**	**0.01**	***PMCA4***	**1.9**	**0.001**
LPL	1.4	0.07	***PMCA4***	**1.4**	**0.04**	***LAT1***	**1.8**	**0.03**
DNMT3b	1.3	0.18	***ASCT1***	**1.4**	**0.01**	*Calbindin D28K*	1.8	0.46
EAAT3	1.3	0.19	*CaT2*	1.4	0.78	*Megalin*	1.6	0.10
SNAT4	1.3	0.33	***ASCT2***	**1.4**	**0.04**	***SNAT2***	**1.5**	**0.03**
VDR	1.2	0.99	*CYP27A1*	1.3	0.51	***PMCA1***	**1.4**	**0.02**
Megalin	1.1	0.99	*SNAT1*	1.3	0.73	***y+LAT2***	**1.3**	**0.003**
LAT4	1.1	0.86	*Cubilin*	1.2	0.97	*ASCT2*	1.3	0.09
NCX1	1.1	0.68	*EAAT2*	1.2	0.41	*Legumain*	1.3	0.09
TAT1	1.1	0.62	*LAT4*	1.2	0.42	*VDR*	1.2	0.26
ASCT2	> 1.0	0.71	*PMCA1*	1.2	0.11	*LAT2*	1.2	0.79
LAT3	> 1.0	0.51	*PTH1R*	1.2	0.70	*y+LAT1*	1.2	0.45
CYP2J2	> 1.0	0.96	*LAT2*	1.1	0.69	*SNAT4*	1.2	0.74
EAAT2	> 1.0	0.70	*CYP2J2*	1.1	0.74	*DNMT1*	1.1	0.11
PTHrP	> 1.0	0.89	*y^+LAT1*	1.1	0.44	*EAAT2*	1.1	0.44
Down-regulated genes			*DNMT1*	1.1	0.69	*CYP2J2*	1.1	0.75
xCT	**0.2**	**0.01**	*LPL*	1.1	0.55	*LAT4*	1.1	0.49
CYP27B1	**0.3**	**0.002**	*y+LAT2*	1.1	0.29	**Down-regulated genes**		
CaT1	**0.3**	**0.004**	*SNAT2*	> 1.0	0.28	***TXNIP***	**0.4**	**0.001**
NCX3	0.3	0.10	*EAAT3*	> 1.0	0.82	*Cubilin*	0.7	0.13

CaT2	0.5	0.47	*TXNIP1*	> 1.0	0.84	***CYP27A1***	**0.7**	**0.02**
LAT1	0.6	0.13	*CaT1*	> 1.0	0.59	*NCX1*	0.7	0.38
SNAT1	0.6	0.06	**Down-regulated genes**			*PTHrP*	0.7	0.47
Legumain	**0.6**	**0.02**	*NCX1*	0.8	0.68	*DNMT3b*	0.7	0.12
SNAT2	0.7	0.87	*PTHrP*	0.8	0.38	*LAT3*	0.8	0.11
PMCA4	**0.7**	**0.03**	*LAT3*	0.8	0.17	*LPL*	0.8	0.06
PMCA1	0.8	0.15	*Legumain*	0.8	0.25	*4F2hc*	0.8	0.21
y^+LAT2	0.8	0.48	*CYP27B1*	0.8	0.58	*TAT1*	0.8	0.45
y^+LAT1	0.9	0.99	*TAT1*	0.9	0.62	*EAAT3*	0.8	0.42
LAT2	0.9	0.97	*NCX3*	< 1.0	0.72	*PTH1R*	0.8	0.03
DNMT1	< 1.0	0.21	*DNMT3b*	< 1.0	0.75	*RXRa*	0.8	0.17
Calbindin D28K	Not detected		*LAT1*	< 1.0	0.55	*ASCT1*	< 1.0	0.88

Results are presented for the 3 comparisons of RNA sequencing data. Data are presented as mean fold change relative to ethanol + albumin control, and relative to 25(OH)D for the 25(OH)D vs 25(OH)D + albumin comparison. Genes above the bold line are those which were up- or down-regulated above the threshold levels of 2 and 0.5, respectively, while genes in bold showed significant alterations in mRNA expression.

5.4. Discussion

The data presented suggest that 25(OH)D is metabolised via the action of CYP27B1 in the placenta and has a transcriptional effect on placental mRNA expression. In addition, this work suggests that vitamin D uptake into the placenta could occur via a specific mechanism. Furthermore, uptake of 25(OH)D and $1,25(OH)_2D$ into the placenta are both demonstrated. Uptake of 25(OH)D may be mediated largely by albumin, whilst $1,25(OH)_2D$ may be less reliant on carrier proteins for uptake. However, uptake of both metabolites may be mediated via the endocytic mechanism of pinocytosis.

5.4.1. Placental uptake of vitamin D

Vitamin D transport into and metabolism within the placenta

No concrete evidence of 25(OH)D or $1,25(OH)_2D$ transport in human placental samples has been published. Furthermore, questions have remained over whether $1,25(OH)_2D$ is transported into the human placenta due to contradictory reports on associations between maternal and fetal $1,25(OH)_2D$ levels (Fleischman *et al.*, 1980; Delvin, 1982; Hollis and Pittard, 1984; Ishida *et al.*, 1988). Additionally $1,25(OH)_2D$ was shown not to cross the rat placenta (Noff and Edelstein, 1978). This study shows uptake of both 25(OH)D and $1,25(OH)_2D$ into the human placenta. The uptake of both major vitamin D metabolites into the placenta means that there is a larger reservoir of vitamin D for the placenta and fetus to access. Due to the lower levels of $1,25(OH)_2D$ within the circulation, it is likely that 25(OH)D will provide the major pool for transport. As $1,25(OH)_2D$ is already in the activated form it is interesting to speculate about differing roles for $1,25(OH)_2D$ and 25(OH)D within the placenta. $1,25(OH)_2D$ could provide a pool of vitamin D with direct actions on placental mRNA expression, as it is active and would have access to VDR within the placenta. Alternatively, if this active form of vitamin D could be protected from breakdown within the placental syncytiotrophoblast, it could provide a source of active vitamin D for the fetus. Intracellular vitamin D binding proteins have been discovered (Willnow and Nykjaer, 2002), which could protect $1,25(OH)_2D$ from metabolism whilst it is transported across the placental syncytiotrophoblast. It is currently unknown whether these are expressed within the human placenta, however due to its hydrophobicity binding proteins would be necessary to transport vitamin D across the syncytiotrophoblast. As contradictory reports have been published on associations between maternal and cord blood $1,25(OH)_2D$ (Fleischman *et al.*, 1980; Delvin, 1982; Hollis and Pittard, 1984; Ishida *et al.*, 1988), it appears more likely that $1,25(OH)_2D$ acts directly upon the placenta, whilst 25(OH)D is the major metabolite transported across the placenta and into the fetal circulation. In addition, as 25(OH)D is likely to provide the bulk of vitamin D transport into the placenta, it may be utilised by both the placenta and fetus. CYP27B1 is expressed and is active in the fetal kidneys, enabling the fetus to convert 25(OH)D into the activated $1,25(OH)_2D$ (Fenton

and Britton, 1980; Ross *et al.*, 1980), and as discussed below, the placenta is also capable of metabolising 25(OH)D.

The increase in *CYP24A1* mRNA expression in placental villous fragments upon exposure to 25(OH)D provides indirect evidence for vitamin D metabolism within the placenta. *CYP24A1* expression is induced upon $1,25(OH)_2D$ binding to VDR. Consequently, this demonstrates activity of CYP27B1 within the placenta converting 25(OH)D to $1,25(OH)_2D$. The notion that the placenta is capable of hydroxylation of 25(OH)D is supported by research showing production of $1,25(OH)_2D$ in cultured human syncytiotrophoblast cells (Diaz *et al.*, 2000; Avila *et al.*, 2007a). This work provides further evidence that the placenta responds to vitamin D. Expression and activity of *CYP27B1* within the placenta has been shown to be altered in pregnancy pathologies including pre-eclampsia (Diaz *et al.*, 2002; Ma *et al.*, 2012) and also decreases in the third trimester (Zehnder, 2002). Specifically, reduced *CYP27B1* mRNA expression was reported in placentas of pre-eclamptic women (Diaz *et al.*, 2002). However, this study used southern blotting to quantify mRNA expression levels, rather than the more robust method of qrt-PCR. Furthermore, statistical analysis was not presented therefore it is unclear whether the results presented were significant. In contrast, an increase in CYP27B1 protein expression was demonstrated in placentas of pre-eclamptic women (Ma *et al.*, 2012). Regardless of the direction of the change in CYP27B1 expression in placental pathologies, the demonstration of enzymatic activity within the placenta indicates that changes in expression may result in pathological outcomes. Furthermore, expression of the enzyme may be modulated to adjust vitamin D transfer to the fetus. Placental expression of *CYP27B1* mRNA was reduced in women with vitamin D deficiency (Vijayendra Chary *et al.*, 2015). It is conceivable that reduced placental metabolism of 25(OH)D via CYP27B1 would leave a greater placental reservoir of 25(OH)D available for fetal transfer when maternal concentrations of the steroid hormone are low. As initial experiments using 100 nmol/l 25(OH)D did not elicit any significant changes in *CYP24A1* mRNA expression, it may be that placental metabolism of 25(OH)D is only stimulated when the steroid hormone is in higher concentrations. This would indicate that transfer to the fetus is prioritised over placental requirements.

Impact of binding proteins on vitamin D transport into the placenta

The free hormone hypothesis states that the role of steroid binding proteins is to regulate the levels of free hormone available for passive diffusion into target cells. In the kidney, where transport of 25(OH)D is essential for the production of $1,25(OH)_2D$, binding proteins have been shown to play an essential role in delivery of the steroid hormone (Nykjaer *et al.*, 1999; Nykjaer *et al.*, 2001). Vitamin D is important for fetal growth and placental function, therefore it seems reasonable that a specific transport system for this steroid hormone could exist within the placenta. In addition, it seems unlikely that DBP and $1,25(OH)_2D$ levels should rise in pregnancy if only the free hormone is active, as this

rise in both components may result in unchanged free 1,25$(OH)_2$D levels (Van Hoof *et al.*, 2001), alongside reduced free 25(OH)D levels (Bouillon, 1981).

As mentioned above, the initial concentration of 100 nmol/l 25(OH)D failed to result in changes to *CYP24A1* mRNA expression, therefore its entry into placental villous fragments could not be assessed. The increase in concentration to 20 μmol/l provided enough 25(OH)D to measure conversion to 1,25$(OH)_2$D through *CYP24A1* expression levels. Subsequent experiments using this higher concentration of 25(OH)D have provided evidence for the role of albumin in mediating entry of the steroid hormone into the placenta. *CYP24A1* expression was increased 49 fold with 25(OH)D and albumin combined compared to a smaller 9 fold increase in expression when placental fragments were exposed to 25(OH)D alone. The identification of the impact of a binding protein for 25(OH)D uptake into the placenta, suggests that a mechanism similar to that in the kidney may be operational within the placenta. In the kidney, DBP and albumin are essential for uptake of 25(OH)D in the proximal convoluted tubule, and the absence of megalin and cubilin, the receptors that these binding proteins interact with, results in loss of 25(OH)D and binding proteins through urinary excretion (Nykjaer *et al.*, 1999; Nykjaer *et al.*, 2001). Alternatively it is possible that the presence of a binding protein serves to increase the solubility of 25(OH)D and deliver it the membrane where transport then occurs via diffusion.

The data obtained for 1,25$(OH)_2$D entry into placental villous fragments is less conclusive as responses showed a high degree of variability. Culture of placental fragments with 1,25$(OH)_2$D alone, with DBP or with albumin all resulted in significant fold changes to *CYP24A1* expression. However, the presence of a carrier protein did not result in further increases to *CYP24A1* expression and the response to 1,25$(OH)_2$D with albumin was variable. It is possible that as 1,25$(OH)_2$D is already active a lower level of transport is required to increase *CYP24A1* mRNA, whilst with 25(OH)D as conversion to 1,25$(OH)_2$D is required, a greater level of transport may be required. This may require carrier protein-mediated transport whereas non carrier protein-mediated transport may provide enough 1,25$(OH)_2$D to increase *CYP24A1* mRNA. Placental fragments were also incubated with 1,25$(OH)_2$D plus an amino acid mix or gelatin as controls for the presence of protein in the buffer. 1,25$(OH)_2$D with the amino acid mix did not result in significantly altered uptake compared to 1,25$(OH)_2$D plus albumin or DBP. However, considering that there was no significant difference between these and 1,25$(OH)_2$D alone this finding is not surprising. Gelatin was also used as an alternative protein control, however, this significantly reduced *CYP24A1* mRNA expression. The gelatin may have sequestered 1,25$(OH)_2$D outside the fragment. Alternatively, the gelatin may have had harmful effects on the placental villous tissue. This is possible as RNA concentrations were lower for placental villous fragments exposed to gelatin.

It is possible that with increased sample numbers an effect of albumin or DBP on $1,25(OH)_2D$ transport may be identified. When looking at the data on an individual basis, some placentas showed a clear up-regulation of *CYP24A1* only when exposed to $1,25(OH)_2D$ with albumin, while *CYP24A1* levels with $1,25(OH)_2D$ alone did not differ from controls. On the other hand, some placentas showed an up-regulation of *CYP24A1* with $1,25(OH)_2D$ alone that was not increased further in the presence of albumin or DBP. This suggests that individual differences between the placentas may account for the variation observed. Expression levels of vitamin D metabolising enzymes within the placenta has been shown to be altered by changes in the maternal environment such as in gestational diabetes (Cho *et al.*, 2013), pre-eclampsia (Diaz *et al.*, 2002; Ma *et al.*, 2012), and in response to pro-inflammatory cytokines (Noyola-Martinez *et al.*, 2014). In addition, differences in maternal vitamin D levels may drive some placentas to increase their uptake of vitamin D. Endocytosis in early embryos has been shown to be altered in response to maternal nutrition, such as a low protein diet (Sun *et al.*, 2014), therefore it seems plausible that uptake of vitamin D could be modulated within the placenta based on maternal signals to the placenta. Furthermore, these experiments were carried out over a year long period. Seasonal variations in vitamin D status in pregnant women have been widely reported (Bowyer *et al.*, 2009; Luque-Fernandez *et al.*, 2014; Zhou *et al.*, 2014a), and therefore this may have contributed to the variation seen within the data. As discussed in section 5.4.4 much larger numbers may be required to identify any impact of albumin or DBP on placental uptake of $1,25(OH)_2D$.

The molar excess of DBP and albumin used in this study should also be compared to that in pregnant women. In comparison to maternal concentrations of 25(OH)D, albumin would be in molar excess by 7000 fold. Whereas, in comparison to maternal concentrations of $1,25(OH)_2D$, albumin would have a molar excess of 700,000,000 and DBP would have a molar excess of 120,000,000. This is based on 25(OH)D levels described by Delvin (1982); Markestad (1984); Salle *et al.* (2000); Crozier *et al.* (2012); Harvey *et al.* (2012a), $1,25(OH)_2D$ levels described by Bouillon (1981); Delvin (1982); Markestad (1984); Salle *et al.* (2000), DBP levels described by Bouillon (1981); Van Hoof *et al.* (2001); Jorgensen *et al.* (2004); Powe *et al.* (2010) and albumin levels described by Schwartz *et al.* (2014). In comparison in these studies, albumin was in 35 fold molar excess to 25(OH)D, and 14,000 fold excess compared to $1,25(OH)_2D$. While, DBP was in 100 fold molar excess in comparison to $1,25(OH)_2D$. Therefore in our studies, whilst the binding protein was always in excess, as occurs naturally, this excess was not as exacerbated as in the natural situation. This could have resulted in increased free concentrations of hormone, particularly with albumin where the binding affinity for vitamin D is significantly lower than that of DBP. The differences observed between the effect of albumin on placental transport of 25(OH)D and $1,25(OH)_2D$ could partially be explained by the differing binding affinities of albumin for these steroid hormones. Albumin has a higher binding affinity for 25(OH)D compared to $1,25(OH)_2D$, 60 μmol/l compared to 540 μmol/, respectively (Bikle *et al.*, 1985; Bikle *et al.*, 1986). Therefore, considering that in these experiments the molar excess of albumin was not as high as that

observed in the natural situation this may explain why there was no significant effect of albumin on $1,25(OH)_2D$ uptake.

Thus far, the data presented suggest there may be differing uptake systems within the placenta for 25(OH)D and $1,25(OH)_2D$. 25(OH)D entry may be facilitated by the presence of albumin, while $1,25(OH)_2D$ entry shows no appreciable difference with albumin or DBP (Figure 5.18). The potential mechanisms for these methods of entry are discussed further below.

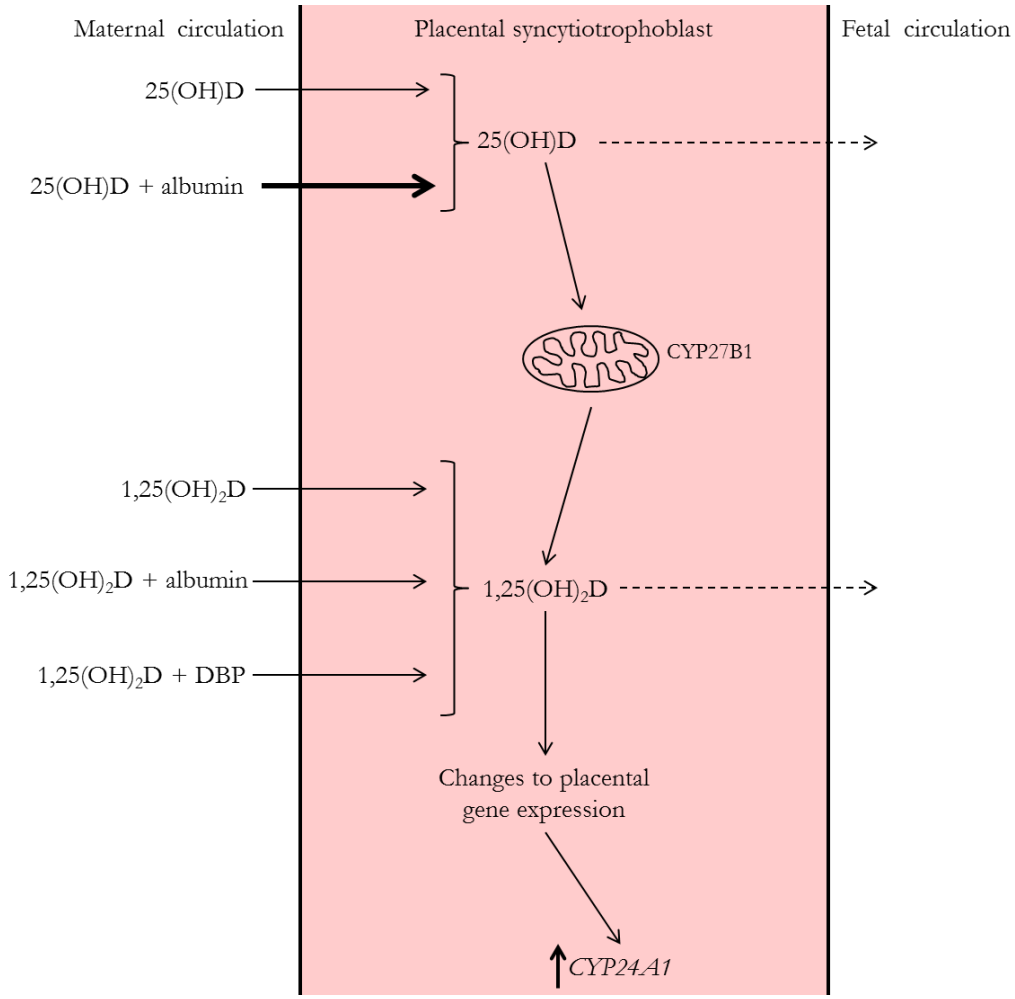

Figure 5.18: Current model of vitamin D transport into the human placenta. Both 25(OH)D and $1,25(OH)_2D$ are transported into the placenta. Uptake of 25(OH)D is greatly increased in the presence of albumin, as indicated by the bold arrow. Uptake of $1,25(OH)_2D$ occurs in the presence of albumin and DBP, but these do not appear to enhance transport. Once inside the placenta, 25(OH)D can be hydroxylated through the action of the mitochondrial enzyme, CYP27B1, to form active $1,25(OH)_2D$. In addition 25(OH)D is likely to be transported into the fetal circulation, as indicated by the dashed arrow. $1,25(OH)_2D$ can cause changes to placental mRNA expression as seen by increased *CYP24A1* expression. It is currently unclear whether $1,25(OH)_2D$ is also transported into the fetal circulation, as indicated by the dashed arrow.

5.4.2. Mechanism of vitamin D entry into the placenta

The differences observed between 25(OH)D and $1,25(OH)_2D$ uptake suggest specific mechanisms of entry as opposed to passive diffusion into the placenta. Furthermore, examination of FITC-albumin

and FITC-dextran entry into placental villous fragments supports the notion of a specific uptake mechanism. Firstly, uptake of FITC-albumin was significantly reduced when the temperature was dropped to 4ºC compared to 37ºC. Secondly, uptake of FITC-dextran was barely detectable compared to FITC-albumin. Both proteins have similar molecular weights; FITC-albumin is 66.4 kDa, compared to 70 kDa for FITC-dextran, therefore if entry was occurring via a passive mechanism entry of both proteins might be expected to occur. Lack of FITC-dextran uptake in human placental perfusions has previously been described (Balakrishnan *et al.*, 2010; Balakrishnan *et al.*, 2011), therefore this supports the notion that protein uptake in the placental villous fragment tissue is occurring in a similar manner to that of whole placental tissue.

It is interesting to note that FITC-albumin uptake was not entirely abolished by incubation at 4ºC. This has previously been observed with uptake of radiolabelled albumin in placental explant culture. As with our data, a significant reduction in uptake was observed by the reduced temperature, but a low level of uptake still occurred. This was suggested to occur as binding to membrane receptors could still occur at the lower temperatures (Lambot *et al.*, 2006), which would result in albumin association with the placental fragment membrane. Confocal microscopy images of FITC-albumin uptake in placental villous fragments also revealed potentially important observations. On lower magnification images (See Figure 5.9), the irregularity of FITC-albumin uptake was starkly apparent. High concentrations of FITC-albumin were observed in some areas of the placental villi, whereas other areas were devoid of FITC-albumin. This phenomenon has previously been described with FITC-BSA uptake in opossum kidney cells, where the uneven distribution of FITC-BSA uptake was closely linked to megalin and cubilin expression at the cell membrane (Zhai *et al.*, 2000). A similar situation could be occurring within the placental villous fragment samples, with the uneven distribution of fluorescence resulting from variation in distribution of the specific receptor on the MVM. Additionally, higher magnification images revealed the presence of FITC in punctate dots within the villous stroma. These dots could indicate the presence of FITC-albumin within vesicles, which would suggest a mechanism of vesicular endocytosis for uptake. As discussed in section 5.4.5, identification of these structures as vesicles could be carried out using antibodies against specific vesicular components.

Mechanism of 25(OH)D uptake into the placenta

Due to time constraints, inhibition of 25(OH)D uptake was not investigated in as much detail as $1,25(OH)_2D$. One experiment using amiloride was conducted, which showed a large decrease in *CYP24A1* mRNA expression when placental villous fragments were cultured with 25(OH)D, albumin and amiloride, compared to 25(OH)D and albumin. As this observation is based on a single experiment, statistical analysis could not be carried out and too much weight should not be put on the results. However, the data obtained do suggest that pinocytosis may have a role in albumin-mediated 25(OH)D uptake into the placenta, and further experiments would clarify whether this is the case.

Mechanism of 1,25(OH)$_2$D uptake into the placenta

The mechanism of 1,25(OH)$_2$D uptake was investigated using a wider range of endocytic inhibitors. Specifically, these inhibitors prevented dynamin-mediated endocytosis, megalin-dependent endocytosis and pinocytosis. Chlorpromazine hydrochloride hydrate, an inhibitor of clathrin-mediated endocytosis, appeared to damage villous fragments, therefore these data were excluded from analysis. A previous study has shown that chlorpromazine hydrochloride hydrate reduced FITC-albumin uptake by 40% compared to controls in placental explant culture (Lambot *et al.*, 2006). This could indicate a partial role for clathrin-mediated endocytosis of albumin, and potentially vitamin D, within the human placenta. However, the modest reduction observed suggests that other clathrin-independent mechanisms may play a more prominent role in albumin uptake. Furthermore, there was no mention of assessment of viability of placental explants following exposure to the inhibitor, which was used at the same concentration that resulted in deterioration of placental villous fragments in this study

Gentamicin was used as a competitive inhibitor of megalin (Dagil *et al.*, 2013), to investigate the role of megalin in receptor-mediated endocytosis of 1,25(OH)$_2$D and albumin. Megalin and cubilin function has been shown to be important for DBP and albumin reabsorption of vitamin D in the renal proximal tubule (Nykjaer *et al.*, 1999; Birn *et al.*, 2000; Zhai *et al.*, 2000; Nykjaer *et al.*, 2001). Gentamicin showed no significant effect on *CYP24A1* mRNA expression alongside 1,25(OH)$_2$D and albumin, suggesting megalin is not responsible for placental uptake of 1,25(OH)$_2$D and albumin. In support of this, a study using chloride channel blockers to reduce megalin-mediated internalisation showed no impact on albumin entry into placental explants (Lambot *et al.*, 2006). This demonstrates that an alternative mechanism may be operating. Furthermore, DBP entry has been observed in cells that do not express megalin or cubilin (Chun *et al.*, 2010), leading to the possibility that another receptor is also responsible for uptake of these proteins. One potential candidate is FcRn which has been shown to bind albumin (Andersen *et al.*, 2012). In support of a role for FcRn in albumin uptake, *FcRn*$^{-/-}$ mice showed increased urinary excretion of albumin (Sarav *et al.*, 2009), analogous to that described for DBP and albumin with *megalin*$^{-/-}$ or *cubilin*$^{-/-}$ mice (Nykjaer *et al.*, 1999; Birn *et al.*, 2000; Nykjaer *et al.*, 2001). Furthermore, FcRn expression has been detected within the placental syncytiotrophoblast (Simister *et al.*, 1996), supporting the notion that this transporter could play in role in albumin bound vitamin D uptake within the placenta. Another potential candidate for vitamin D uptake in the placenta could be cholesterol transporters. Cholesterol transporters within the intestine have been shown to be involved in vitamin D uptake. However, in these studies vitamin D was delivered to the transporters in micelles and albumin or DBP were not present (Reboul *et al.*, 2011), so these are unlikely to be the transporters whose action we are witnessing.

The binding affinity of gentamicin for megalin is reported to be moderately low, and therefore may not completely abrogate megalin-mediated uptake of substrates. In addition, it is unclear whether

gentamicin would eliminate the megalin-cubilin interaction. Subsequently, if $1,25(OH)_2D$ and albumin entry into the placenta is mediated via cubilin binding to albumin, followed by a cubilin and megalin interaction and internalisation, gentamicin may not inhibit this process. Cubilin binding to albumin and DBP is thought to provide the major ligand-receptor interaction involved in albumin and DBP reabsorption in the kidney (Birn *et al.*, 2000; Nykjaer *et al.*, 2001), therefore, these data have not entirely ruled out a role for megalin and cubilin in vitamin D uptake into the placenta. However, megalin- and cubilin-mediated endocytosis is thought to be clathrin-dependent as specific components of the clathrin pits, Low density lipoprotein receptor adapter protein 1 and Disabled 2, have been shown to bind megalin (Saito *et al.*, 2010). As discussed below, experiments with dynasore, indicate a clathrin-independent mechanism of entry into the placenta, which may mean entry is not driven by megalin and/or cubilin function.

The use of dynasore, an inhibitor of dynamin-mediated endocytosis, alongside $1,25(OH)_2D$ with albumin or DBP, had no significant impact on *CYP24A1* mRNA expression compared to $1,25(OH)_2D$ with albumin or DBP without the inhibitor. Furthermore, dynasore exposure showed no major impact on FITC-albumin uptake into placental villous fragments. This experiment was only conducted once so statistical analysis could not be carried out, but it supports mRNA expression data demonstrating that dynamin does not play a role in $1,25(OH)_2D$ entry into the placenta. If $1,25(OH)_2D$ uptake occurs independently of dynamin, uptake should also be clathrin-independent (Figure 5.1) Dynamin-independent endocytosis mediated by clathrin has not previously been described, therefore based on current literature it appears that uptake of $1,25(OH)_2D$ is both clathrin- and dynamin-independent. Unfortunately, due to the issues with chlorpromazine hydrochloride, clathrin-independence could not be confirmed.

The use of amiloride to block pinocytosis of $1,25(OH)_2D$ with albumin resulted in significantly reduced *CYP24A1* mRNA expression, indicating reduced uptake of $1,25(OH)_2D$ and albumin. Furthermore, the change in mRNA expression was also significantly lower than $1,25(OH)_2D$ alone, which may indicate a role for pinocytosis in uptake of albumin bound $1,25(OH)_2D$ as well as unbound $1,25(OH)_2D$. In addition, FITC-albumin uptake in the placental villous fragments was significantly reduced by amiloride, providing further support for inhibition of albumin bound $1,25(OH)_2D$ entry. Amiloride has previously been shown to partially block albumin uptake in opossum kidney cells (Gekle *et al.*, 1999), yet albumin uptake into placental explants was not inhibited by the pinocytosis inhibitor (Lambot *et al.*, 2006). However the concentration of amiloride used by Lambot *et al.* (2006) was 10 fold lower than that used in our studies, so this could explain the discrepancy in the results. These data suggests that albumin and $1,25(OH)_2D$ transport into the placenta is at least partially mediated via the mechanism of pinocytosis, as may also be the case for 25(OH)D. Interestingly, FcRn has been shown to play a role in salvaging of pinocytosed IgG. IgG molecules that could not bind FcRn were targeted

for lysosomal degradation, whilst those that could bind FcRn were recycled or transcytosed (Ward *et al.*, 2003). If FcRn functions in a similar capacity with albumin, this raises an interesting possibility that FcRn function within the placenta may mediate the fate of pinocytosed vitamin D-albumin complexes. Further experiments are required to confirm the uptake of $1,25(OH)_2D$ via pinocytosis, as concerns have been raised over the efficacy of amiloride (see section 5.4.4)

Entry of $1,25(OH)_2D$ by caveolin-dependent endocytosis was not studied specifically in these experiments, however, as amiloride inhibited uptake this implies caveolin-dependent uptake is not involved. Further experiments using specific inhibitors of this pathway are required to definitively rule out this mechanism. Albumin has been reported to be internalised via caveolae in some cell types (Minshall *et al.*, 2002), however, in placental explants, albumin uptake was not affected by inhibition of the caveolae system (Lambot *et al.*, 2006). Furthermore, while caveolin-1 has been identified in the placental endothelium, it does not appear to be present in the syncytiotrophoblast layer of the placenta (Lyden *et al.*, 2002; Linton *et al.*, 2003). This indicates that caveolae-mediated mechanisms of endocytosis are unlikely to play a major role in uptake of vitamin D into the placenta.

5.4.3. Effect of vitamin D, DBP and albumin on placental mRNA expression

RNA sequencing and qrt-PCR data showed that 25(OH)D with albumin resulted in a larger fold increase in *CYP24A1* than 25(OH)D alone. In addition, expression of a number of the genes of interest in this thesis, including amino acid and calcium transporters, was altered following exposure to 25(OH)D or 25(OH)D with albumin. This suggests that vitamin D could alter the transport of essential nutrients to the fetus, which has implications for fetal growth as well as for the health of the individual in later life (see Chapter 7.7).

Differences in mRNA expression were observed following exposure of placental villous fragments to 25(OH)D alone compared to 25(OH)D with albumin. Specifically, *xCT*, *CaT1* and *CYP27B1* were significantly up-regulated in samples treated with 25(OH)D and albumin, whilst *TXNIP* was significantly down-regulated. The differences between the two 25(OH)D conditions could result either directly from the presence of albumin or through increased exposure to 25(OH)D, directed via increased uptake of albumin bound 25(OH)D. The larger fold increase in *CYP24A1* mRNA levels in 25(OH)D and albumin treated villous fragments compared to those treated with 25(OH)D alone would suggest that vitamin D entry is enhanced and that effects are mediated via the presence of additional 25(OH)D being converted into $1,25(OH)_2D$ within the samples. However, it is interesting that other vitamin D responsive genes were regulated differently in placental villous fragments treated with 25(OH)D and albumin compared to 25(OH)D alone. Specifically, *TXNIP* was down-regulated while *CYP27B1* was up-regulated. In the classical $1,25(OH)_2D$-mediated feedback loop, *CYP27B1*

mRNA expression is down-regulated to prevent further rises in $1,25(OH)_2D$ levels, therefore the addition of albumin has in this case resulted in a reversal of the expected vitamin D-mediated effects. This could result from increased entry of 25(OH)D into the samples as if albumin is mediating increased entry of 25(OH)D into the placental fragments, an increased *CYP27B1* would be required to activate the 25(OH)D. Most studies investigate vitamin D-mediated effects on mRNA expression through addition of the active $1,25(OH)_2D$. This could explain why this response is not commonly reported. However, a similar finding has been reported in kidneys of cattle receiving a vitamin D_3 supplementation. The supplementation resulted in increased mRNA levels of both *CYP27B1* and *CYP24A1* (Rezende *et al.*, 2013), and this would support the notion that the effect on *CYP27B1* expression is due to treatment with a precursor form of vitamin D, possibly as a result of up-regulation in order to metabolise the increased levels of inactive vitamin D. It would be interesting to study the effect on mRNA expression at a later time point, as it seems probable that a longer term exposure to increased $1,25(OH)_2D$ via the 25(OH)D would result in a down-regulation of *CYP27B1*. In addition, the results could indicate differences in splice variants. Differences in *CYP27B1* splice variants have been reported with a human kidney cell line in response to 25(OH)D compared to $1,25(OH)_2D$ (Wu *et al.*, 2007). It is possible that differences in *CYP27B1* splice variants may underlie the differences observed between 25(OH)D alone and 25(OH)D with albumin. More in-depth analysis of the data obtained from RNA sequencing would enable this to be investigated. Otherwise the enhanced up-regulation of *CYP24A1* in response to 25(OH)D and albumin could explain some of the differences in mRNA expression between 25(OH)D alone and 25(OH)D with albumin. The increase in *CYP24A1* would result in increased catabolism of both 25(OH)D and $1,25(OH)_2D$, therefore over time a smaller proportion of $1,25(OH)_2D$ may be available to exert effects on mRNA expression. This could explain why genes such as *TXNIP*, which were up-regulated in the presence of 25(OH)D alone were not up-regulated in response to 25(OH)D with albumin.

Alternatively, the differences in mRNA expression could be a result of the presence of albumin. Once inside the placental villous fragment albumin could be hydrolysed within the lysosome into its respective amino acids. The release of these amino acids into the placental fragment could then result in amino acid-mediated effects. For example, mRNA and protein expression of LAT3 increased in murine liver and skeletal muscle following a period of 24 h starvation, which was proposed to occur due to increased levels of branched chain amino acids (Fukuhara *et al.*, 2007). In addition, albumin itself has been shown to increase arginine transporter expression and arginine uptake in renal cells (Ashman *et al.*, 2005; Ashman *et al.*, 2006). Alternatively, albumin-mediated effects could be driven by fatty acids. The albumin used in the experiments was ≥ 96% pure, therefore it is possible that the presence of fatty acids with the albumin may have resulted in the albumin-mediated effects. We now need measures of $1,25(OH)_2D$ concentrations within the placental villous fragments to conclusively

show whether the observed effects on mRNA expression are a result of increased 25(OH)D uptake and conversion to $1,25(OH)_2D$, or whether they are the result of albumin itself.

qrt-PCR data revealed similar patterns of mRNA expression to those observed in RNA sequencing. mRNA expression of *LAT3* measured by qrt-PCR was significantly lower upon exposure to 25(OH)D and albumin, and the ethanol with albumin control in comparison to 25(OH)D alone. This could result from 25(OH)D-mediated up-regulation or albumin-mediated down-regulation of mRNA levels. In RNA sequencing no significant effect on *LAT3* was observed, however, in the presence of 25(OH)D and albumin *LAT3* mRNA expression showed a slight fold reduction of 0.8 compared to both control, and 25(OH)D alone. In these studies the albumin present in the control buffer did not have the same impact as the albumin combined with 25(OH)D suggesting that the effect is mediated via the combination of 25(OH)D and albumin, rather than albumin itself. As *LAT3* may be up-regulated by branched chain amino acids which would be released alongside other amino acids upon the breakdown of albumin (Fukuhara *et al.*, 2007), an albumin-mediated effect is plausible for this gene. Further experiments with increased sample numbers would allow the albumin or 25(OH)D effects on this gene to be investigated more thoroughly. Interestingly, the same pattern was also observed for *TAT1* mRNA expression in qrt-PCR and RNA sequencing suggesting a similar method of regulation for this transporter.

The effect of $1,25(OH)_2D$ on placental mRNA expression was also investigated with qrt-PCR. In response to $1,25(OH)_2D$ alone *TAT1* mRNA expression was significantly increased compared to the ethanol + albumin control and $1,25(OH)_2D$ + albumin, while *PMCA1* mRNA expression was significantly increased with $1,25(OH)_2D$ + albumin compared to $1,25(OH)_2D$ alone and $1,25(OH)_2D$ + DBP but not compared to the ethanol + albumin control. These findings suggest active vitamin D may be mediating expression of specific nutrient transporters within the placenta. As these effects were not seen with 25(OH)D, it supports the notion of differing roles for $1,25(OH)_2D$ and 25(OH)D within the human placenta. In addition, as effects differed depending on the presence or absence of, and type of carrier protein, it may be that function of $1,25(OH)_2D$ is modulated by the presence of a carrier protein. For example, FcRn salvages IgG for recycling, while unbound IgG is marked for degradation (Ward *et al.*, 2003). A similar sorting pathway may be present within the syncytiotrophoblast, and $1,25(OH)_2D$ may be marked for differing roles depending on the mode of entry or presence of a carrier protein. If this is the case, this would add further complexity to the relationship between maternal vitamin D, DBP and albumin and placental function. $1,25(OH)_2D$ has previously been shown to result in changes to taurine transporter expression but only in the presence of retinoic acid (Chesney and Han, 2013). It was suggested that the combination of retinoic acid binding to RXRα and $1,25(OH)_2D$ binding to VDR, created the optimal conformation of the nuclear receptor heterodimer. In our studies, retinoic acid was not included in the buffer therefore it is possible that further changes

to mRNA expression may have been observed if the combination of retinoic acid and vitamin D had been used.

5.4.4. Limitations

There are a number of limitations to this work. Firstly, due to the exciting discovery of 1,25$(OH)_2$D uptake into the placenta, the active form of vitamin D was focussed on more in uptake and endocytic inhibition experiments. Following data analysis, it is now clear that uptake of the two forms of vitamin D may occur through different mechanisms. In hindsight, it would have been interesting to focus more on albumin-mediated uptake of 25(OH)D. Secondly, sample numbers are low particularly for some aspects of the study, for example experiments with 25(OH)D and amiloride were only conducted once. As individual variation between the placentas may impact results, the data would be made more robust through a significant increase in sample numbers. In addition, analysis of FITC-albumin uptake in placental villous tissue revealed non-responders. These samples showed only negligible uptake of the FITC-albumin. It is possible that these samples were damaged during the experimental process. As these experiments were only carried out in single and numbers were low, non-responders were included in the data set and subsequent analysis. With an increase in sample numbers it may be possible to accurately identify non-responsive fragments and remove them from the data set. Alternatively, with increased numbers the variation resulting from these unresponsive samples would be minimised. A further limitation of this work is that uptake of vitamin D into the placental villous tissue was assessed indirectly through *CYP24A1* induction. A more precise measurement of entry, such as measurement of 1,25$(OH)_2$D levels within the placental fragments, may have reduced the variation observed in the data. Furthermore, due to time restrictions only a handful of genes were studied with qrt-PCR. Additional qrt-PCR is required to verify effects observed by RNA sequencing.

There are some weaknesses with the experiments using inhibitors of the different forms of endocytosis. Namely, no positive controls for each of the forms of endocytosis were used. Therefore, in instances where the inhibitor showed no impact on vitamin D uptake, the efficacy of the inhibitor is unknown. To prove that the inhibitor had worked a positive control for that form of endocytosis would be needed. For example, the transferrin receptor is commonly used as a positive control for clathrin-dependent endocytosis (Akula *et al.*, 2003; Cortese *et al.*, 2008), while HRP is commonly used for pinocytosis (Jones, 2007). Furthermore, the inhibition of one form of endocytosis may result in the up-regulation of an alternative form of vitamin D entry. This could prevent identification of the primary mechanism of vitamin D uptake in the physiological situation. Additionally, a number of concerns have been expressed in the literature regarding amiloride. Amiloride has been demonstrated to result in changes to the actin cytoskeleton (Lagana *et al.*, 2000), as well as altering the distribution of the endosomal markers, early endosome antigen 1 and Lamp1 (Fretz *et al.*, 2006). In addition,

questions have arisen over the specificity of amiloride, as it was shown to result in changes to albumin uptake in kidney cells where uptake is thought to occur primarily via clathrin-dependent mechanisms (Gekle *et al.*, 1999). However, in our studies we have shown that albumin uptake is not mediated via dynamin (and presumably clathrin-dependent) mechanisms, but is sensitive to amiloride. Amiloride may also have non-specific effects on cellular function, as it has been reported to inhibit DNA, RNA and protein synthesis, as well as protein kinase function (Kleyman and Cragoe, 1988).

5.4.5. Future work

Additional work for this study could include measurement of vitamin D uptake into placental tissue via mass spectrometry. This would have a major advantage, as it would give a direct measure of vitamin D uptake. This may reduce some of the variation in the data, which could have arisen from differences in *CYP27B1* expression within the placentas used. Furthermore, measurement of $1,25(OH)_2D$ and 25(OH)D within placental fragments incubated with 25(OH)D could give a measure of the percentage conversion to $1,25(OH)_2D$. Measurement of $1,25(OH)_2D$ within the placental villous fragments would enable analysis of whether the effects observed with combined treatment with 25(OH)D and albumin were due to increased conversion of 25(OH)D into $1,25(OH)_2D$ or whether they were mediated by the presence of albumin. In addition, this work could be extended using the placental perfusion system. This system would allow investigation of uptake of the various forms of vitamin D, as well as examination of the forms transported into the fetal circulation. As mentioned in section 5.4.1 it is currently unclear whether $1,25(OH)_2D$ is transported across the placenta to the fetus. Investigation of transfer of this metabolite in the placental perfusion system would answer this question, and may provide clues as to whether 25(OH)D and $1,25(OH)_2D$ perform differing roles during pregnancy. Additionally, as with future placental fragment experiments, the placental perfusion system could also be used to calculate a percentage of $1,25(OH)_2D$ conversion within the placenta from maternal 25(OH)D.

A more thorough investigation into 25(OH)D uptake would be interesting. The preliminary data presented here suggest pinocytosis may play a role in albumin-mediated uptake of 25(OH)D. However, other endocytic blockers were not investigated. In addition, the role of DBP on 25(OH)D uptake was not assessed. DBP has a higher affinity for 25(OH)D than albumin, therefore even more profound effects on uptake may be present. DBP-mediated uptake may occur via a different mechanism to albumin-mediated uptake, so investigation of the endocytic mechanisms of this uptake would provide a nice comparison with albumin-mediated 25(OH)D uptake. A wider range of endocytic inhibitors could also be used to clarify mechanisms of both 25(OH)D and $1,25(OH)_2D$ entry into the placenta. Gentamicin is a relatively low affinity competitive inhibitor of megalin, therefore a more potent inhibitor, such as RAP (Nykjaer *et al.*, 2001), may clarify whether megalin plays a role in placental

vitamin D uptake. The role of FcRn could also be investigated, as this has previously been shown to bind albumin (Sarav *et al.*, 2009; Andersen *et al.*, 2012). It is possible that this receptor, rather than megalin or cubilin, may mediate albumin bound vitamin D entry into the placenta. Clathrin-dependent endocytosis was not directly inhibited due to the issues with chlorpromazine hydrochloride, therefore another inhibitor for this form of endocytosis could prove useful. Hypertonic treatment of cells has been shown to inhibit clathrin-mediated endocytosis (Cortese *et al.*, 2008), while methyl-β-cyclodextrin inhibits clathrin- and caveolin-dependent endocytosis (Lambot *et al.*, 2006).

Confocal microscopy work could also be expanded upon to further dissect the endocytic mechanisms of vitamin D entry into the placenta. Fluorescent staining of vesicular components, such as clathrin, in villous fragments incubated with FITC-albumin would help to elucidate the mechanisms involved in endocytic uptake of vitamin D in the placenta. Fluorescent staining of vesicular components would give clues as to which endocytic mechanism is prevalent when the endocytic pathways in the cell have not been modified by inhibitors. Additionally, immunofluorescent staining of megalin and cubilin within the placental fragments would be interesting, as a previous study described a relationship between the distribution of FITC-albumin uptake and megalin and cubilin localisation (Zhai *et al.*, 2000). In addition, these experiments could be repeated using fluorescently tagged vitamin D and DBP, to explore potential differing mechanisms of uptake.

Further work on the actions of vitamin D on placental mRNA expression could include verification of RNA sequencing data by qrt-PCR. In addition, due to time constraints, RNA sequencing data were only analysed for genes of interest for this thesis. RNA sequencing revealed many more genes with altered expression in response to 25(OH)D or 25(OH)D with albumin. Pathway analysis on RNA sequencing data would reveal specific pathways that may be altered in response to 25(OH)D, as opposed to the single gene approach taken here. In addition, data from RNA sequencing also shows differences in splice variants of genes in response to the different 25(OH)D treatments, therefore analysis of these data would provide more information on the actions of 25(OH)D on placental mRNA expression.

5.4.6. Conclusions

In conclusion, this study has shown that 25(OH)D is metabolised within the human placenta and that uptake of vitamin D into the placenta is likely to occur via a specific mechanism. Specifically, 25(OH)D uptake is greatly increased by the presence of albumin, implying uptake of 25(OH)D-albumin complexes. Moreover, uptake of $1,25(OH)_2D$ into the placenta has been demonstrated. Uptake of $1,25(OH)_2D$ does not appear to be increased via albumin or DBP. Investigation of the potential mechanisms for uptake have revealed that pinocytosis may play a role in the uptake of both

25(OH)D and 1,25$(OH)_2$D. However, further work is needed to explore the mechanisms of uptake in much greater detail.

Chapter 6:

The effect of maternal vitamin D supplementation during pregnancy on placental mRNA expression

6.1. Introduction

6.1.1. Maternal vitamin D supplementation during pregnancy

Maternal vitamin D levels during pregnancy have been associated with increased fetal growth and improved bone development, as well as childhood lung function, neurocognitive development and bone mass (Javaid *et al.*, 2006; Bowyer *et al.*, 2009; Mahon *et al.*, 2010; Hart *et al.*, 2015). However, as discussed in a recent systematic review, these associations are not consistently found. Although, a meta-analysis of studies which were adjusted for potential confounders did reveal a positive relationship between maternal vitamin D levels and offspring birth weight (Harvey *et al.*, 2014a).

The prevalence of vitamin D deficiency among women of childbearing age is relatively high and varies with country of residence, ethnicity and season of sampling. 61% of mothers of Arab or South Asian origin in the United Arab Emirates had serum 25(OH)D levels below 25 nmol/l (Dawodu *et al.*, 2001). In a Southampton cohort, 21.2 % of women had levels below 27.5 nmol/l, while 28.3 % had levels between 28.3 to 50 nmol/l (Gale *et al.*, 2008). This suggests that in the region of the UK being studied here ~50% of women of childbearing age have vitamin D levels below the Institute of Medicine recommended guidelines for vitamin D sufficiency (IOM, 2011). Maternal and cord serum 25(OH)D levels are highly correlated (Markestad, 1984; Bowyer *et al.*, 2009; Hollis *et al.*, 2011; Dawodu *et al.*, 2013), therefore if the mother is vitamin D deficient this means the offspring is at high risk of vitamin D deficiency. Vitamin D supplementation during pregnancy could be used to prevent maternal and therefore fetal vitamin D deficiency. Trials of vitamin D supplementation during pregnancy have shown increased 25(OH)D levels in both maternal and cord serum (Brooke *et al.*, 1980; Hollis *et al.*, 2011; Dawodu *et al.*, 2013). However, other maternal and neonatal outcomes from these trials are contradictory. One study showed a trend for increased birth weight with maternal vitamin D supplementation alongside a non-significant reduction in SGA offspring (Brooke *et al.*, 1980), while another study showed no effect of vitamin D supplementation on offspring birth weight, length or head circumference (Dawodu *et al.*, 2013). Maternal vitamin D supplementation does appear to have a positive association on neonatal calcium levels (Brooke *et al.*, 1980; Harvey *et al.*, 2014a), however this effect is not always observed (Dawodu *et al.*, 2013; Yang *et al.*, 2015). The results of maternal vitamin D supplementation on many aspects of fetal and neonatal growth are summarised in Harvey *et al.* (2014a). This systematic review shows that there are currently no clear effects of vitamin D supplementation on neonatal parameters.

The seasonal aspect of vitamin D is well documented, and needs to be taken into consideration when interpreting data from vitamin D supplementation studies. As one of the major sources of vitamin D_3 is sunlight, circulating 25(OH)D levels tend to peak in summer and dip in winter months. This has

been observed in numerous studies of vitamin D levels in pregnant women and their neonates (Marwaha *et al.*, 2011; Walsh *et al.*, 2013; Dovnik *et al.*, 2014; Godang *et al.*, 2014; Lechtermann *et al.*, 2014; Luque-Fernandez *et al.*, 2014; Gidlof *et al.*, 2015) and reflects variation in vitamin D_3 as opposed to vitamin D_2 levels (Luque-Fernandez *et al.*, 2014). Associations between cord blood 25(OH)D levels and measures of fetal growth differ depending on season of delivery. For neonates born in winter months, cord blood 25(OH)D was positively associated with head circumference at 20 weeks of gestation, and femur length at 20 and 34 weeks of gestation. While early pregnancy and 28 weeks of gestation maternal 25(OH)D levels were positively associated with femur length at 20 and 34 weeks of gestation, respectively. For neonates born in summer months, only a positive association between cord blood 25(OH)D and head circumference was observed (Walsh *et al.*, 2013), suggesting that during the summer vitamin D is not limiting for fetal growth. The relationship between pre-eclampsia and 25(OH)D levels is also altered by season of delivery. In summer, patients with pre-eclampsia had significantly lower 25(OH)D levels at delivery compared to healthy controls. During winter months, this difference was not observed and pre-eclamptic patients did not show the typical seasonal variation in 25(OH)D levels that was observed in healthy controls (Lechtermann *et al.*, 2014). This seasonal variation may mean that effects of supplementation are more prominent in winter compared to summer months. Analysis of the effects of supplementation will be separated by season in this study.

Both maternal and cord blood 25(OH)D levels are consistently increased following maternal vitamin D supplementation, however inconsistent effects on outcomes such as fetal growth or bone development are observed. This leads to the question of what may be modifying the effects of the supplement. Differences in methodology are likely to underlie some of the differences observed, such as the differing populations studied, doses of supplement and dosing regimen. However, there are currently no data for the effect of maternal vitamin D supplementation on the placenta. *Ex vivo* studies using cell culture and placental explants have looked at the effect of adding vitamin D on mRNA expression, and observational studies report associations between maternal vitamin D levels and placental mRNA expression (see Chapter 1 section 1.6.3). However, there are currently no studies which investigate the effects of maternal vitamin D supplementation on placental mRNA expression. It could be that the differences in fetal and neonatal outcomes observed in many studies are a result of variation in placental function, and the placental response to and mediation of vitamin D effects.

6.1.2. Potential actions of vitamin D on placental function

Vitamin D could affect expression of genes involved in nutrient transport, epigenetics and metabolism as well as those involved in the transport of and response to vitamin D itself. These effects on placental function may alter fetal growth (Figure 6.1). Whilst the effect of vitamin D supplementation on these pathways within the human placenta has not previously been studied, animal and cell culture

studies as well as human observational studies have linked maternal vitamin D status with expression of a wide range of genes.

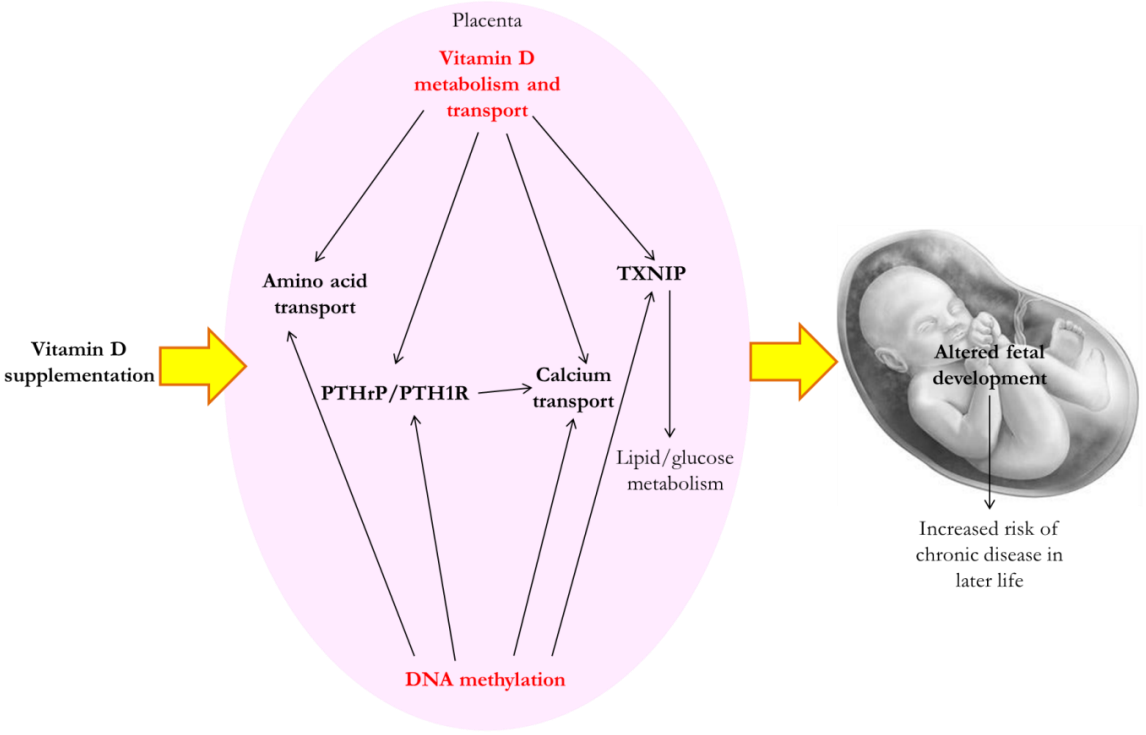

Figure 6.1: Potential effects of maternal vitamin D supplementation on placental function. Vitamin D supplementation could directly affect placental transport and metabolism of vitamin D, amino acid and calcium transport, PTHrP, PTH1R and TXNIP expression and DNA methylation. Furthermore, these effects may have consequences on each other. As indicated in red DNA methylation and vitamin D transport and metabolism within the placenta could affect all the other aforementioned functions, while PTHrP may also affect calcium transport. These effects may then impact upon fetal growth which has implications for the health of the offspring in later life.

Vitamin D transport and metabolism

Vitamin D generally operates via a negative feedback system where increased $1,25(OH)_2D$ results in increased mRNA expression of *CYP24A1*, the 25(OH)D and $1,25(OH)_2D$ catabolism enzyme, and reduced expression of *CYP27B1*, the vitamin D activation enzyme (Avila *et al.*, 2007b; Turunen *et al.*, 2007). Genes not involved in this feedback loop may be regulated by vitamin D as *VDR* and *megalin* mRNA have been shown to be up-regulated by vitamin D (Chlon *et al.*, 2008; Pojednic *et al.*, 2015). Associations between maternal 25(OH)D levels and placental mRNA expression of *CYP24A1*, *CYP27B1*, *VDR* and *RXRa* have also been described (Cho *et al.*, 2013; Lechtermann *et al.*, 2014; O'Brien *et al.*, 2014; Vijayendra Chary *et al.*, 2015), suggesting that vitamin D may also regulate expression of placental vitamin D-related genes. Furthermore, placental expression of these genes has been associated with fetal growth (Young *et al.*, 2014; Nguyen *et al.*, 2015), suggesting that vitamin D handling by the placenta may be involved in some pregnancy pathologies and may impact fetal growth and development. These impacts on fetal growth and development may lead to an increased risk of chronic disease in offspring in later life.

Nutrient transport

Vitamin D may have effects on amino acid and calcium transporter expression within the placenta. Placental amino acid and calcium transport are critical for normal fetal growth and development (Jansson *et al.*, 2006; Martin *et al.*, 2007; Cleal *et al.*, 2011). Links between vitamin D and amino acid transport first emerged due to observations of aminoaciduria in rachitic children (Jonxis *et al.*, 1952; Jonxis and Huisman, 1953) and transporter regulation via vitamin D may be tissue-specific. This idea is supported by studies on the effects of vitamin D on calcium transporter expression, for example *PMCA1* mRNA was increased in intestine, kidney and osteoblasts cells in response to active vitamin D, but no effect was observed in cochlea cells (Glendenning *et al.*, 2000, 2001; Walters *et al.*, 2007; Balesaria *et al.*, 2009). In Chapter 3, data were presented demonstrating associations between maternal vitamin D and DBP levels and placental *CYP24A1*, a vitamin D responsive gene, with mRNA expression of amino acid transporters and *PMCA1*. This indicates that vitamin D may mediate changes to calcium and amino acid transport within the placenta.

DNA methylation

Vitamin D may play a role in mediation of DNA methylation. This could occur through vitamin D-directed changes to DNMT mRNA expression. In this study mRNA expression of *DNMT1* will be investigated as it was positively associated with *CYP24A1* mRNA expression in placentas from the SWS cohort. This DNMT will be focused on as opposed to DNMT3b, as DNMT1 plays a larger role in maintenance methylation (Bird, 2002). As we are investigating placental samples at term, maintenance methylation is likely to play a larger role in the DNA methylation status of genes. This idea is supported by the fact that *DNMT1* mRNA expression increases in third trimester placenta samples in comparison to first trimester samples (He *et al.*, 2014).

Regulatory systems

Vitamin D has been linked with TXNIP and PTHrP. Both of these molecules can affect a variety of processes which could result in further alterations to placental function. TXNIP is involved in mediation of mRNA transcription and regulation of insulin secretion, glucose homeostasis and fatty acid metabolism (Chung *et al.*, 2006; Chong *et al.*, 2014), while PTHrP may play a role in regulation of calcium transport. Vitamin D has been shown to increase mRNA expression of *TXNIP* (Nishiyama *et al.*, 1999; Hamilton *et al.*, 2014) and reduce protein expression of PTHrP and PTH1R (Kovacs *et al.*, 2005; Bach *et al.*, 2014). In addition, PTHrP has been shown to increase *VDR* expression, therefore increasing the responsiveness to vitamin D (Bach *et al.*, 2014). If these genes are also responsive to vitamin D within the placenta they may mediate additional effects of vitamin D within the placenta

6.1.3. Aims

The aim of this study is to investigate the effects of vitamin D supplementation during pregnancy on placental mRNA expression. Expression levels of genes involved in vitamin D handling, amino acid and calcium transport, DNA methylation and metabolic functions will be measured in placentas from mothers on a randomised placebo controlled trial of vitamin D supplementation during pregnancy. Specifically the aims of this study are:

1. Does vitamin D supplementation during pregnancy alter expression of placental genes involved in vitamin D, nutrient transport and epigenetic functions?
2. Does mRNA expression within the placenta associate with maternal 25(OH)D or albumin levels?
3. Does mRNA expression within the placenta associate with neonatal outcomes, such as birth weight?

6.2. Methods

qrt-PCR was used to measure mRNA levels of vitamin D-related genes as well as amino acid and calcium transporter genes in placenta samples from a randomised, double-blind placebo controlled trial of vitamin D supplementation during pregnancy. The effects of maternal vitamin D levels and vitamin D supplementation on placental mRNA expression were investigated. Placental mRNA expression was also tested for associations with fetal and neonatal growth measures. These studies were conducted according to the guidelines in the Declaration of Helsinki, and the Southampton and South West Hampshire Research Ethics Committee approved all procedures. Written informed consent was obtained from all participating women and by parents or guardians with parental responsibility on behalf of children.

6.2.1. MAVIDOS

qrt-PCR analysis was carried out on placental samples from MAVIDOS, a randomised, double-blind placebo controlled trial of vitamin D supplementation during pregnancy (see section 2.2.2). Women were recruited to the study around 12 weeks of gestation and circulating 25(OH)D levels were measured. Those with 25(OH)D levels between 25-100 nmol/l were then recruited to the study and randomised to receive placebo or vitamin D supplementation (1000 iu daily) from 14 weeks of gestation until delivery of the baby. Maternal blood samples were taken at 14 and 34 weeks of gestation for measurement of serum 25(OH)D, albumin and calcium. Following delivery, placental weight was recorded and 10 samples from each placenta were collected using a random sampling grid and snap frozen within 30 min of delivery. The samples from each placenta were pooled and ground together in a frozen tissue press. Neonatal anthropometry measurements (weight, crown-heel length, subscapular and triceps skinfold thicknesses, and head, mid-upper arm, chest and abdominal circumferences) were taken and neonatal DXA was carried out to measure whole body BA, BMC, and BMD as well as lean and fat mass. Maternal, fetal and infant measurements were taken by the MAVIDOS Study Group (Harvey *et al.*, 2012a). A total of 72 placental samples were collected from MAVIDOS, as described in General methods 2.2.3. Following exclusion for samples which did not have serum vitamin D measures a total of 69 samples remained, which were collected from both vaginal delivery and caesarean section delivery (Table 6.1). The proportion of natural and caesarean section deliveries did not differ significantly between the two treatment groups.

Table 6.1: Total number of placental samples collected from MAVIDOS with mode of delivery.

Treatment	Total number	Natural delivery	Caesarean section	Unknown
Placebo	38	25	11	2
Vitamin D	31	19	12	N/A
TOTAL	69	44	23	2

6.2.2. mRNA expression

RNA was extracted using the miRNeasy kit as described in section 2.5.2. RNA quality was determined through bioanalyser measurements. cDNA was synthesised from 0.5 μg RNA using the Promega M-MLV rt method as described in Chapter 2 section 2.5.4. NECs were synthesised at the same time as cDNA.

qrt-PCR was carried out to determine expression levels of genes of interest as described in section 2.5.8 using Roche UPL and Primer Design Perfect Probe assays. geNorm analysis was conducted on 5 placebo treated samples and 5 vitamin D treated samples to investigate the effect of maternal vitamin D supplementation on HKG expression and stability. The HKGs tested were *ATP5B*, *β-actin*, *CYC1*, *GAPDH*, *RPL13A*, *SDHA*, *TOP1*, *UBC* and *YWHAZ*. For each qrt-PCR assay, samples (4 ng) were run alongside a standard curve, CV controls, NECs and NTCs in triplicate (Table 2.6). UPL qrt-PCR cycling conditions were 95°C for 10 min; 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 s; 50°C for 30 s, with data collection at the 72°C step. Perfect Probe target gene qrt-PCR cycling conditions were 95°C for 10 min; 50 cycles of 95°C for 10 s and 60°C for 60 s and; 50°C for 30 s, with data collection at the 60°C step. Perfect Probe HKG qrt-PCR cycling conditions were 95°C for 10 min; 50 cycles of 95°C for 10 s, 50°C for 30 s and 72°C for 15 s; 50°C for 30 s, with data collection at the 50°C step. Cp values were determined by the second derivative method and were converted to DNA concentration (ng/5 μl) using a standard curve (see General methods 2.5.8). Target gene primer sets are displayed in Table 6.2. Expression levels of genes of interest were normalised to the geometric mean of the HKGs selected following geNorm analysis.

Table 6.2: Primers and probes used for qrt-PCR on MAVIDOS samples.

Gene	Accession number	Primer sequence (5'-3')	UPL number	Amplicon (bp)
		Amino acid transporter genes		
ASCT1	NM_001193493.1,	F: TTTGCGACAGCATTTGCTAC	78	1189 – 1248
	NM_003038.4	R: GCACTTCATCATAGAGGGAAGG		1289 – 1348
ASCT2	NM_001145144.1,	F: GAGGAATATCACCGGAACCA	43	114 – 179
	NM_001145145.1,	R: AGGATGTTCATCCCCTCCA		304 – 369
	NM_005628.2			1250 – 1315
LAT1	NM_003486.5	F: GTGGAAAAACAAGCCCAAGT	25	1503 – 1573
		R: GCATGAGCTTCTGACACAGG		
LAT3	NM_001198810.1,	F: GCCCTCATGATTGGCTCTTA	29	623 – 642
	NM_003627.5	R: CCGGCATCGTAGATCAGC		794 – 864
LAT4	NM_001284498.1,	F: ACAAGTATGGCCCGAGGAA	3	673 – 742
	NM_152346.2	R: GCAATCAGCAAGCAGGAAA		471 – 540
SNAT1	NM_001077484.1,	F: ATTTTGGGACTCGCCTTTG	47	1034 – 1110
	NM_001278387.1,	R: AGCAATGTCACTGAAGTCAAAAGT		1496 – 1572
	NM_001278388.1,			1258 – 1334
	NM_001278389.1,			523 – 599
	NM_001278390.1,			1236 – 1312
	NM_030674.3			1373 – 1449
TAT1	NM_018593.4	F: GGTGTGAAGAAGGTTTATCTACAGG	6	1238 – 1329
		R: AGGGCCCCAAAGATGCTA		
		Calcium transporter genes		
Calbindin-D9K	NM_004057.2	F: CCCAGTGAAGGAGAAAACAAA	69	287 – 380
		R: AGAGACTTTGGGGGATTCCA		
Calbindin-D28K	NM_004929.2	F: AGATCTGGCTCCATTTCGAC	2	237 – 315
		R: TGGAGCTCCTGGATCAAGTT		
CaT1	NM_018646.5	F: TCTGCAGATGGTTCCAGAGA	62	285 – 380
		R: GAAGGAGAGGAGACTCCCAGA		
CaT2	NM_019841.6	F: GGCAACCGCACTCATTCT	9	1333 – 1413
		R: GATATCTTCACGTGTCTCATAGGC		
NCX1	NM_001112800.1,	F: GCCCTTGTGGTTGGGACT	78	2215 – 2306
	NM_001112801.1,	R: CCACATTCATCGTCGTCATC		2191 – 2282
	NM_001112802.1,			2209 – 2300
	NM_001252624.1,			2206 – 2297
	NM_021097.2			2230 – 2321
NCX2	NM_015063.2	F: CTACGTGGACTACCGCACTG	53	1559 – 1650
		R: TCTCGCCTGGTTTGAACAC		
NCX3	NM_001130417.2,	F: GGCATTTGGCACCTCTGT	74	836 – 910
	NM_033262.4,	R: GAGGCGTCTGCATATACATCCT		3133 – 3207
	NM_058240.3,			3130 – 3204
	NM_182932.2,			3121 – 3195
	NM_182936.2,			732 – 806
	NM_183002.2			3139 – 3213
PMCA1	NM_001001323.1,	F: CCATAGTATCATTGGGCCTTTC	75	534 – 620
	NM_001682.2	R: CTTCTTCCTCCCCAACAGAA		534 – 620
PMCA4	NM_001001396.2,	F: GCACACCCTGGTGAAAGG	9	3180 – 3275
	NM_001684.4	R: AGAGCAGGCCCGTCATTT		3180 – 3275
		Vitamin D genes		
Cubilin	NM_001081.3	F: GGACAATGTCAGAATAGCTTCGT	10	8624 – 8696
		R: CAGTGGCTAGCAGGGCTTT		
CYP24A1	NM_001128915.1,	F: GAAAGAATTGTATGCTGCTGTCA	78	1337 – 1408
	NM_000782.4	R: CACATTAGACTGTTTGCTGTCGT		1337 – 1408
CYP27B1	NM_000785.3	F: CATTCATGTGGGTGACTATATTATCC	Perfect	1334 – 1435
		R: AAAGAATTTGGCTCTGGGAACT	Probe	
Megalin	NM_004525.2	F: TTGTTTTGATGCCTCTGATGA	34	8877 – 8937
		R: AGCTAGGCATGTTCGCTCAG		
RXRα	NM_001291920.1,	F: ACATGCAGATGGACAAGACG	26	1917 – 1994
	NM_001291921.1,	R: TCGAGAGCCCCTTGGAGT		1708 – 1785
	NM_002957.5			1245 – 1322
VDR	NM_001017535.1,	F: TCTGTGACCCTAGAGCTGTCC	43	928 – 1055
	NM_001017536.1,	R: TCCTCAGAGGTGAGGTCTCTG		1197 – 1324
	NM_000376.2			806 – 933
		Other genes		
DNMT1	NM_001130823.1,	F: CGATGTGGCGTCTGTGAG	66	2182 – 2225
	NM_001379.2	R: TGTCCTTGCAGGCTTTACATT		2134 – 2197

PTH1R	NM_001184744.1,	F: CCTGAGTCTGAGGAGGACAAG	62	331 – 416
	NM_000316.2	R: CACAGGATGTGGTCCCATT		468 – 553
PTHrP	NM_002820.2,	F: CTCGGTGGAGGGTCTCAG	25	397 – 477
	NM_198964.1,	R: TGGATGGACTTCCCCTTGT		378 – 458
	NM_198965.1,			397 – 477
	NM_198966.1			378 – 458
TXNIP	NM_006472.4	F: AACATCCCTGATACCCCAGA	52	1332 – 1401
		R: TCTCCAATCGGTGATCTTCA		

F = forward primer, R = reverse primer.

rt-PCR was carried out on primer sets that did not amplify any product in placental tissue to ensure that lack of amplification was as a result of an absence of gene expression. rt-PCR was run on placental tissue and a positive control tissue in which the target gene was known to be highly expressed, as described in section 2.5.5. PCR products were run on 4% agarose gels and visualised with UV light (see section 2.5.6) to determine the presence or absence of product amplification.

6.2.3. Data analysis

geNorm analysis was carried out in qbase+ software (as described in section 2.5.8) to determine the stability of expression of each HKG in the sample set and the number of HKGs required for accurate normalisation of target genes. geNorm data were further analysed in SPSS. Cp values were tested for normal distribution and log transformed or square rooted if not normally distributed. A t-test with Levene's equality of variance test was used to investigate whether mRNA expression differed according to treatment group. For HKGs that were still not normally distributed following transformation a Kruskal Wallis test was performed. A t-test of the three selected HKGs on the full sample set revealed a trend for a change in *RPL13A* mRNA expression with vitamin D supplementation, therefore mRNA expression data were normalised to the geometric mean of the remaining two HKGs.

The remainder of the MAVIDOS data analysis was carried out by S. D'Angelo (MRC LEU, Southampton) in Stata (StataCorp LP, Texas, USA). Data were tested for normal distribution and log transformed if non-normally distributed. Data were analysed as a whole set and separately by season of birth. Season of birth was defined as spring: March-May, summer: June-August, autumn: September-November and winter: December-February. Linear regression was used to analyse the effect of vitamin D supplementation on placental gene expression, with sex and gestational age included in the model. Data are presented as mean + SEM or β value with 95% CIs. Pearson's correlation was used to test mRNA expression data for associations with maternal plasma vitamin D, calcium and albumin measures at 14 and 34 weeks of gestation, as well as with *CYP24A1* mRNA expression as a potential measure of active vitamin D. Gene expression values were adjusted for sex and gestational age prior to correlation analysis. Placental mRNA expression data were also tested for associations with neonatal anthropometry and measurements of bone indices using Pearson's correlation. Correlation data are

presented as Pearson's correlation r values. $p < 0.05$ was considered statistically significant and $p < 0.1$ was considered a trend. Significant results and trends displayed, and the full data set is presented in Appendix 6.

Sample size

No previous data were available on MAVIDOS placental samples to conduct power calculations prior to investigation, therefore retrospective power calculations were conducted using Sigma Plot version 12.5. These power calculations showed that within this data set there is a power of 0.92 to detect an r value of 0.4 at a p value of 0.05, while there is a power of 0.6 to detect an r value of 0.3 at a p value of 0.05.

6.3. Results

6.3.1. Housekeeper gene analysis

geNorm analysis compared the stability of nine HKGs in a sub-set of MAVIDOS placental samples. To determine the appropriate HKGs for data normalisation geNorm was carried out on placebo treated samples, vitamin D treated samples and placebo and vitamin D treated samples combined. V values demonstrated that two or three HKGs were adequate for normalisation of qrt-PCR data (Figure 6.2), as the V value was below 0.15. The addition of further HKGs did not really affect the V value, indicating that the addition of further HKGs was of no benefit.

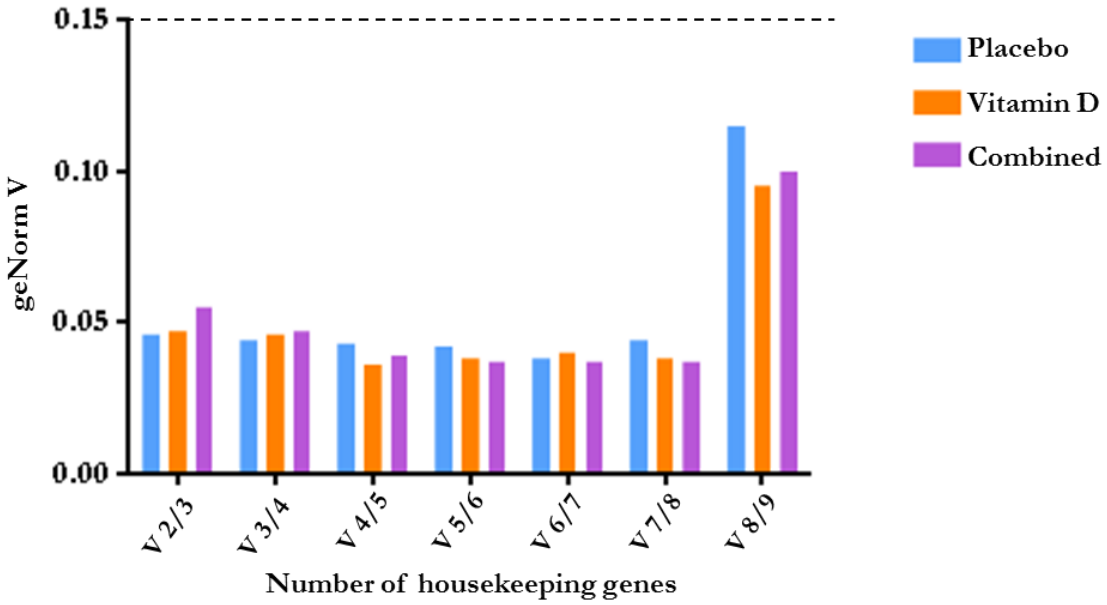

Figure 6.2: The number of genes required for normalisation of MAVIDOS placenta qrt-PCR data. The graph indicates that the use of two or three of the most stable HKGs creates a normalisation factor which is not significantly improved by the addition of further HKGs. A V value below 0.15, as indicated by the dashed line, is deemed a suitable normalisation factor.

HKG stability was slightly different between the three conditions tested but largely showed the same patterns of most and least stable HKGs (Figure 6.3).

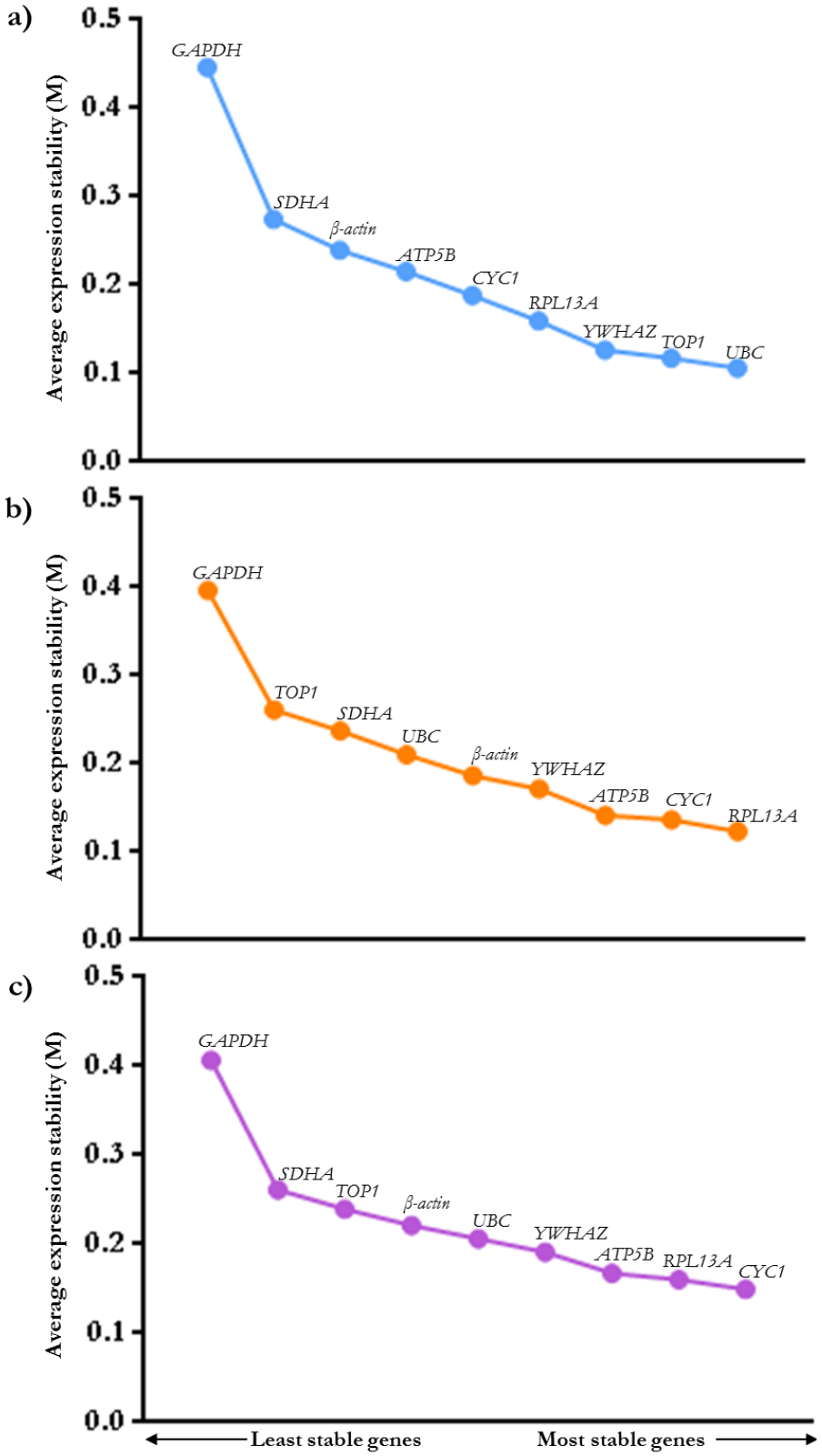

Figure 6.3: The most stably expressed HKGs for a) placebo treated samples, b) vitamin D treated samples, and c) placebo and vitamin D treated samples combined. The average expression stability value (M) for each HKG ranked according to increasing stability with the most stable genes on the right.

The difference in cp values for each HKG was tested to ensure that supplementation did not alter HKG expression levels. None of the HKGS tested were significantly altered by maternal vitamin D

supplementation (Figure 6.4). This information combined with the geNorm M and V values led to the choice of three HKGs for qrt-PCR data normalisation: *CYC1*, *RPL13A* and *YWHAZ*. However, on the full placental sample set *RPL13A* showed a trend (p = 0.099) for reduced mRNA expression with vitamin D supplementation therefore data were adjusted to the geometric mean of the two remaining HKGs.

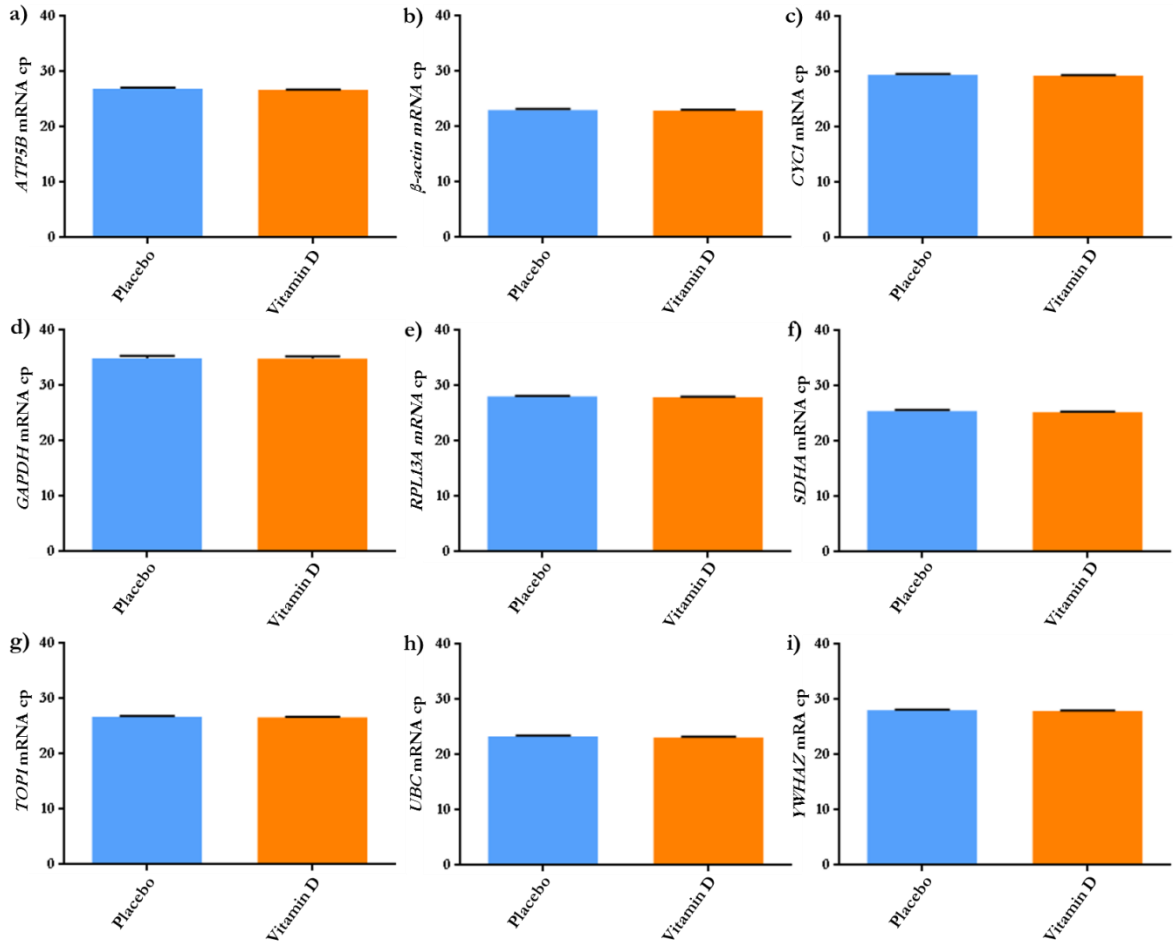

Figure 6.4: Expression levels of HKGs in placebo and vitamin D treated MAVIDOS placenta samples. None of the HKGS tested were significantly affected by maternal vitamin D supplementation during pregnancy in this sub-set of samples. a) *ATP5B*, b) *β-actin*, c) *CYC1*, d) *GAPDH*, e) *RPL13A*, f) *SDHA*, g) *TOP*, h) *UBC* and i) *YWHAZ*. Data are presented as mean cp value + SEM.

6.3.2. The effect of maternal vitamin D supplementation on placental mRNA expression

qrt-PCR assays for *calbindin-D9K*, *calbindin-D28K*, *CaT2*, *NCX2* and *NCX3* failed to amplify any product in placental tissue. rt-PCR was carried out for *calbindin-D9K*, *calbindin-D28K* and *CaT2* on placental and liver (positive control) cDNA. *Calbindin-D28K* was clearly amplified in liver, whilst *calbindin-D9K* and *CaT2* showed faint amplification in liver cDNA. No amplification in placental cDNA was observed for any of the three genes tested (Figure 6.5).

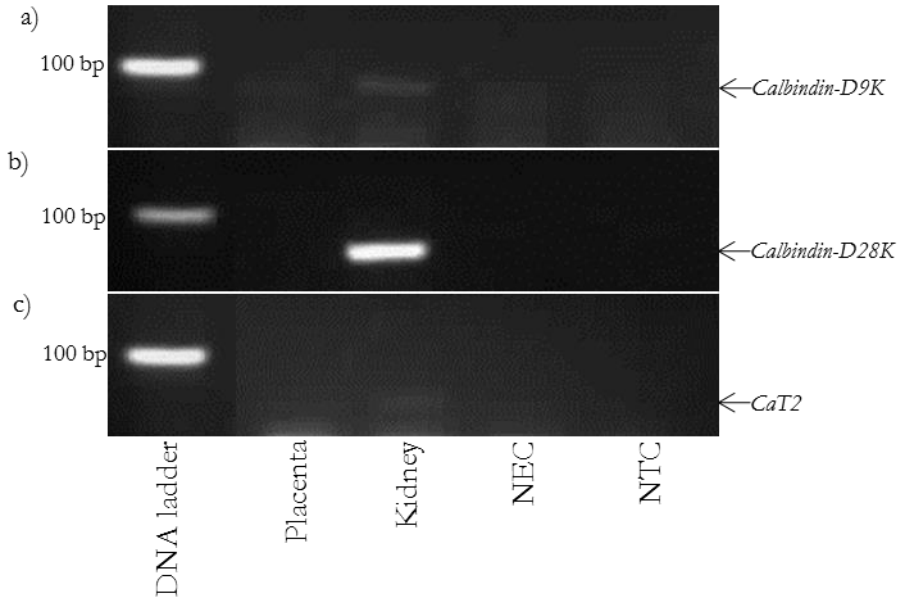

Figure 6.5: rt-PCR for a) *calbindin-D9K*, b) *calbindin-D28K* and *CaT2* in placenta and kidney cDNA. *Calbindin-D9K* product size = 94 bp, *Calbindin-D28K* product size = 79 bp and *CaT2* product size = 81 bp.

Primer sets for *NCX2* and *NCX3* could not be tested, due to the lack of positive control tissue (brain). The assay for *PTH1R* did amplify product in placental tissue, but the slope of the amplification curve was not very steep, -1.8 compared to the ideal value of -3.3, therefore it was decided that this primer set required further optimisation before use.

In this sub-set (n = 69) of the MAVIDOS cohort mean (SD) maternal vitamin D levels at baseline were 48.16 (18.83) and 49.14 (18.47) nmol/l for placebo and vitamin D supplemented, respectively. At 14 weeks of gestation 25(OH)D levels ranged from 15.3 to 93.95 nmol/l in placebo treated women, and 17.5 to 94.4 nmol/l in vitamin D treated women. At 34 weeks of gestation mean (SD) maternal vitamin D levels were 44.85 (20.42) and 75.77 (17.82) nmol/l for placebo and vitamin D treated, respectively. 25(OH)D levels ranged from 9.57 to 100.65 nmol/l in placebo treated women and from 46.2 to 107.5 nmol/l in vitamin D supplemented women. Initial analysis revealed no significant differences in placental mRNA expression in response to maternal vitamin D supplementation during pregnancy. After the analysis was stratified by season of birth, maternal vitamin D supplementation was shown to affect placental mRNA expression of *ASCT2* and *cubilin* in specific seasons, while *SNAT1* mRNA showed a trend ($p = 0.07$) for reduced expression in winter with supplementation (Figure 6.6).

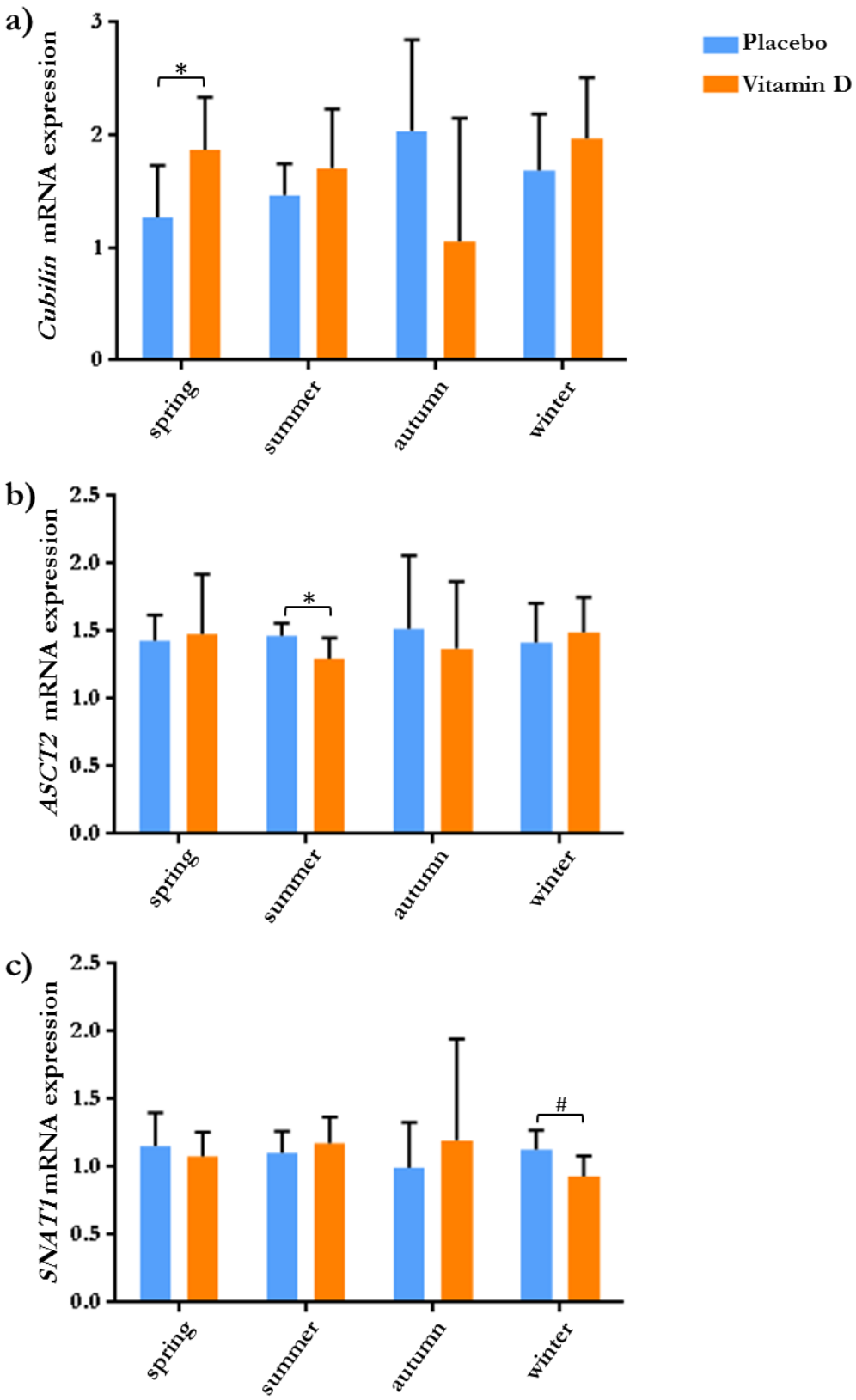

Figure 6.6: Effect of vitamin D supplementation on placental relative mRNA expression of a) *cubilin*, b) *ASCT2* and c) *SNAT1* separated by season of birth. a) *Cubilin* mRNA was significantly up-regulated by vitamin D supplementation during spring, b) *ASCT2* mRNA was significantly reduced by maternal vitamin D supplementation in summer and c) *SNAT1* mRNA showed a trend for reduced expression with maternal vitamin D supplementation in winter. Sample numbers for placebo treated samples are: spring n = 8, summer n = 12, autumn n = 7 and winter n = 11. Sample numbers for vitamin D treated samples are: spring n = 9, summer n = 11, autumn n = 3 and winter n = 8. Data are presented as mean + SEM. * $p < 0.5$, # $p < 0.1$.

6.3.3. Associations between maternal vitamin D, albumin and calcium and placental mRNA expression

Placental mRNA expression showed one association with maternal 25(OH)D levels. *CaT1* was positively associated with maternal 25(OH)D levels at 34 weeks of gestation. Maternal serum albumin at 14 weeks of gestation was negatively associated with placental mRNA expression of *cubilin*, *LAT3*, *TAT1*, *NCX1*, *PMCA4*, and *TXNIP*, and showed a negative trend with *PTHrP* expression. 34 week maternal serum albumin showed trends for negative associations with *megalin* and *PTHrP* mRNA expression. Maternal 14 week serum calcium showed trends for negative associations with mRNA expression of *cubilin*, *RXRa*, *ASCT1*, *PMCA4* and *TXNIP*, while a negative trend was observed between maternal 34 week serum calcium and *SNAT1* mRNA expression. Placental mRNA expression was also tested for associations with *CYP24A1* mRNA expression as a potential measure of active vitamin D levels. *CYP24A1* mRNA expression showed strong positive associations with mRNA levels of *cubilin*, *ASCT2*, *TAT1*, *PMCA4* and *DNMT1* as well as a negative association with *ASCT1* (Table 6.3).

Table 6.3: Associations between placental relative mRNA expression and maternal 25(OH)D, albumin and calcium levels.

Gene		14 week 25(OH)D levels (nmol/l)	34 week 25(OH)D levels (nmol/l)	14 week albumin levels (g/l)	34 week albumin levels (g/l)	14 week calcium levels (mmol/l)	34 week calcium levels (mmol/l)	*CYP24A1* mRNA expression
Vitamin D genes								
Cubilin	r	0.08	0.12	**-0.30**	0.11	*-0.22*	0.14	**0.38**
	p	0.50	0.37	**0.01**	0.43	*0.07*	0.31	**0.001**
CYP24A1	r	-0.06	0.08	-0.17	-0.08	-0.01	-0.12	
	p	0.64	0.54	0.16	0.54	0.93	0.39	
CYP27B1	r	-0.05	0.01	0.08	-0.13	0.02	-0.11	-0.004
	p	0.66	0.93	0.49	0.34	0.87	0.41	0.98
Megalin	r	-0.07	0.05	-0.03	*-0.24*	-0.13	-0.06	0.19
	p	0.58	0.73	0.82	*0.08*	0.28	0.68	0.11
RXRα	r	-0.04	-0.10	0.04	0.12	*-0.2*	-0.12	-0.08
	p	0.77	0.48	0.76	0.37	*0.09*	0.37	0.52
VDR	r	0.03	0.18	-0.01	0.03	-0.00	0.18	0.13
	p	0.82	0.18	0.95	0.85	0.98	0.17	0.27
Amino acid transporter genes								
ASCT1	r	-0.01	0.06	-0.17	-0.15	*-0.21*	0.14	**-0.25**
	p	0.94	0.65	0.16	0.28	*0.08*	0.29	**0.04**
ASCT2	r	0.06	-0.09	-0.12	0.07	-0.13	0.06	**0.33**
	p	0.65	0.51	0.31	0.60	0.29	0.66	**0.01**
LAT1	r	0.08	0.18	-0.05	0.12	-0.14	-0.09	-0.15
	p	0.53	0.19	0.69	0.36	0.26	0.50	0.20
LAT3	r	-0.04	0.06	**-0.29**	-0.05	-0.2	-0.06	0.16
	p	0.74	0.66	**0.02**	0.7	0.1	0.64	0.17
LAT4	r	0.09	0.01	-0.03	0.21	-0.08	-0.03	-0.20
	p	0.45	0.95	0.79	0.13	0.51	0.80	0.10
SNAT1	r	-0.14	0.06	0.13	0.03	-0.15	*-0.25*	-0.01
	p	0.26	0.68	0.31	0.83	0.22	*0.06*	0.94
TAT1	r	-0.04	-0.07	**-0.38**	-0.17	0.02	-0.10	**0.46**
	p	0.75	0.60	**0.001**	0.20	0.86	0.47	**0.001**
Calcium transporter genes								
CaT1	r	0.09	**0.30**	0.11	0.20	-0.06	-0.08	-0.04
	p	0.45	**0.02**	0.37	0.14	0.64	0.57	0.74
NCX1	r	-0.13	0.02	**-0.27**	-0.15	-0.14	0.03	0.19
	p	0.30	0.9	**0.03**	0.27	0.26	0.83	0.11

PMCA1	r	-0.04	0.01	-0.04	0.11	-0.14	-0.02	0.19
	p	0.72	0.93	0.78	0.40	0.24	0.90	0.11
PMCA4	r	-0.05	-0.1	**-0.25**	-0.04	*-0.24*	-0.11	**0.40**
	p	0.71	0.46	**0.04**	0.75	*0.05*	0.42	**0.001**
Other genes								
DNMT1	r	-0.09	0.04	-0.004	-0.09	-0.19	-0.11	**0.40**
	p	0.49	0.74	0.98	0.50	0.12	0.43	**0.001**
PTHrP	r	0.18	-0.10	*-0.21*	*-0.24*	-0.11	0.15	0.03
	p	0.13	0.45	*0.08*	*0.08*	0.35	0.28	0.79
TXNIP	r	-0.05	-0.17	**-0.28**	-0.07	*-0.20*	-0.06	0.19
	p	0.67	0.21	**0.02**	0.62	*0.09*	0.68	0.12

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

6.3.4. Associations between placental mRNA expression and neonatal anthropometry and bone indices

Placental mRNA expression of *CYP24A1* and *megalin* was positively associated with placental weight, while *ASCT1* mRNA levels were negatively associated with placental weight. *TXNIP* mRNA showed a trend for a negative association with placental weight. No associations were observed between placental mRNA expression and birth weight or mid-upper arm circumference of the neonate at birth. *SNAT1* mRNA expression was positively associated with neonatal head circumference, while *megalin* mRNA was positively associated with abdominal circumference and showed a positive trend with chest circumference of the neonate. Neonatal triceps thickness was positively associated with *PMCA4* mRNA, and showed positive trends with *cubilin* and *NCX1* mRNA expression, while *LAT4* and *LAT1* mRNA showed strong negative associations with triceps thickness. *LAT4* mRNA also showed a trend for a negative association with neonatal subscapular thickness. Crown-heel length of the neonate at birth showed a negative trend with *ASCT1* mRNA expression (Table 6.4).

Table 6.4: Associations between placental relative mRNA expression and neonatal anthropometry.

Gene		Placental weight (g)	Birth weight (g)	Head circ (cm)	Chest circ (cm)	Abdominal circ (cm)	Mid-upper arm circ (cm)	Triceps thickness (mm)	Subscapular skinfold thickness (mm)	Crown-heel length (cm)
Vitamin D genes										
Cubilin	r	0.02	0.07	0.08	0.01	-0.02	0.10	*0.22*	0.09	0.08
	p	0.86	0.56	0.54	0.96	0.89	0.45	*0.08*	0.51	0.52
CYP24A1	r	**0.31**	0.16	0.11	0.18	0.17	0.08	0.18	0.13	0.20
	p	**0.01**	0.19	0.38	0.16	0.17	0.51	0.16	0.30	0.12
CYP27B1	r	-0.15	-0.02	-0.11	-0.11	-0.08	-0.01	0.02	-0.13	0.002
	p	0.23	0.90	0.38	0.38	0.51	0.95	0.90	0.30	0.99
Megalin	r	**0.35**	0.17	0.15	*0.22*	*0.25*	0.16	-0.04	-0.04	0.15
	p	**0.003**	0.17	0.24	*0.08*	*0.05*	0.22	0.74	0.78	0.25
RXRα	r	-0.07	-0.11	-0.01	-0.08	0.01	-0.10	-0.15	-0.15	-0.09
	p	0.57	0.39	0.93	0.51	0.95	0.42	0.23	0.25	0.51
VDR	r	-0.04	-0.05	-0.15	-0.02	0.004	0.01	0.06	0.08	-0.05
	p	0.74	0.72	0.23	0.86	0.97	0.94	0.65	0.52	0.70
Amino acid transporter genes										
ASCT1	r	**-0.27**	-0.19	-0.03	-0.20	-0.18	-0.11	-0.04	0.11	*-0.25*
	p	**0.03**	0.12	0.79	0.10	0.16	0.40	0.75	0.37	*0.05*
ASCT2	r	0.05	-0.02	0.02	-0.02	-0.01	0.07	0.19	0.08	0.08
	p	0.66	0.88	0.85	0.87	0.96	0.61	0.13	0.52	0.52
LAT1	r	-0.03	-0.10	0.05	-0.10	-0.17	-0.15	**-0.26**	-0.21	-0.09
	p	0.78	0.43	0.70	0.44	0.19	0.25	**0.04**	0.10	0.50
LAT3	r	-0.12	-0.14	-0.11	-0.17	-0.09	0.03	0.20	0.09	-0.09
	p	0.31	0.25	0.41	0.17	0.50	0.79	0.10	0.49	0.47
LAT4	r	-0.10	-0.14	0.01	-0.09	-0.14	-0.04	**-0.33**	*-0.22*	-0.10
	p	0.41	0.26	0.93	0.50	0.27	0.77	**0.01**	*0.08*	0.44
SNAT1	r	-0.005	0.04	**0.25**	0.04	0.09	-0.02	-0.05	0.04	0.17
	p	0.97	0.75	**0.04**	0.75	0.47	0.88	0.68	0.78	0.20
TAT1	r	0.02	-0.08	0.02	-0.08	-0.02	-0.02	0.17	0.08	-0.10
	p	0.89	0.50	0.91	0.54	0.88	0.87	0.18	0.52	0.46
Calcium transporter genes										
CaT1	r	0.06	0.03	0.18	0.10	0.08	-0.03	-0.06	0.07	0.08
	p	0.60	0.79	0.16	0.44	0.54	0.80	0.63	0.56	0.55
NCX1	r	-0.11	-0.16	-0.16	-0.18	-0.10	-0.07	*0.23*	0.08	-0.13
	p	0.37	0.19	0.20	0.16	0.42	0.58	*0.07*	0.55	0.31

PMCA1	r	-0.13	-0.10	-0.11	-0.14	-0.16	-0.03	0.15	0.02	-0.03
	p	0.28	0.41	0.39	0.25	0.21	0.82	0.25	0.85	0.79
PMCA4	r	0.02	-0.003	-0.08	-0.05	-0.03	0.10	**0.31**	0.15	0.06
	p	0.88	0.98	0.54	0.72	0.83	0.41	**0.01**	0.24	0.62
Other genes										
DNMT1	r	0.17	0.03	0.03	0.004	0.05	0.12	0.12	-0.03	0.13
	p	0.17	0.81	0.79	0.98	0.67	0.33	0.33	0.84	0.31
PTHrP	r	-0.04	0.01	-0.13	-0.08	-0.17	0.08	-0.05	0.01	0.03
	p	0.75	0.94	0.30	0.55	0.17	0.55	0.71	0.95	0.81
TXNIP	r	*-0.23*	-0.20	-0.10	-0.16	-0.20	-0.05	0.18	0.01	-0.12
	p	*0.06*	0.10	0.41	0.21	0.12	0.68	0.17	0.96	0.37

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Placental mRNA expression was also tested for associations with measures of neonatal body composition and bone indices from DXA. *CYP24A1* mRNA levels showed strong positive associations with BMC and BA, as well as trends for positive associations with BMD, lean and fat mass of the neonate. *Megalin* mRNA was positively associated with neonatal lean mass, and showed a trend for a positive association with neonatal BMC, while *RXRa* mRNA showed a trend for a negative association with neonatal BMD. Associations observed with neonatal DXA measurements and placental amino acid transporter expression were negative; *ASCT1* mRNA was negatively associated with neonatal BMC and BA, while *LAT1* mRNA expression was negatively associated with neonatal BMC and BMD. *DNMT1* mRNA expression was positively associated with BMC and showed positive trends with BA and lean mass of the neonate (Table 6.5).

Table 6.5: Associations between placental relative mRNA expression and neonatal DXA measurements of body composition and bone indices.

Gene		BMC (g)	BA (cm²)	BMD (g)	Lean mass (g)	Fat mass (g)
Vitamin D genes						
Cubilin	r	0.15	0.12	0.14	0.15	0.04
	p	0.28	0.39	0.32	0.31	0.77
CYP24A1	r	**0.34**	**0.33**	*0.23*	*0.24*	*0.23*
	p	**0.01**	**0.01**	*0.09*	*0.09*	*0.09*
CYP27B1	r	-0.02	0.01	-0.06	-0.04	0.13
	p	0.88	0.95	0.66	0.75	0.34
Megalin	r	*0.24*	0.22	0.18	**0.29**	-0.03
	p	*0.08*	0.11	0.20	**0.04**	0.82
RXRα	r	-0.19	-0.11	*-0.26*	-0.12	-0.14
	p	0.17	0.43	*0.06*	0.41	0.31
VDR	r	0.08	0.07	0.03	0.03	0.11
	p	0.58	0.59	0.82	0.85	0.43
Amino acid transporter genes						
ASCT1	r	**-0.29**	**-0.29**	-0.20	-0.17	-0.12
	p	**0.04**	**0.04**	0.15	0.22	0.39
ASCT2	r	0.15	0.10	0.14	0.04	0.09
	p	0.27	0.47	0.31	0.77	0.50
LAT1	r	**-0.28**	-0.18	**-0.32**	-0.15	-0.21
	p	**0.04**	0.20	**0.02**	0.28	0.13
LAT3	r	-0.04	-0.04	-0.02	-0.08	0.08
	p	0.79	0.82	0.86	0.54	0.55
LAT4	r	-0.21	-0.15	-0.23	-0.16	-0.26
	p	0.12	0.27	0.10	0.24	0.25
SNAT1	r	-0.01	0.06	-0.12	0.12	0.06
	p	0.93	0.65	0.40	0.39	0.66
TAT1	r	-0.05	-0.05	-0.03	-0.15	0.07
	p	0.70	0.72	0.81	0.28	0.61
Other genes						
DNMT1	r	**0.28**	*0.26*	0.23	*0.24*	0.08
	p	**0.04**	*0.06*	0.10	*0.08*	0.55
PTHrP	r	0.13	0.06	0.15	-0.001	0.05
	p	0.34	0.65	0.28	0.99	0.69
TXNIP	r	-0.02	-0.08	0.04	-0.09	0.03
	p	0.89	0.57	0.75	0.52	0.85

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

6.4. Discussion

This study has shown that placental mRNA expression of specific genes was associated with *CYP24A1* mRNA expression within the placenta. *CYP24A1* mRNA may be a biomarker for active vitamin D within the placenta, therefore these associations could indicate regulation of these genes via active vitamin D. Placental expression of genes involved in nutrient transport was largely unaffected by maternal vitamin D supplementation during pregnancy. *CYP24A1* mRNA also showed positive associations with measures of neonatal bone health, which could indicate effects of active vitamin D on fetal development.

6.4.1. Genes not detected in placental tissue

Calbindin-D9K, *Calbindin-D28K*, *NCX2*, *NCX3* and *CaT2* mRNA expression was not detected in human placental tissue by qrt-PCR. Primer sets for *NCX2* and *NCX3* could not be tested further due to a lack of positive control tissue (brain or muscle). However, both genes were identified in RNA sequencing of placental villous fragment samples (Chapter 5 section 5.3.3), therefore it appears they are expressed within human placenta.

Primer sets for *calbindin-D9K*, *calbindin-D28K* and *CaT2* were verified using rt-PCR with fetal kidney. *Calbindin-D28K* was clearly amplified in the control tissue while no amplification was observed in placental tissue. *Calbindin-D28K* expression has previously been observed in human placenta at both the mRNA and protein level (Belkacemi *et al.*, 2003) and was observed in some placental villous fragment samples by RNA sequencing (Chapter 5 section 5.3.3). Our primer set was designed to cover all known transcript variants, therefore differences in transcript variants should not underlie the discrepancies observed between the studies. Belkacemi *et al.* (2003) used a different preparation of placental cells than was used here. In our study RNA was extracted from whole placental tissue, whilst Belkacemi *et al.* (2003) purified cytotrophoblasts from full term human placenta and cultured them for 4 days until a syncytium was formed. These different methodologies could explain the contrasting results as whole placental tissue will contain a mixture of cell types as opposed to the more purified syncytiotrophoblast. In support of this, cytotrophoblasts show a lower calcium transport capacity than syncytiotrophoblast cells (Belkacemi *et al.*, 2005a). Furthermore, the culture of placental cells for 4 days following delivery of the placenta could lead to mRNA expression changes. The cells were originally cytotrophoblasts at the time of delivery, and it is not clear how similar regenerated syncytiotrophoblast in culture is to the actual placental syncytiotrophoblast. *Calbindin-D9K* and *calbindin-D28K* mRNA were also detected in placental cells by Halhali *et al.* (2010), however, the authors did note that due to low expression cDNA levels in qrt-PCR had to be increased from 1 μl to 5 μl.

Both *calbindin-D9K* and *CaT2* showed no amplification in placenta by rt-PCR and minimal amplification in kidney. *CaT2* was barely detectable in kidney by rt-PCR. Expression of both of these genes has also previously been demonstrated in human placenta with the same methodologies used for *Calbindin-D28K* (Moreau, 2002b; Belkacemi *et al.*, 2004), therefore the differences in methodology may again explain the variation in results. Differences could also result from low expression of these genes within placenta, as while *CaT2* mRNA has been detected within placental tissue its expression was much lower than that of *CaT1* (Peng *et al.*, 2001). However, the amplification of both genes in kidney was minimal, therefore the lack of detection in placenta may also be a result of the primer set used. In support of this, *CaT2* was detected in placental villous fragments by RNA sequencing (Chapter 5 section 5.3.3). Alternatively, the minimal detection of *CaT2* and *calbindin-D9K* within kidney tissue could mean that this is not an ideal positive control tissue for these genes. An intestinal cell line was used as a positive control for *calbindin-D9K* mRNA by Belkacemi *et al.* (2004), as *calbindin-D9K* may not be expressed in kidney (Belkacemi *et al.*, 2005a), while *CaT2* mRNA was detected at much lower levels in kidney compared to *CaT1* (Peng *et al.*, 2001). As there are queries over the expression of these genes within kidney, the lack of conclusive mRNA detection within this tissue does not unambiguously prove or disprove efficacy of the primer set.

The primer set for *PTH1R* showed presence of the gene within placental tissue, however the primer set was deemed not to amplify the product efficiently enough due to the shallow slope of the standard curve. Due to time constraints optimisation of the primer set could not be carried out, however, it does demonstrate the presence of *PTH1R* mRNA in human placental tissue. Expression has previously been demonstrated in mouse placenta (Qian *et al.*, 2003), but to the best of our knowledge this is the first demonstration of *PTH1R* expression in human placenta.

6.4.2. The effect of maternal vitamin D supplementation on placental mRNA expression

Placental expression of the genes tested appeared largely unaffected by maternal supplementation of vitamin D during pregnancy. Initial analysis revealed no differences, except for a trend in reduced expression of one HKG. However, when analysis was stratified by season of birth a small number of genes were affected by vitamin D supplementation but only in specific seasons. Maternal 25(OH)D levels in this sub-set of the cohort were increased following supplementation, therefore it appears that this increased circulating vitamin D had no major effects on placental mRNA expression. This could mean that vitamin D supplementation during pregnancy does not affect placental mRNA expression. Alternatively, the results could have occurred for a number of reasons. Firstly, we have only looked at 20 genes, it is possible that expression of genes not investigated in this study may be altered. However, *CYP24A1* is known to respond to vitamin D

(Turunen *et al.*, 2007), therefore this gene would be expected to increase in mRNA expression following increased 1,25$(OH)_2$D. Women were supplemented with 25(OH)D and showed increased circulating 25(OH)D. The conversion to the active form, 1,25$(OH)_2$D may not have been increased, resulting in no increase in active vitamin D within the placenta to exert additional effects on mRNA expression. As we do not currently have measures of active vitamin D in maternal or cord serum this idea cannot presently be investigated further. As previously mentioned in this thesis, *CYP24A1* is a vitamin D responsive gene and may provide a biomarker for active vitamin D levels within the placenta, therefore in the absence of circulating 1,25$(OH)_2$D levels placental mRNA expression was tested for associations with *CYP24A1* mRNA (see section 6.4.3).

A further reason for the lack of effect of vitamin D supplementation on placental mRNA expression could be due to the long-term exposure leading to desensitisation. Studies have presented data showing effects of vitamin D on mRNA expression in placental cell culture following exposure for 18 h (Avila *et al.*, 2007b) or 24 h (Diaz *et al.*, 2009). Differing effects of short and long-term active vitamin D treatment on placental cell culture have previously been described. Treatment of human trophoblast for 6 h with 1,25$(OH)_2$D resulted in increased *hCGβ* mRNA while following 24 h of treatment the effect was not observed. Furthermore, when treatment was further increased to 48 h inhibitory effects of 1,25$(OH)_2$D on *hCGβ* mRNA were observed (Barrera *et al.*, 2008). However, 48 h is still a relatively short time frame compared to the months of supplementation in this study. It is possible that the long-term vitamin D supplementation in MAVIDOS altered homeostatic mechanisms within the placenta, possibly to encourage increased transfer of 25(OH)D across the placenta. In support of this idea, *cubilin* mRNA was increased by vitamin D supplementation but only in placentas of infants born in spring.

Compared to cell culture data showing effects of vitamin D on placental mRNA expression in a controlled environment, MAVIDOS involves a much more physiological setting where many factors are likely to interact resulting in increased variation in the data. Due to this natural variation it may be harder to detect effects, particularly small effects. While it is clear that vitamin D supplementation increased maternal circulating 25(OH)D levels, cord blood levels have not yet been measured, therefore it is unclear whether any of this 25(OH)D was transferred to the placenta and fetus. Additionally, women classed as vitamin D deficient at 14 weeks of gestation were excluded from the study and referred to their general practitioners to receive vitamin D supplementation. In this study vitamin D deficiency was classified as 25(OH)D levels < 25 nmol/l. Different cut off points for vitamin D deficiency, inadequacy and sufficiency are often described but the ones used in this study are close to the Institute of Medicine guidelines which state that 25(OH)D ≤ 30 nmol/l is classed as deficient, 31 – 50 nmol/l is insufficient and levels > 50 nmol/l are sufficient (IOM, 2011). The average vitamin D levels at 14 weeks of gestation were 48.16

nmol/l and 49.14 nmol for placebo and vitamin D treated, respectively, so on average supplementation was being received by those at the high levels of vitamin D insufficiency. This study therefore does not address the effects of supplementation during pregnancy on those who are vitamin D deficient. It is likely that this is the group of individuals in which the largest effects of supplementation would be observed. Furthermore, the Institute of Medicine guidelines are based on the levels of vitamin D required for bone health. Different tissues may have different vitamin D requirements, for example, the levels of vitamin D required to support placental function may be higher or lower than those required to support bone health.

Vitamin D supplementation was initiated at 14 weeks of gestation, by this time in gestation the placenta and fetus are already formed and the placenta is receiving its blood supply from the mother (see Chapter 1 sections 1.3 and 1.4.1). It is possible that vitamin D supplementation may have had a larger impact on placental mRNA expression if it was implemented when the placenta was being formed in the initial phases of gestation. Alterations to placental development during this critical window could result in altered placental function throughout gestation. In addition, if vitamin D is involved in mediating mRNA expression via methylation changes these changes may be more likely to occur during early gestation, as this is when placental methylation status is initially established. During the early stages of development a genome-wide demethylation process removes the methylation marks on the DNA of the blastocyst. This is followed by a period of *de novo* methylation where new methylation marks are established on the embryonic and placental DNA (Reik *et al.*, 2001). In placental samples from the SWS associations were observed between maternal 11 week 25(OH)D levels and *DMNT3b* mRNA levels, while *CYP24A1* mRNA was positively associated with both *DNMT1* and *DNMT3b* mRNA expression (Chapter 3). If vitamin D is indeed regulating DNMT expression and activity, the observed effects may be much more profound if treatment were administered at these early stages of gestation.

In the previous chapter, data were presented showing that 25(OH)D transport into the placenta was greatly facilitated by the presence of a carrier protein and mRNA expression in SWS placentas showed associations with maternal DBP levels (Chapter 3). It is therefore possible that maternal vitamin D supplementation may not hugely increase placental transport of vitamin D unless adequate serum concentrations of DBP and albumin are available. DBP and albumin are generally in large molar excess compared to 25(OH)D (Bouillon, 1981; Delvin, 1982; Markestad, 1984; Crozier *et al.*, 2012; Schwartz *et al.*, 2014), so unless a woman showed specifically low levels of the carrier proteins they are unlikely to limit transport of vitamin D into the placenta.

Analysis of mRNA expression differences by season of birth revealed significant effects of vitamin D supplementation on two genes. Specifically, vitamin D supplementation resulted in increased

cubilin mRNA expression in placentas of neonates born in spring, while *ASCT2* was reduced by vitamin D supplementation in placentas of neonates born in summer. Analysis of maternal vitamin D levels in the entire MAVIDOS cohort revealed that vitamin D supplementation prevented the dip in 25(OH)D levels observed in winter and spring (Prof. C. Cooper, unpublished observations). For *ASCT2* mRNA expression this suggests that this increased 25(OH)D during the winter and spring had lasting impacts on mRNA expression that were observed in the summer. This is perhaps due to the fact that the vitamin D supplementation increased maternal 25(OH)D during winter and spring. Therefore, infants born during the summer have received a more prolonged exposure to the increased maternal vitamin D, while those born in winter may perhaps only see one or two months of increased maternal vitamin D prior to delivery. *Cubilin* expression showed a different seasonal effect. The increased expression during spring may result from the increased 25(OH)D over winter and through spring culminating in the need to increase the placental capacity for vitamin D uptake. Although, it is unclear why this would also not be observed in winter following increased maternal 25(OH)D. Alternatively, as cubilin is known to mediate transport of other ligands such as fatty acids (Hammad, 2000), the increase in *cubilin* expression may be aimed at increasing transport of other factors essential for fetal development. However, as cubilin works in concert with megalin (Nykjaer *et al.*, 1999; Nykjaer *et al.*, 2001) it is difficult to see how this increased expression could translate into increased uptake of nutrients unless megalin is either highly expressed or shows increased expression in response to the same stimuli as cubilin. In these placental samples, megalin was amplified at a lower cp value than cubilin, indicating a higher expression level, therefore it is possible that the basal level of megalin may be able to support increased cubilin-mediated uptake.

It is interesting that the effect on *ASCT2* mRNA was negative with maternal vitamin D supplementation. At the molecular level, this could be due to the presence of a nVDRE within the *ASCT2* promoter region. However, at the physiological level it is less certain why this effect would occur. None of the other amino acid transporter genes tested showed any impacts of vitamin D supplementation, so it seems sensible to assume that overall the placenta may be able to compensate for the reduction in *ASCT2*. In support of this *ASCT2* mRNA levels showed no associations with any measures of neonatal size, body composition or bone development.

6.4.3. Associations between maternal vitamin D and albumin and placental mRNA expression

Maternal 25(OH)D levels at 14 weeks of gestation showed no significant associations with mRNA expression of any of the placental genes investigated, while 25(OH)D at 34 weeks of gestation showed just one significant association, a positive relationship with *CaT1* mRNA. Differing effects of vitamin D on *CaT1* mRNA expression have previously been reported. In human duodenal

biopsies incubation with 1,25$(OH)_2$D resulted in increased *CaT1* mRNA, while patient analysis showed that *CYP27B1* mRNA levels and patient 1,25$(OH)_2$D levels associated with duodenal *CaT1* mRNA expression (Walters *et al.*, 2007; Balesaria *et al.*, 2009). In rat cochlea cells incubation with 1,25$(OH)_2$D showed no impact on *CaT1* mRNA expression (Yamauchi *et al.*, 2010). These contrasting results could represent tissue- or species-specific differences. The effect of vitamin D on mRNA expression of the taurine transporter, *TauT*, has previously been shown to differ depending on the cell type used for investigation (Chesney and Han, 2013).

The fact that few associations were observed with 25(OH)D levels and placental mRNA expression could have resulted from the vitamin D supplementation increasing circulating 25(OH)D in half of the study population. Furthermore, in this study 25(OH)D was assessed at 14 weeks of gestation and those with low circulating vitamin D were excluded. 25(OH)D levels ranged from 15.3 to 94.4 in the cohort as a whole at 14 weeks of gestation, and from 9.57 to 107.5 nmol/l in the cohort at 34 weeks of gestation. While this study is therefore covering a wide range of vitamin D concentrations, the proportions of participants with lower levels of 25(OH)D is likely to be reduced, which may result in difficulty in detecting associations with lower vitamin D levels.

CYP24A1 mRNA levels were used as a potential biomarker for active vitamin D within the placenta, as we previously did in the SWS cohort (Chapter 3 section 3.3.2). *CYP24A1* mRNA was positively associated with *cubilin*, *ASCT1*, *TAT1*, *PMCA4* and *DNMT1*, and showed a negative relationship with *ASCT1* mRNA expression. *PMCA4* was not measured in the SWS placental samples, but all the other genes identified here showed associations with *CYP24A1* in the SWS cohort as well. Similarities and differences between the results from the two cohorts are discussed further in General Discussion section 7.5. These results could be indicative of regulation of these specific placental genes via 1,25$(OH)_2$D. This cannot be confirmed without measures of maternal 1,25$(OH)_2$D but it seems reasonable to think that active vitamin D levels within the placenta can play a role in regulation of placental mRNA expression. It is the active 1,25$(OH)_2$D that binds and activates the VDR-RXRα heterodimer to initiate changes to mRNA expression (Lin and White, 2004). In support of this, in human placental samples *CYP27B1* mRNA expression was positively associated with maternal mid-gestation 1,25$(OH)_2$D, but not with 25(OH)D (O'Brien *et al.*, 2014), while in human duodenal biopsies, the patients levels of 1,25$(OH)_2$D but not 25(OH)D were associated with *CaT1* mRNA expression (Walters *et al.*, 2007). However, an alternative explanation could be that both the target gene and *CYP24A1* mRNA expression are regulated by a common factor resulting in the observed associations.

Maternal serum albumin at 14 weeks of gestation was negatively associated with placental mRNA expression of *cubilin*, *LAT3*, *TAT1*, *NCX1*, *PMCA4* and *TXNIP* in term placenta. Interestingly, the

negative association between albumin with gene expression of *LAT3* and *TAT1* was also observed in placental villous fragments where the addition of albumin in the Tyrodes buffer resulted in a significant reduction in gene expression. The potential impact of albumin is discussed further in section 7.2.4.

None of these associations with 14 week serum albumin were observed at 34 weeks of gestation, suggesting there may be windows of susceptibility, whereby at specific points in gestation mRNA expression is more pliable to environmental influences. The notion that fetal development undergoes critical periods where development of specific organ systems is particularly vulnerable to environmental perturbations at specific time points during gestation has long been established (Widdowson and McCance, 1975; Barker and Clark, 1997). More recent studies have suggested that the placenta may also undergo critical periods of development. For example, explants from placentas of mice fed a low-protein diet in early gestation showed reduced trophoblast giant cell outgrowths and reduced trophoblast proliferation rates compared to placentas of mice fed a normal diet throughout gestation. The same effect was also seen in placentas exposed to the low-protein diet for the entire length of gestation. The fact that the short-term gestational exposure to the experimental diet had the same effect as exposure to the diet throughout gestation suggests that the mouse placenta is particularly susceptible in early gestation (Watkins *et al.*, 2015). In the same mouse model exposure to the low-protein diet in early gestation resulted in increased endocytosis in the trophectoderm cells of the blastocyst (Sun *et al.*, 2014). These studies support the idea that the placenta may be particularly susceptible to maternal signals during the earlier stages of gestation and could explain why maternal albumin at 14 weeks, but not 34 weeks, of gestation was associated with placental mRNA expression.

It is interesting that all the associations with mRNA expression and albumin were negative. If these relationships were indicative of increased vitamin D transport via albumin-mediated transfer of vitamin D, we may expect some of the associations to be positive, as was observed with maternal DBP levels in SWS placentas. However, albumin is not such a high affinity vitamin D carrier as DBP is. Only ~10% of circulating 25(OH)D is bound to albumin compared to ~88% bound to DBP (Bikle *et al.*, 1985; Bikle *et al.*, 1986; White and Cooke, 2000). Furthermore, albumin is a carrier protein for a wide range of substrates including cortisol and fatty acids (van der Vusse, 2009; Perogamvros *et al.*, 2012). Therefore, associations between albumin and mRNA expression are likely to result from a variety of functions, of which vitamin D will only constitute a small portion. In this sense it is therefore not surprising that the associations between albumin and placental mRNA expression did not mirror that between DBP and placental mRNA expression observed in Chapter 3 section 3.3.2.

6.4.4. Associations between placental mRNA expression and neonatal anthropometry and bone indices

mRNA expression of some placental genes was associated with neonatal anthropometry, body composition and bone measures. Some genes showed associations with a single measure, for example *SNAT1* mRNA expression was positively associated with head circumference, while *LAT1* and *LAT4* mRNA expression were both inversely related to triceps thickness. The most consistent observation was with *CYP24A1* mRNA expression which was positively associated with placental weight, as well as showing strong correlations with neonatal BMC and BA. *CYP24A1* mRNA expression also showed trends for positive associations with BMD and lean and fat mass of the neonate. As *CYP24A1* expression is increased by $1,25(OH)_2D$, these associations could represent the actions of vitamin D on these outcomes. Vitamin D status during pregnancy has been linked with bone development and body composition. As previously described in this thesis, low maternal vitamin D was associated with increased femur splaying at 19 and 34 weeks of gestation (Mahon *et al.*, 2010), while maternal vitamin D status during late pregnancy was positively associated with BMC, BA and BMD at 9 years of age (Javaid *et al.*, 2006). In relation to body composition, maternal late pregnancy vitamin D levels have previously been positively associated with fat mass at birth. Interestingly, this association was reversed at 6 years of age (Crozier *et al.*, 2012). Late pregnancy 25(OH)D levels have also been linked with lean mass of the offspring at 4 years of age, which translated into increased grip strength (Harvey *et al.*, 2014b). Furthermore, vitamin D supplementation resulted in increased neonatal BMC in those born during winter in MAVIDOS (Prof. C. Cooper, unpublished observations). This observational data alongside the placental mRNA expression data present a strong case for the role of vitamin D in fetal body composition and bone development. However, measures of $1,25(OH)_2D$ within the cohort are required to confirm whether *CYP24A1* is a biomarker for active vitamin D levels.

An alternative explanation for the associations between *CYP24A1* and neonatal bone measures could be linked to $24,25(OH)_2D$ or $1,24,25(OH)_3D$, the products of CYP24A1 action. $24,25(OH)_2D$ treatment of chicks prevented development of rachitic lesions in bone that were evident following vitamin D deficiency (Ornoy *et al.*, 1978) and it has been suggested that $24,25(OH)_2D$ produced from the placenta accumulates in bone and may play a direct role in ossification of the fetal skeleton (Shin *et al.*, 2010). This suggests that the actions of CYP24A1 itself may play a role in bone development. This could also explain the associations between maternal 25(OH)D and bone development, as $24,25(OH)_2D$ is formed from the action of CYP24A1 on 25(OH)D. Although the study by Ornoy *et al.* (1978) did note that treatment with $24,25(OH)_2D$ alone still left chicks with low plasma calcium and phosphate levels, whilst co-treatment with $24,25(OH)_2D$ and $1,25(OH)_2D$ restored bone morphology as well as serum calcium and phosphate.

This suggests that while 24,25$(OH)_2$D may play a role in bone development, the classical 1,25$(OH)_2$D is also likely to play an important role too.

6.4.5. Limitations

A major limitation of this study is the small sample size. This study uses a sub-set of samples from a much larger human cohort. The sample numbers are limited due to the difficulties in collecting fresh human placental tissue. The small numbers are particularly emphasised when the analysis is stratified by treatment group and will limit the power to detect small associations between placental mRNA expression and maternal vitamin D supplementation or neonatal outcomes. The effect of sample size is highlighted by the results we observed for expression of the HKG *RPL13A*. Following geNorm analysis on a small sub-set of the placenta samples this was selected as a good HKG as it was stably expressed in placentas from placebo and vitamin D treated mothers, and was shown not to be altered by the supplementation. However, when this gene was measured in the entire sample set, we observed a trend for reduced expression with supplementation which could not be detected in the small initial sample set. A number of the significant correlations observed have an r value greater than 0.3, while a number of the trends have r values around 0.2. It is possible that with larger sample numbers these lower level correlations may also reach significance. As the system we are investigating is complex, a large number of factors are likely to impact the overall outcome, therefore the smaller associations are also of interest. The sub-group analysis undertaken when samples were stratified by season of birth amplifies the chance that multiple comparisons will result in false positives, however, due to the fact that season is a confounder for vitamin D levels this sub-group analysis was required. In addition, this study uses a target gene approach, where genes of interest have been selected and studied. The supplementation may have resulted in changes in expression of genes that have not been considered in this study, as discussed in 6.4.6 RNA sequencing would give a much more comprehensive analysis of mRNA expression changes within the placenta samples.

The study is limited by the current serum measures that have been carried out on the cohort. Measures of maternal serum 1,25$(OH)_2$D and DBP would prove useful for this cohort to provide a fuller picture of the effect of maternal vitamin D supplementation on maternal circulating levels of vitamin D. Furthermore, measures of 25(OH)D and 1,25$(OH)_2$D in cord serum would provide a good measure of the level of transfer of vitamin D from the mother to the fetus, and could therefore provide some idea of the level of vitamin D transferred to the placenta. In addition, due to obvious ethical constraints women identified as vitamin D deficient at 14 weeks of gestation were excluded from the study and referred to their general practitioners for supplementation. While it is clearly unethical to prevent a pregnant woman from receiving supplementation when she is

identified as vitamin D deficient, this does mean that in this study we do not observe the effects of supplementation on this population group. Furthermore, in this study supplementation commences at 14 weeks of gestation. At this stage in pregnancy the placenta is already formed, which may limit the extent of the impact of the supplementation on placental function.

This study is also limited by the fact that placentas were from both caesarean section and vaginal deliveries. The process of labour has been shown to effect expression of some genes within the placenta. For example, mRNA expression of *RXRa* increased in placental tissues following labour compared to placental tissues from elective caesarean sections in which the process of labour is never initiated (Holdsworth-Carson *et al.*, 2009). In this study it would have resulted in a severe reduction in sample numbers to restrict the method of delivery for placenta collection, nevertheless it should be considered that this may increase the variation in expression of specific genes within the data set. However, this should not impact on the effect on treatment or placebo, but may result in less power to detect differences between the groups as a result of increased variation.

As with the data presented in Chapter 3 from the SWS cohort, the data presented describing associations between placental mRNA expression with maternal serum measures and neonatal outcomes are correlations and therefore do not prove causation. Further studies are required to investigate the associations presented. For example, associations observed between placental mRNA expression and neonatal anthropometry may indicate a direct relationship between the two factors. Alternatively, the placental mRNA expression measure may be regulated by factors which are also resulting in impacts on neonatal bone development. In this study, it is not possible to untangle these relationships further, therefore more mechanistic studies are required to investigate whether the associations observed are causative.

6.4.6. Future work

Further work for this study could include measurement of DBP and $1,25(OH)_2D$ in the maternal serum. As mentioned above these data combined with the calcium, 25(OH)D and albumin measurements would provide a much more well-rounded picture of the effects of maternal vitamin D supplementation on maternal circulating vitamin D levels. This information may aid the interpretation of the placental mRNA expression data, and may confirm whether *CYP24A1* mRNA levels do provide a potential biomarker for active vitamin D within the placenta. Furthermore, cord blood samples are available for this cohort, so the measurement of these metabolites in cord serum would prove useful. With these measures we could explore the relationship between placental mRNA expression with measures of maternal and fetal vitamin D metabolites and binding proteins. In addition, as we have data on both calcium and albumin for participants in this cohort, an

albumin adjusted calcium level could be calculated. This would give an indication of the free calcium concentrations, which could then be tested for associations with placental gene expression.

Data on infants in MAVIDOS are continuing to be collected. Data on 4 year old infants are currently being collected; this includes data on anthropometry and DXA measurements of bone indices and body composition. When the data collection is completed it would be interesting to see if the associations observed between placental mRNA expression and neonatal anthropometry are also observed with 4 year anthropometry. This would be of particular interest with *CYP24A1* expression which showed strong associations with neonatal bone indices.

The associations between placental mRNA expression and neonatal outcomes are correlations, and further studies are required to establish whether these are causative. It is not really possible to establish this in human studies but animal studies could be utilised to investigate the mechanistic links between placental-specific gene expression and neonatal outcomes. This could be achieved through using placental-specific knockout models. This process has previously been used to generate a placental-specific *IGF2* knockout in mice, and was effective in establishing the importance of placental expression of this gene (Constancia *et al.*, 2002). A similar process could be utilised to investigate the link between *CYP24A1* and infant bone development further. The use of an animal model to pursue this investigation would also allow for detailed exploration of the impact on fetal bone development, which obviously cannot be conducted in human studies.

We did not observe many changes to placental gene expression with maternal vitamin D supplementation. This study investigated gene expression of 20 candidate genes. RNA sequencing would enable non-candidate based examination of mRNA expression changes within the placental samples and would enable a much wider range of genes to be examined. mRNA expression changes observed with RNA sequencing could then be analysed using pathway analysis to identify specific biological pathways within the placenta that may be affected. In addition, as mentioned in relation to the SWS cohort, it would also be interesting to split the data set by sex of the offspring and analyse whether maternal vitamin D levels show different associations with gene expression in male and female placentas.

In MAVIDOS pregnant women were randomised to vitamin D supplementation at 14 weeks of gestation. At this time the placenta and fetus are already formed and the placenta is already receiving maternal blood. It would be interesting to investigate the effects of vitamin D supplementation prior to conception and throughout the entire gestational period as the early stages of gestation when the placental and fetus are initially formed may be particularly sensitive to intervention. The PREPARE trial is currently being conducted in Southampton where women

undergoing IVF are randomised to receive a daily supplement drink enriched with vitamin D and omega-3 fatty acids or a non-enriched daily supplement drink for 6 weeks prior to conception (Kermack *et al.*, 2014). It would be interesting to investigate the effects of this vitamin D intervention on placental samples from this study.

6.4.7. Conclusions

In conclusion, this study has revealed that maternal vitamin D supplementation from 14 weeks of gestation onwards had little impact on placental expression of the genes examined. However, *CYP24A1* mRNA expression was positively associated with a number of placental genes and showed positive associations with measures of neonatal body composition and bone structure (Figure 6.7). We now need to measure $1,25(OH)_2D$ levels in this cohort to establish whether the associations with *CYP24A1* mRNA expression are indicative of the actions of active vitamin D within the placenta.

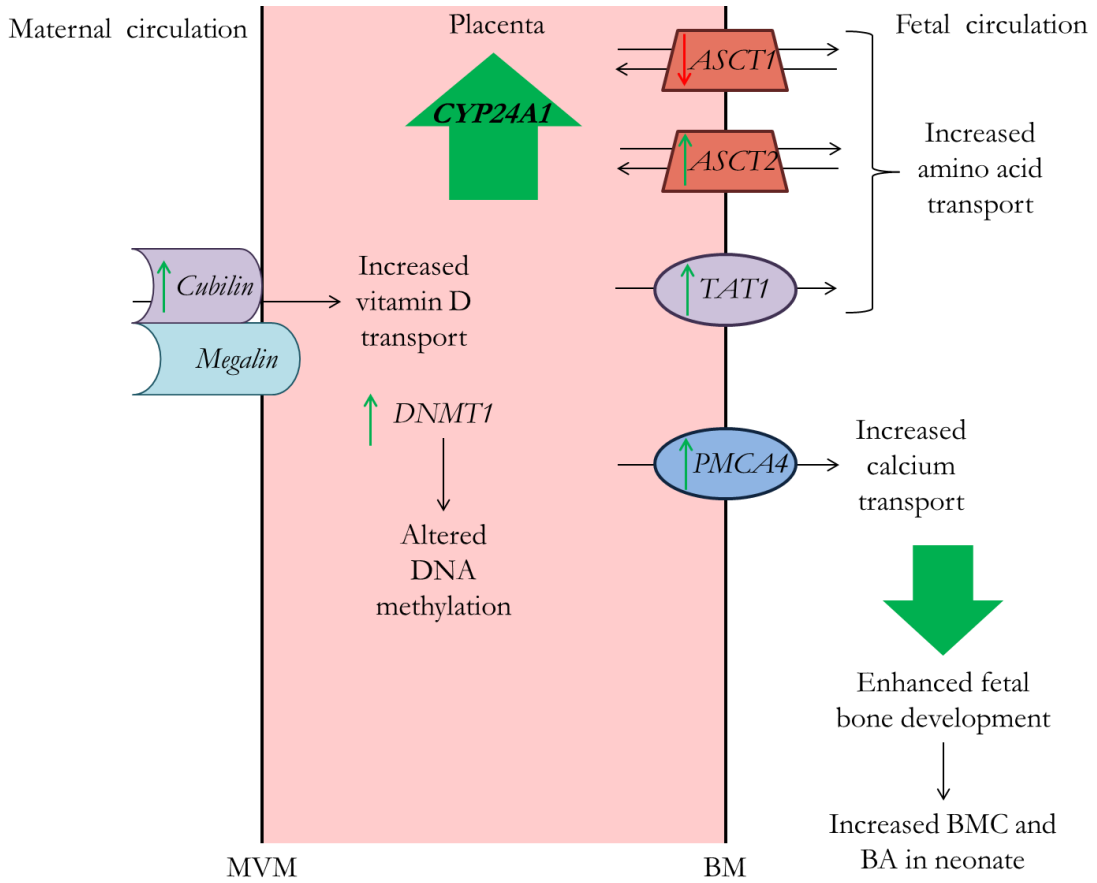

Figure 6.7: Associations with placental mRNA expression of *CYP24A1*. Placental expression of *CYP24A1* was positively associated with increased mRNA expression of *cubilin*, *DNMT1*, *ASCT2*, *TAT1* and *PMCA4*. Collectively, these mRNA expression changes could increase transport of vitamin D into the placenta, and increase export of amino acids and calcium from the placenta into the fetal circulation. These mechanisms could then result in the associations observed between *CYP24A1* mRNA levels and BMC and BA in the neonate. It is yet to be seen whether these changes are mediated by $1,25(OH)_2D$.

Chapter 7:
General Discussion

7.1. Overview

This thesis aimed to investigate links between vitamin D, the placenta and fetal growth. Positive associations have been described in some epidemiological studies between maternal vitamin D levels and fetal growth. In order to reach the fetus, vitamin D must be transferred across the placenta. The precise mechanisms of vitamin D uptake into the placenta are unknown. Vitamin D was thought to passively diffuse across the placenta, however, in light of the reduction in free 25(OH)D and $1,25(OH)_2D$ in pregnancy it seemed unlikely that this small pool of free vitamin D would be adequate for fetal needs. The data presented in this thesis suggest that uptake of vitamin D occurs via a specific process, possibly involving receptor-mediated endocytosis. In addition, these data show that vitamin D is metabolised within the placenta and acts upon placental mRNA expression. Investigation into the placental metabolism and transport of vitamin D is important as these factors will determine the amount of vitamin D reaching the fetus and subsequent fetal growth. The actions of vitamin D upon the placenta could also alter placental function which could impact fetal growth.

This study has shown that in *ex vivo* placental villous fragments both 25(OH)D and $1,25(OH)_2D$ are taken up into the human placenta. Uptake of 25(OH)D is increased greatly by the presence of a binding protein such as albumin, whilst uptake of $1,25(OH)_2D$ appears to be largely independent of albumin and DBP. Furthermore, our placental villous fragment studies revealed that 25(OH)D is converted to $1,25(OH)_2D$ within the human placenta. Work on the human cohorts, MAVIDOS and SWS, has shown that maternal 25(OH)D levels were associated with placental mRNA expression of specific nutrient transporters. Also, in the SWS cohort a number of associations were observed with maternal DBP levels and mRNA expression of placental nutrient transporters. This supports the *ex vivo* work showing that a carrier protein is important for 25(OH)D uptake into the placenta. Placental mRNA expression of *CYP24A1*, a known vitamin D responsive gene, has been used as a proxy for placental exposure to $1,25(OH)_2D$. In both of the human cohorts, placental mRNA expression of this enzyme was positively associated with measures of fetal growth and bone development. This suggests that vitamin D may be important for fetal growth and development, which has implications not only for the immediate health of the neonate, but also for health of the individual in later life. Furthermore, it is possible that some of the effects on neonatal size are driven via vitamin D-mediated changes in placental nutrient transporter expression, as *CYP24A1* mRNA was positively associated with placental gene expression of specific amino acid and calcium transporters.

7.2. Placental uptake of vitamin D

7.2.1. Uptake of 25(OH)D and 1,25$(OH)_2$D into the human placenta

Placental uptake of vitamin D is important as it will determine the amount of vitamin D available not only to reach the fetus but also to act on placental mRNA expression. Uptake of both 25(OH)D and 1,25$(OH)_2$D was demonstrated in placental villous fragments. This *ex vivo* human placental study is the first demonstration of 1,25$(OH)_2$D uptake into the human placenta. Previously, 1,25$(OH)_2$D was not thought to be transported into the placenta due to contradictory data on correlations between maternal and cord serum 1,25$(OH)_2$D levels (Fleischman *et al.*, 1980; Delvin, 1982; Hollis and Pittard, 1984; Ishida *et al.*, 1988). It is possible that the 1,25$(OH)_2$D transported into the placenta acts directly upon the placenta and is not transported out into the fetal circulation (see section 7.3). This could explain the lack of association between maternal and cord serum 1,25$(OH)_2$D seen in some studies. This study has also revealed that uptake of 25(OH)D is greatly facilitated by the presence of the carrier protein, albumin, while 1,25$(OH)_2$D uptake is not affected by the presence of a carrier protein. Associations observed in the SWS between placental mRNA expression and maternal DBP levels at 34 weeks of gestation support the notion the carrier protein-mediated uptake of vitamin D is important for vitamin D delivery to the placenta.

The data presented in this thesis contradicts the free hormone hypothesis for vitamin D. This hypothesis states that only unbound vitamin D would be available to exert biological actions (Mendel, 1989), for example, changes to mRNA expression. In the case of 25(OH)D, *CYP24A1* fold induction was greatly increased by the presence of albumin with 25(OH)D, suggesting that not only did albumin not inhibit the activity of vitamin D, it greatly enhanced its effects. This is likely due to its action in mediating enhanced uptake of albumin bound 25(OH)D into the placental villous fragment. For 1,25$(OH)_2$D, while transport was not increased by the action of either carrier protein tested, its actions on induction of *CYP24A1* expression were not abrogated by the presence of these carrier proteins. The free hormone hypothesis would predict that in the presence of increased levels of binding protein, *CYP24A1* fold induction via 25(OH)D or 1,25$(OH)_2$D would decrease, due to a reduction in free vitamin D. Once within the placenta, this is likely to be the case, as 25(OH)D sequestered to a carrier protein would be unavailable for conversion to 1,25$(OH)_2$D. Whilst, 1,25$(OH)_2$D bound to a carrier protein would not be available for binding to and activation of the VDR/RXRα heterodimer. Therefore, these data would suggest that the placenta possesses adequate systems to release vitamin D from its carrier protein once it has been internalised.

- **Both 25(OH)D and 1,25$(OH)_2$D are transported into the placenta. Transport of 25(OH)D is facilitated largely by the carrier protein, albumin; therefore megalin and**

cubilin may be involved in placental uptake of carrier protein bound vitamin D, in a similar manner to their function within the kidney.

7.2.2. Megalin- and cubilin-mediated uptake of vitamin D

The fact that 25(OH)D and 1,25$(OH)_2$D uptake were mediated differently by carrier proteins suggests that the mechanism of entry into the placenta may differ for each type of vitamin D. In addition, the reduction in uptake of FITC-albumin into placental villous fragments at 4°C suggests that carrier protein-mediated uptake occurs via a specific mechanism not involving diffusion. One candidate mechanism for albumin bound 25(OH)D uptake into the human placenta is via megalin- and cubilin-mediated endocytosis. These receptors play an important role in the reabsorption of albumin and DBP in the glomerular filtrate, with their absence resulting in urinary excretion of DBP and albumin (Nykjaer *et al.*, 1999; Birn *et al.*, 2000; Zhai *et al.*, 2000; Nykjaer *et al.*, 2001). Immunofluorescent microscopy studies in opossum kidney cells revealed co-localisation of megalin and cubilin. Furthermore, following incubation of cells with FITC-BSA, the FITC-BSA showed punctate labelling which was localised to the areas of megalin and cubilin expression (Zhai *et al.*, 2000). In our placental villous fragment microscopy work, FITC-albumin showed a similar punctate pattern to that described by Zhai *et al.* (2000). This could mean that the albumin was localising to areas of megalin and cubilin expression on the placental membrane. Alternatively, a different receptor, such as FcRn, could be involved in the observed uptake, as uptake of DBP has been described in cells which do not express megalin (Chun *et al.*, 2010). Further investigation using *ex vivo* placental villous fragments with megalin- and cubilin-specific antibodies would prove useful in pursuing these questions further.

Data from the human cohorts, MAVIDOS and SWS, have revealed associations between placental mRNA expression of *CYP24A1* and *megalin* and *cubilin* mRNA levels. Specifically, in the SWS cohort, placental mRNA levels of both *megalin* and *cubilin* were associated with *CYP24A1* mRNA expression. Whilst in MAVIDOS, *cubilin* mRNA expression showed a positive association with *CYP24A1* mRNA levels. An association with *megalin* and *CYP24A1* was not observed in MAVIDOS, however, the p value of 0.11 indicated a near trend association, which may have reached significance with a larger sample set. These associations could be interpreted in two ways. Firstly, increased placental 1,25$(OH)_2$D could result in VDR-mediated effects on both *CYP24A1*, *megalin* and *cubilin* mRNA levels within the placenta, therefore the positive relationship between *CYP24A1* and the potential vitamin D transporters is indicative of a common up-regulation of their expression via vitamin D. Secondly, the associations could mean that increased placental expression of *megalin* and *cubilin* are responsible for increased transport of vitamin D into the placenta, which can then activate the VDR/RXRα heterodimer and drive increased *CYP24A1* mRNA expression

(Figure 7.1). In MAVIDOS, *cubilin* mRNA expression within the placenta was significantly increased by maternal vitamin D supplementation in those who had spring births. Furthermore, in a breast cancer cell line combined treatment with $1,25(OH)_2D$ and retinoic acid resulted in increased megalin mRNA and protein expression (Chlon *et al.*, 2008). These data would support the former hypothesis, and could mean that vitamin D acts on placental mRNA expression to enhance its uptake into the placenta in a positive feedback loop (Figure 7.1).

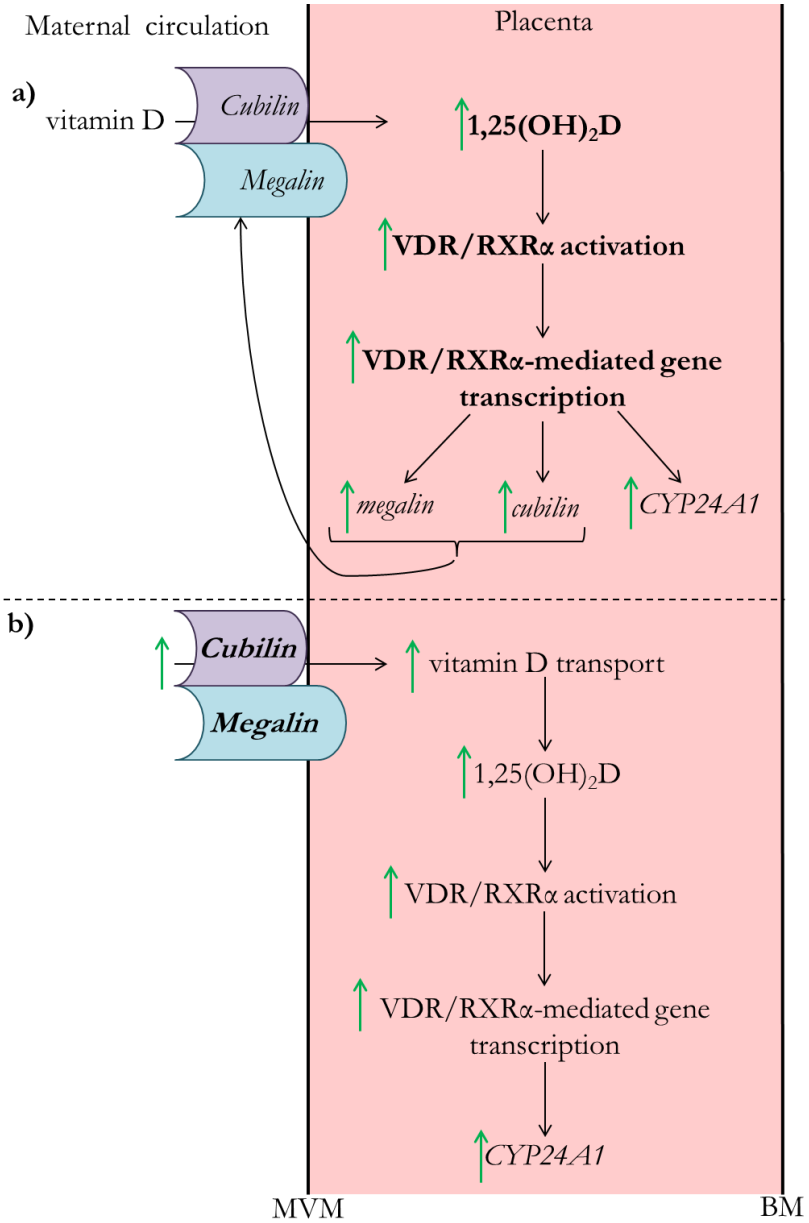

Figure 7.1: Possible explanations for the associations between placental mRNA expression of *CYP24A1* with *megalin* and *cubilin* mRNA. a) Increased mRNA expression of the 3 genes is driven by vitamin D-mediated effects on mRNA expression (highlighted in bold). This results in increased mRNA expression of *megalin* and *cubilin* in a positive feedback loop, which would further increase transport of vitamin D into the placenta. b) In this second option, the driving force between the associations in mRNA expression is an already elevated mRNA expression of *megalin* and *cubilin* (highlighted in bold) driven by an unknown factor. The elevated expression of the endocytic receptors results in increased transport of vitamin D into the placenta, which drives vitamin D induced mRNA expression changes, including the up-regulation in *CYP24A1* mRNA.

- **Megalin and cubilin may be involved in placental uptake of 25(OH)D. Active vitamin D may also act to increase placental expression of these endocytic receptors to drive enhanced placental uptake of 25(OH)D into the placenta.**

7.2.3. Other potential mechanisms of vitamin D uptake

Uptake of $1,25(OH)_2D$ may not be mediated by megalin and cubilin as exposure to gentamicin, a competitive inhibitor of megalin, did not significantly decrease the $1,25(OH)_2D$-mediated increase in *CYP24A1* mRNA expression. However, as discussed in Chapter 5 section 5.4.2 gentamicin is a relatively low affinity inhibitor so may not have prevented the interaction between albumin bound $1,25(OH)_2D$ and megalin to a large enough extent. Furthermore, if albumin bound vitamin D is transported via binding to cubilin followed by cubilin interaction with megalin it is unclear whether gentamicin would prevent this interaction. High specificity blockers to disrupt the function of megalin and cubilin individually are required to fully explore whether these receptors are involved in placental uptake of 25(OH)D and $1,25(OH)_2D$.

Uptake of both 25(OH)D and $1,25(OH)_2D$ with albumin was inhibited in the presence of the pinocytic inhibitor, amiloride. However, the effect on 25(OH)D is currently based on a single experiment and as discussed in Chapter 5 section 5.4.4, this inhibitor has been shown to affect other cellular processes. For example, the distribution of endosomal markers was altered following treatment of HeLa cells with amiloride (Fretz *et al.*, 2006). Endosomes and lysosomes are also products of clathrin-mediated endocytosis, therefore, it is possible that this effect may be due to disturbances in other endocytic pathways. More specific experiments are now required to determine whether the inhibitory effect observed with amiloride is linked to pinocytosis or other cellular processes. In addition, if albumin uptake does occur via pinocytosis, this does not necessarily disprove megalin- and cubilin-mediated uptake, as in renal proximal tubule cells, albumin reabsorption occurs via both clathrin-dependent and fluid-phase endocytosis (Gekle *et al.*, 1999; Birn *et al.*, 2000; Dickson *et al.*, 2014).

7.2.4. Carrier proteins

The fact that 25(OH)D uptake was enhanced by the presence of albumin, while $1,25(OH)_2D$ uptake was not impacted by either albumin or DBP could arise partly due to the differences in binding affinity of the carrier proteins for these two forms of vitamin D. The binding affinity of albumin for $1,25(OH)_2D$ is reported to be 540 μmol/l, while the affinity constant for albumin binding to 25(OH)D is 60 μmol/l (Bikle *et al.*, 1985; Bikle *et al.*, 1986). These dissociation constants show that the affinity of albumin for 25(OH)D is higher than that for $1,25(OH)_2D$ as a lower

concentration of 25(OH)D is required before the albumin binding site is half occupied. DBP has an even higher affinity for 25(OH)D than albumin (70 nmol/l), therefore it is possible that if 25(OH)D uptake were investigated in the presence of DBP instead of albumin, even more profound impacts on transport may be observed.

While there appear to be differences in the uptake of 25(OH)D and $1,25(OH)_2D$ into the human placenta, there may also be differences involved in uptake by the two carrier proteins, DBP and albumin. Unfortunately, we do not currently have serum measures of both of these carriers in a single cohort. 34 week serum DBP measures in the SWS showed positive associations with placental mRNA expression of *4F2hc*, *EAAT3*, *LAT1*, *LAT3*, *LAT4*, *SNAT1*, *SNAT2* and *y+LAT2*. In the MAVIDOS cohort, serum albumin at 14 weeks of gestation was negatively associated with placental mRNA expression of *cubilin*, *LAT3*, *TAT1*, *NCX1*, *PMCA4* and *TXNIP*. While serum albumin at 34 weeks of gestation showed trends for negative associations with placental mRNA expression of *megalin* and *PTHrP*. In addition, in placental villous fragments cultured with 25(OH)D and albumin, and ethanol vehicle control with albumin, there was a significant reduction in *LAT3* mRNA. The same trend was observed with *TAT1* mRNA levels. This suggests there may be a specific albumin-mediated effect on placental mRNA expression, as in *ex vivo* placental fragment culture this effect was observed in the absence of vitamin D. There are no specific data showing albumin influencing mRNA expression directly. As albumin also binds calcium (Husain and Mughal, 1992), it is possible that the effects of albumin occur as a result of an interaction between albumin and calcium in the Tyrodes buffer. The addition of albumin into the buffer may have resulted in a reduction in free calcium and therefore reduced the calcium concentrations available to the placental villous fragments. This potential reduction in available calcium may have then been responsible for the changes in gene expression. However, the genes altered by calcium in the placental villous fragments were not associated with maternal serum albumin in MAVIDOS placentas, which could suggest regulation via an alternative mechanism.

It is possible that the associations observed with albumin may be mediated via its breakdown once it has been transported into the placenta. Following endocytosis, the cargo is delivered to an endosome. The acidic pH within the endosome results in disassociation of albumin from megalin and/or cubilin, and presumably with 25(OH)D too. Cargo within the endosomes can then be directed down a lysosomal pathway for degradation, where the albumin would be broken down into amino acids and the 25(OH)D released. In support of this, cubilin-mediated uptake of albumin in renal proximal cells resulted in recycling of megalin and cubilin back to the luminal membrane, whilst albumin was directed into the lysosomal pathway (Birn *et al.*, 2000). The increased levels of amino acids within the placenta as a result of albumin degradation could then be responsible for the negative associations observed with albumin and *LAT3* mRNA expression in both placental villous

fragment culture and the MAVIDOS human cohort. *LAT3* mRNA and protein levels have been shown to be increased in murine liver and skeletal muscle as a result of 24 h starvation. This was proposed to occur as a result of the increased production of branched chain amino acids (Fukuhara *et al.*, 2007). While this is the opposite situation to what we are proposing within the placenta, this reveals that amino acid levels may regulate *LAT3* expression. Within the mouse starvation model *LAT3* expression was thought be increased to enhance delivery of the branched chain amino acids to the bloodstream so that they can reach the organs. However, within the placenta, in the case of maternal starvation, *LAT3* mRNA levels may be down-regulated to preserve resources for the mother over the fetus. In addition, alterations in the levels of branched chain amino acids that an embryo was exposed to from the 2 cell until the blastocyst stage altered the level of endocytosis, with reduction in amino acid levels resulting in increased endocytosis in the blastocyst (Sun *et al.*, 2014). This study supports the notion that alterations in amino acid levels could cause adjustment to nutrient transport systems within the placenta.

Interestingly, at the acidic pH of the endosome the affinity of FcRn for albumin is greatly increased and can drive albumin down a route of transcytosis (Andersen *et al.*, 2012; Dickson *et al.*, 2014). It is interesting to speculate that if the albumin-25(OH)D association is not disrupted by the acidic pH of the endosome that FcRn may play a role in direct delivery of albumin bound 25(OH)D across the cytosol of the syncytiotrophoblast and to the BM for extrusion into the fetal circulation. This theory could be supported by the lack of significant associations observed between maternal serum albumin and placental mRNA expression in MAVIDOS samples compared to the numerous significant positive associations observed between maternal serum DBP at 34 weeks of gestation and placental mRNA expression in SWS placentas. As the direct trafficking of albumin bound 25(OH)D would mean that the placenta would not be able to access that particular pool of vitamin D, and therefore the vitamin D would not exert any effects on placental mRNA expression.

The route for disassociation of DBP with vitamin D may differ from that of albumin. Some DBP is likely to be degraded within the lysosomes (Willnow and Nykjaer, 2010), resulting in the release of vitamin D as well as amino acids. Activity of the lysosomal protease legumain has previously been described in human placenta (Chen *et al.*, 1997). In addition *legumain* mRNA expression in SWS placentas was positively associated with maternal 34 week DBP levels. It is currently unclear whether legumain is involved in cleavage of both albumin and DBP. DBP cleavage by legumain has been described (Yamane *et al.*, 2002), however, opposing results have been described for cleavage of albumin by legumain (Chen *et al.*, 1997; Yamane *et al.*, 2002).

7.2.5. Summary

One of the primary aims of this thesis was to investigate the entry of vitamin D into the human placenta. *Ex vivo* work has demonstrated uptake of both 25(OH)D and 1,25$(OH)_2$D into the placenta. Furthermore, uptake of 25(OH)D is facilitated by the presence of albumin, as summarised in Figure 7.2. It now needs to be established whether both 25(OH)D and 1,25$(OH)_2$D are transported out of the placenta into the fetal circulation. Furthermore, the differing uptakes of 25(OH)D and 1,25$(OH)_2$D and both forms of carrier protein need to be investigated in more detail. The specific mechanism for carrier protein mediated uptake of vitamin D is still unclear, although placental fragment culture suggests the mechanism does not involve simple diffusion, as reduction in temperature led to decreased FITC-albumin uptake. Further *ex vivo* studies are now required to establish the mechanism(s) of 25(OH)D and 1,25$(OH)_2$D entry into the placenta.

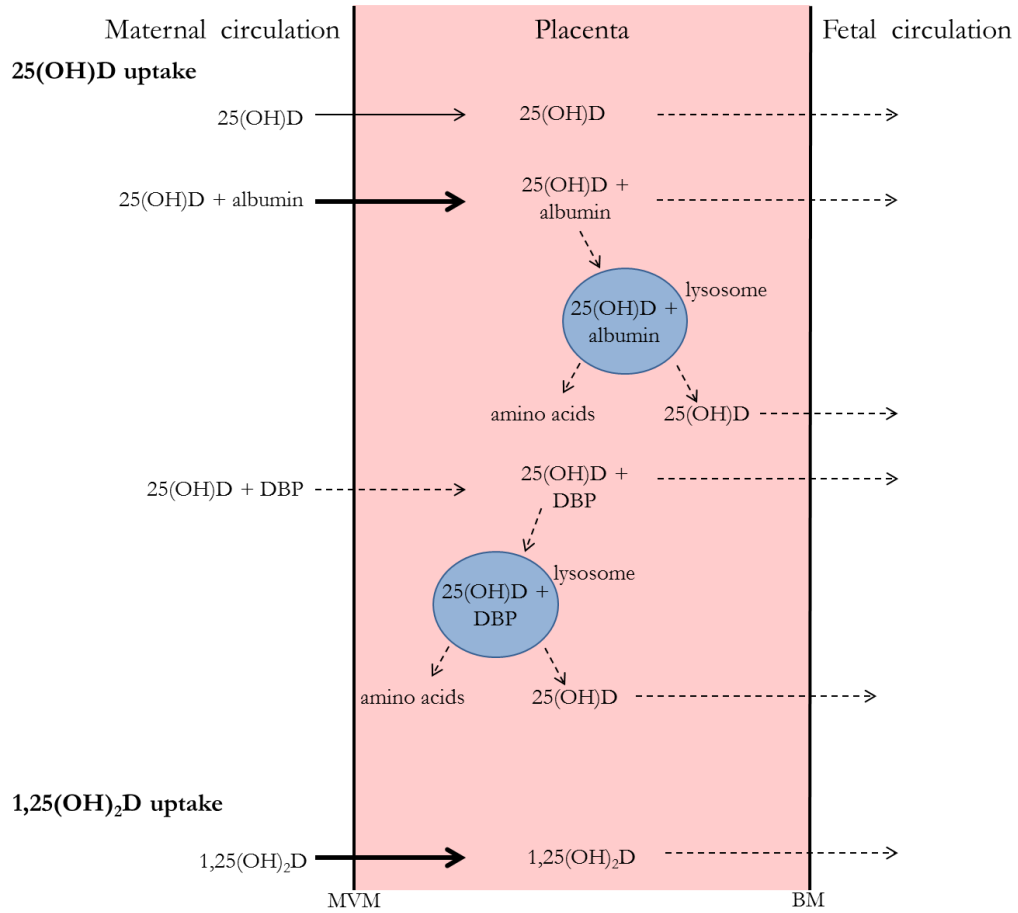

Figure 7.2: Summary of vitamin D uptake and potential routes of vitamin D transport into the fetal circulation. 25(OH)D entry is greatly facilitated by the presence of albumin. It is currently unknown whether 25(OH)D entry into the placenta is similarly affected by DBP. Once inside the placenta 25(OH)D may pass directly into the fetal circulation. Albumin or DBP bound 25(OH)D could be dissociated from the carrier protein by degradation of the carrier within the lysosome resulting in release of free 25(OH)D and amino acids. This lysosomal degradation may be partly mediated by the lysosomal enzyme, legumain. 1,25$(OH)_2$D entry into the placenta is not enhanced by the presence of a binding protein, therefore it appears uptake is mainly of unbound 1,25$(OH)_2$D. Dashed arrows indicate pathways that require further investigation.

7.3. Placental handling and metabolism of vitamin D

7.3.1. Placental production of $1,25(OH)_2D$

In order for vitamin D to act upon the placenta either $1,25(OH)_2D$ entry into the placenta is required or the placenta must be able to metabolise 25(OH)D to generate the active form of vitamin D. Furthermore, expression of the receptors VDR and RXRα are required for $1,25(OH)_2D$ to exert effects on mRNA expression. This study confirms previous investigations reporting the production of $1,25(OH)_2D$ from 25(OH)D within the human placenta (Halhali *et al.*, 1999). The fact that we saw a large fold increase in *CYP24A1* mRNA expression when placental villous fragments were cultured with 25(OH)D means that within the placental fragment CYP27B1 was active and acted upon 25(OH)D to generate the active form of vitamin D, $1,25(OH)_2D$. $1,25(OH)_2D$ could then bind and activate the VDR/RXRα heterodimer resulting in vitamin D-mediated changes to mRNA expression. Primarily, this resulted in the increased expression of the vitamin D catabolism enzyme, *CYP24A1* (Figure 7.3). *CYP24A1* has been reported to be heavily methylated within the placenta, which was proposed to result in abrogation of the classic $1,25(OH)_2D$-mediated up-regulation in *CYP24A1* mRNA expression within this tissue (Novakovic *et al.*, 2009). The data presented here and by Halhali *et al.* (1999) show that *CYP24A1* mRNA is responsive to $1,25(OH)_2D$ in placental tissue.

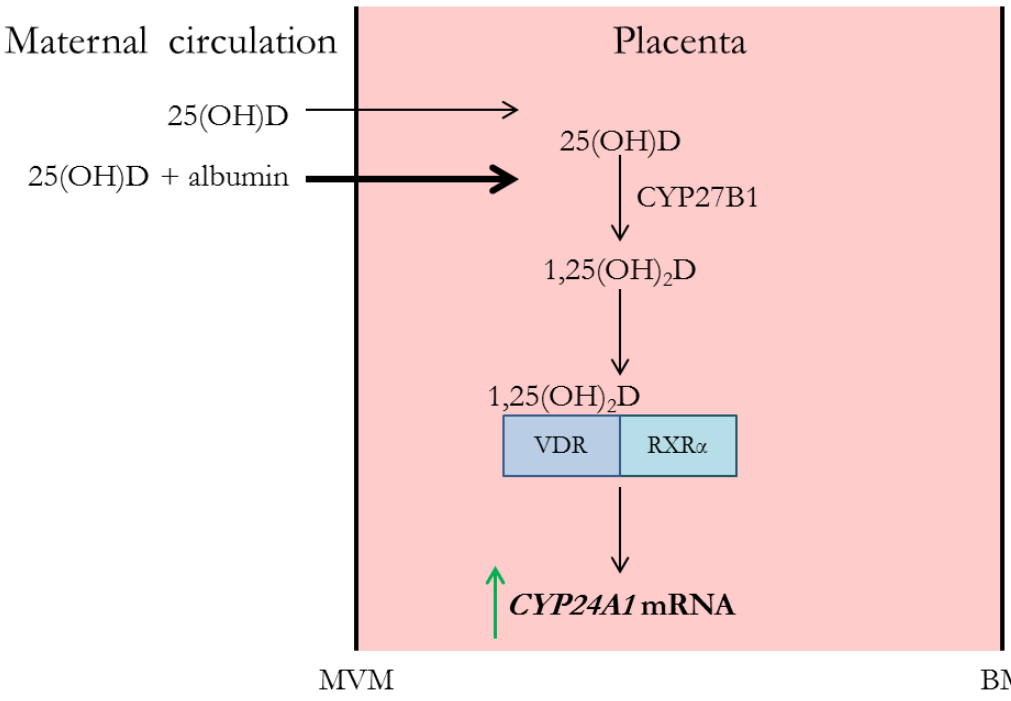

Figure 7.3: *CYP24A1* mRNA expression is induced following active vitamin D binding to and activating the VDR/RXRα heterodimer. 25(OH)D is converted to the active $1,25(OH)_2D$ via the action of CYP27B1. $1,25(OH)_2D$ can then bind and activate the VDR/RXRα heterodimer, resulting in vitamin D-mediated effects on mRNA expression. One of the classical effects of vitamin D on mRNA expression is the increase in mRNA expression of the vitamin D catabolism enzyme *CYP24A1*.

This conversion of 25(OH)D into $1,25(OH)_2D$ was not observed in the HEK293 cell model, as no increase in *CYP24A1* mRNA expression was observed following incubation with increasing amounts of 25(OH)D. Furthermore, due to the amplification of genomic DNA the presence of *CYP27B1* mRNA could not be clearly ascertained in this cell line. This result confirms the conclusion from the cell culture studies presented in Chapter 4, that an *ex vivo* placental model was required to investigate the transport of vitamin D and its effects on the placenta. However, the highest concentration used with HEK293 cells was 100 nmol/l 25(OH)D, while in human placental villous fragment culture a higher concentration of 20 μmol/l 25(OH)D was used. It is possible that *CYP24A1* up-regulation may have occurred in HEK293 cells with this higher concentration of 25(OH)D.

The demonstration of $1,25(OH)_2D$ production within the placenta, leads to the question of the function of this active vitamin D within the placenta. There are three possibilities for the fate of active vitamin D within the placenta; i) it acts locally upon the placenta itself, ii) it is transported into the fetal circulation, or iii) it is transported back into the maternal circulation. Experimental evidence has been published which supports all of these possibilities to an extent. The increase in *CYP24A1* mRNA expression observed in placental villous fragments supports the notion that $1,25(OH)_2D$ acts locally upon the placenta to alter mRNA expression. Furthermore, in this *ex vivo* placental model, *TAT1* mRNA expression was also increased in response to $1,25(OH)_2D$. The effect of vitamin D on placental mRNA expression is discussed in more detail in section 7.4. Some studies have reported positive associations between maternal and cord serum levels of $1,25(OH)_2D$ (Hollis and Pittard, 1984), providing support for transfer of this metabolite from the placenta and into the fetal circulation. However, other studies has reported no association between maternal and cord serum $1,25(OH)_2D$ (Fleischman *et al.*, 1980; Delvin, 1982; Ishida *et al.*, 1988), leading to the currently accepted view that $1,25(OH)_2D$ is not transported across the human placenta (Adams and Hewison, 2012). In support of the third option, there is evidence showing transport of $1,25(OH)_2D$ produced in the placenta into the maternal circulation. In pregnant nephrectomised rats, injection of 25(OH)D led to the appearance of $1,25(OH)_2D$ in the maternal circulation. This effect was not seen in non-pregnant rats (Weisman *et al.*, 1978). However, the evidence for transport of placental $1,25(OH)_2D$ into the maternal circulation is not consistent, as *CYP27B1*$^{-/-}$ sows showed no increase in $1,25(OH)_2D$ during pregnancy when carrying a *CYP27B1*$^{+/-}$ fetus (Lachenmaier-Currle and Harmeyer, 1989). In addition, a case study of a pregnant woman in chronic renal failure described a small increase in $1,25(OH)_2D$ levels during pregnancy, however, her vitamin D levels remained much lower than those of a normal pregnant woman (Turner *et al.*, 1988). Nevertheless, the transfer of $1,25(OH)_2D$ into the maternal circulation has not been investigated in a non-pathological pregnancy state. It is currently unclear whether the placenta would supply the mother with

$1,25(OH)_2D$, if she is able to produce an adequate supply herself. This question could be addressed in human placental perfusion studies (see section 7.6).

- **The placenta can metabolise 25(OH)D into the active $1,25(OH)_2D$ through the action of CYP27B1. This active vitamin D has been shown to be able to exert effects on placental expression of *CYP24A1*, indicating that the placenta is able to metabolise and respond to vitamin D.**

7.3.2. Differing roles for 25(OH)D and $1,25(OH)_2D$ within the placenta?

It is interesting to speculate that $1,25(OH)_2D$ and 25(OH)D may play differing roles in the placenta and in fetal development. $1,25(OH)_2D$ may act upon the placenta and cause changes in placental mRNA expression and function, while 25(OH)D may primarily provide a source of vitamin D for the fetus (Figure 7.4). CYP27B1 is active within the fetal kidneys (Fenton and Britton, 1980), therefore the fetus can generate its own source of active vitamin D from 25(OH)D. In addition, fetal nephrectomy in sheep resulted in a significant reduction in fetal plasma $1,25(OH)_2D$, implying that fetal CYP27B1 activity may be the primary source of active vitamin D for the fetus (Ross *et al.*, 1980). The notion that 25(OH)D may mostly be transported across the placenta and into the fetal circulation is supported by the fact that to see significant changes in *CYP24A1* mRNA expression in human placental fragments in response to 25(OH)D the concentrations of inactive vitamin D had to be increased. This could demonstrate that delivery to the fetus is prioritised over local actions on the placenta. It may only be when 25(OH)D is in excess that placental production of $1,25(OH)_2D$ occurs. This could also explain the relatively low expression levels of *CYP27B1* within the placenta. In order to detect this gene reliably the amount of cDNA in the qrt-PCR reaction was increased from 3 to 5 μl. If placental production of $1,25(OH)_2D$ was crucial for the fetus, we would expect this enzyme to be expressed at higher levels. The maternal supply of $1,25(OH)_2D$ into the placenta may provide the placenta with a source of active vitamin D. The notion that this form acts locally upon the placenta, rather than being transported into the fetal circulation is supported by studies describing no association between maternal and cord serum $1,25(OH)_2D$ (Fleischman *et al.*, 1980; Delvin, 1982; Ishida *et al.*, 1988), while significant positive associations between maternal and cord serum 25(OH)D are widely reported (Bouillon, 1981; Delvin, 1982; Hollis and Pittard, 1984; Markestad, 1984; Novakovic *et al.*, 2012).

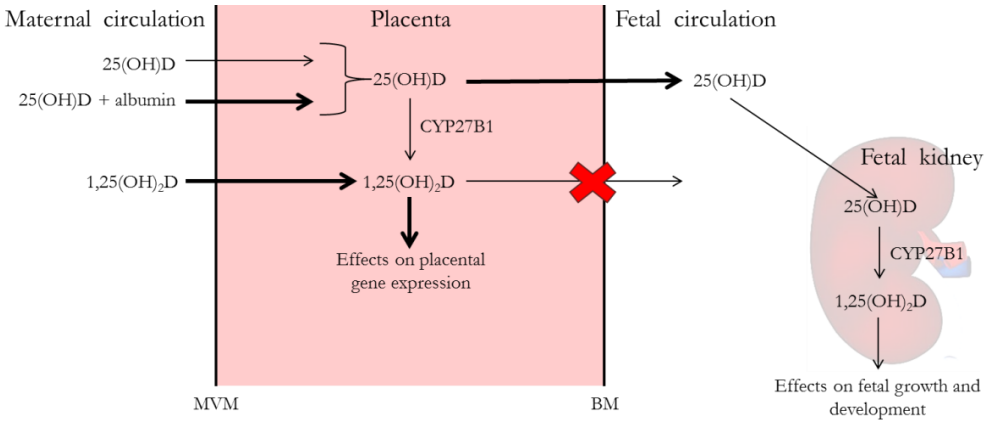

Figure 7.4: Proposed differing roles of 25(OH)D and 1,25(OH)$_2$D in the human placenta and for fetal growth. 25(OH)D from the maternal circulation may primarily provide a source of vitamin D for transport into the fetal circulation. This vitamin D can then undergo hydroxylation in the fetal kidneys via CYP27B1 and exert vitamin D-mediated effects on fetal growth and development. In situations of 25(OH)D excess, hydroxylation in the placenta may be stimulated. 1,25(OH)$_2$D from the maternal circulation may primarily provide a source of vitamin D for local actions on placental mRNA expression.

7.3.3. Placental expression of vitamin D metabolism and signalling genes relates to fetal growth

Associations observed between placental expression of vitamin D-related genes with fetal, neonatal and infant growth in the SWS and MAVIDOS cohorts, suggest that the placental handling of vitamin D may have implications for fetal growth and development. For example, an increased expression of the *VDR* would presumably result in a placenta that is more responsive to vitamin D. In SWS placentas, *VDR* mRNA expression was negatively associated with measures of fetal and neonatal size, including head circumference, femur length, lean and fat mass. These associations were still present at 4 years of age, with placental *VDR* showing negative associations with weight and percentage lean and fat mass, as well as a positive association with BMD. Associations between placental expression of VDR and fetal growth have previously been described (Young *et al.*, 2014; Nguyen *et al.*, 2015), while, maternal vitamin D deficiency was shown to be associated with low birth weight only with specific *VDR* genotypes (Morley *et al.*, 2009). Placental *VDR* expression in SWS placentas showed the most extensive associations with infant size from the fetus until 4 years of age. Other associations were observed with other vitamin D-related genes, for example, *CYP27B1* mRNA expression in SWS placentas was negatively associated with percentage fat mass at 6 years of age. Placental *CYP24A1* mRNA expression also showed associations with a number of measures of fetal and neonatal size and body composition, suggesting positive associations between vitamin D and fetal and infant growth. These are discussed further in section 7.4. Altered expression of vitamin D-related genes has been demonstrated in pregnancy pathologies including pre-eclampsia (Diaz *et al.*, 2002; Ma *et al.*, 2012; Anderson *et al.*, 2015). This could mean that the placentas ability to metabolise and respond to vitamin D is involved in the development of specific

pathologies. However, it is currently unclear whether these alterations in placental mRNA expression are a cause or a consequence of the adverse intrauterine environment.

The placental associations observed with *VDR* suggest an increased responsiveness to vitamin D within the placenta results in reductions in both lean and fat mass at 4 years of age. A similar association has been described for 25(OH)D within the same cohort, as late pregnancy serum 25(OH)D was negatively associated with fat mass at 6 years of age (Crozier *et al.*, 2012). However, the same maternal measure of vitamin D was associated positively with grip strength and percentage lean mass at 4 years of age (Harvey *et al.*, 2014b), while showing positive trends with BA and BMC in female neonates (Harvey *et al.*, 2008). These associations suggest positive associations with maternal vitamin D status and lean mass, while the *VDR* mRNA expression data suggest a negative association between the placental response to vitamin D and lean mass. The differing associations obtained from the same cohort could provide support for the notion of the differing roles of the two forms of vitamin D within the placenta; the maternal 25(OH)D being the form that is largely transported into the fetal circulation and influencing fetal growth and development, while $1,25(OH)_2D$ mediates placenta-specific effects. It could then be these placenta-specific actions of vitamin D that we are observing with the associations between placental mRNA expression and infant anthropometry. In SWS placentas, *cubilin* mRNA expression was positively associated with fetal femur length, as well as crown-heel length and head circumference of the neonate. *Megalin* mRNA expression in SWS placentas was positively associated with crown-heel length of the fetus in early gestation, and showed positive associations with placental weight and lean mass of the neonate in MAVIDOS placentas. If megalin and cubilin are involved in transport of 25(OH)D into the placenta, these associations could be indicative of the positive effects of vitamin D on growth observed in the SWS.

If placental expression of specific vitamin D-related genes impacts the placentas response to vitamin D, this leads to the question of what may regulate these differences in mRNA expression. Many factors have been described that could alter expression of vitamin D-related genes, such as cAMP (Avila *et al.*, 2004), IGF1 (Halhali *et al.*, 1999), pro-inflammatory cytokines and leptin (Noyola-Martinez *et al.*, 2014; O'Brien *et al.*, 2014). It is also possible that some of the differences in placental expression of vitamin D-related genes may be mediated via maternal factors. In SWS placentas, *CYP24A1* mRNA expression was positively associated with measures of maternal fat mass both before and during pregnancy, while *CYP27B1* mRNA showed inverse relationships with maternal fat mass before and during pregnancy. These data suggest that maternal body composition may play a role in determination of placental handling of vitamin D. In addition, methylation levels at CpG -1062 in the *TAT1* promoter region in SWS placental DNA were negatively associated with numerous measures of maternal fat mass prior to pregnancy. This suggests that one way in which

maternal body composition could change placental mRNA expression is via altered methylation status. In support of the role of methylation in mediating expression of placental genes, differential methylation of *CYP27B1*, *VDR* and *RXRa* was reported in placentas from pre-eclamptic pregnancies compared to healthy controls (Anderson *et al.*, 2015). Furthermore, differential DNA methylation of *CYP24A1* measured in human serum was shown to occur in those who responded to vitamin D supplementation compared to those who did not (Zhou *et al.*, 2014b). It is currently unclear what may cause this altered methylation, as mRNA expression of *DNMT1* and *DNMT3b* in SWS placentas was not affected by maternal body composition either prior to or during pregnancy. One possibility is that associations with fat mass may be mediated via vitamin D as circulating vitamin D levels are reported to be lower in those with higher BMIs due to sequestration of the vitamin D within excess adipose tissue (Wortsman *et al.*, 2000; Bodnar *et al.*, 2007a; Zhao *et al.*, 2012; Vimaleswaran *et al.*, 2013). In support of this, methylation of the *TAT1* promoter CpG -1062 in DNA from SWS placentas showed a trend for a negative association with placental expression of *CYP24A1*, a potential marker for active vitamin D within the placenta. However, in the SWS cohort we did not see associations between maternal serum 25(OH)D and *CYP27B1* or *CYP24A1* mRNA expression within the placentas, which would suggest that the association between maternal fat mass and expression of these placental genes is driven by a currently unknown factor. Altered expression of vitamin D metabolising enzymes has been reported in pregnancy pathologies (Diaz *et al.*, 2002; Ma *et al.*, 2012) but this is the first study to report associations with maternal body composition in normal uncomplicated pregnancies. We now need to investigate what maternal signals may result in the altered expression of these enzymes in normal human pregnancy.

- **Placental expression of genes involved in the metabolism and response to vitamin D was associated with fetal growth. This could indicate that the placentas ability to respond to vitamin D is important for fetal development.**

7.3.4. Summary

The second major aim of this thesis was to investigate expression of genes involved in placental metabolism, transport and response to vitamin D. This study has confirmed the activity of the vitamin D activating enzyme, CYP27B1, within human placental samples, and proposes that 25(OH)D is the major form of vitamin D transported to the fetus, while $1,25(OH)_2D$ may predominantly act upon the placenta. The placentas capability to respond to and metabolise vitamin D may impact placental function, resulting in effects on fetal growth and development. Furthermore, this ability to metabolise vitamin D may be mediated by maternal body composition both prior to and during pregnancy.

7.4. Vitamin D effects on placental mRNA expression

7.4.1. Effects of vitamin D on expression of placental nutrient transporter genes

While vitamin D may affect fetal growth directly it is also possible that vitamin D may act upon the placenta causing altered expression of nutrient transporters. This alteration in placental nutrient transporter expression could then result in alterations to fetal growth as a result of differential nutrient transport to the fetus. In the human cohorts, MAVIDOS and SWS, there were some, although not many associations between maternal 25(OH)D levels and placental mRNA expression. In SWS placentas, maternal 34 week 25(OH)D was positively correlated with *LAT3* and *y+LAT1*, while in MAVIDOS placentas maternal 34 week 25(OH)D was positively associated with *CaT1* mRNA. Furthermore, in MAVIDOS vitamin D supplementation during pregnancy did not have a large effect on placental mRNA expression. mRNA levels of only 2 genes were significantly altered following maternal vitamin D supplementation; *cubilin* was increased in placentas of spring births, and *ASCT2* was decreased in placentas of summer births. These data are in line with the theory that maternal, and possibly a small portion of placentally produced, $1,25(OH)_2D$ may have larger impacts on placental mRNA expression, while 25(OH)D is predominantly used for supply to the fetus. It is possible that the associations observed between maternal 25(OH)D and placental mRNA expression may reflect an indirect effect of 25(OH)D on placental mRNA expression. Maternal vitamin D levels may regulate a factor which in turn then regulates placental mRNA expression. Alternatively, maternal vitamin D levels and placental nutrient transporter expression may be regulated by the same common factor. One possibility is that they are regulated by maternal body composition, as higher BMI and fat mass have been linked with a lower vitamin D status (Wortsman *et al.*, 2000; Bodnar *et al.*, 2007a; Zhao *et al.*, 2012; Vimaleswaran *et al.*, 2013). In addition, *LAT3* mRNA expression in SWS placentas was negatively associated with maternal calf circumference before and during pregnancy (Cleal, J.K, unpublished observations). Activity of the system A amino acid transporters within placentas from the SWS was shown to be positively associated with maternal arm muscle area (Lewis *et al.*, 2010a). This suggests that the placenta can respond to maternal body composition through alteration of its transport capabilities. *ASCT1* and *SNAT1* placental mRNA expression were positively associated with maternal late pregnancy 25(OH)D levels but these associations were lost following adjustment for maternal confounding factors, which included a measure of fat mass. This suggests that these associations were driven through maternal confounding factors as opposed to being driven by maternal vitamin D levels. The associations between *LAT3* and *y+LAT1* in SWS placentas remained following this adjustment for confounding factors, suggesting that it may be vitamin D rather than maternal body composition that is driving these associations.

Placental expression of *CYP24A1* has been used a marker of placental exposure to active vitamin D, as *CYP24A1* mRNA expression is up-regulated following binding of $1,25(OH)_2D$ to the VDR/RXRα heterodimer. In the human cohort studies, *CYP24A1* mRNA expression was associated with mRNA levels of many more placental genes than maternal levels of 25(OH)D were. In SWS placentas *CYP24A1* mRNA was positively associated with mRNA expression of *ASCT2*, *cubilin*, *CYP27A1*, *megalin*, *PMCA1*, *DNMT1* and *DNMT3b* and showed positive trends with *TAT1* and *y+LAT2*. *LAT1* and *LAT2* mRNA expression levels were inversely correlated with *CYP24A1* mRNA. In MAVIDOS placentas, a number of these associations were observed again. Specifically, *CYP24A1* mRNA was positively associated with *cubilin*, *ASCT2*, *TAT1* and *DNMT1*. A positive association with *PMCA4* and a negative association with *ASCT1* with *CYP24A1* mRNA were also observed in MAVIDOS placentas. These associations suggest that active vitamin D levels within the placenta may affect a range of placental genes, including those involved in amino acid and calcium transport and DNA methylation (Figure 7.5). The fact that these associations were observed in both cohorts provides strong evidence for their importance. Furthermore, placental *CYP24A1* mRNA expression was positively associated with a number of measures of fetal and infant growth in both cohorts, suggesting that the effect of vitamin D on placental mRNA expression may be impacting fetal growth. Specifically, *CYP24A1* mRNA expression in SWS placentas was associated with fetal and neonatal head circumference, and showed a positive trend with birth weight. In MAVIDOS placentas, *CYP24A1* mRNA was positively associated with placental weight, and showed positive associations with BMC and BA, and positive trends with BMD, lean and fat mass of the neonate. In addition, placental *CYP24A1* mRNA expression was positively associated with measures of maternal fat mass prior to and during pregnancy (Figure 7.5). This could mean that maternal body composition mediates the placental response to vitamin D. In this case the associations were positive, suggesting that with higher maternal fat mass, the placenta was responding more to vitamin D. This could be a reflection of the lower vitamin D levels that are common with increased fat mass (Wortsman *et al.*, 2000; Bodnar *et al.*, 2007a; Zhao *et al.*, 2012; Vimaleswaran *et al.*, 2013), resulting in up-regulation of the vitamin D signalling pathway within the placenta. Placental *CYP27B1* mRNA expression did not show any associations with fetal or neonatal size or body composition. If placental production of active vitamin D was driving the increased *CYP24A1* expression, we may also have seen associations with expression of this activating enzyme. The lack of associations could mean that the enzyme expression level is not rate limiting for production of $1,25(OH)_2D$ in the placenta or that maternally-derived $1,25(OH)_2D$ plays a larger role in vitamin D-mediated actions on the placenta. As we found that placental mRNA expression of this enzyme was quite low, it seems more likely that maternal $1,25(OH)_2D$ is the primary source of active vitamin D for the placenta. Interestingly, none of the genes associated with placental *CYP24A1* mRNA were associated with maternal 25(OH)D. This suggests that these two sets of genes are regulated in different ways. It could be that placental expression of *CYP24A1* and

the genes it was associated with are regulated by a factor that is not vitamin D. In both cohorts, we need measures of 1,25(OH)$_2$D in both the maternal and cord serum to explore whether *CYP24A1* mRNA expression provides a good biomarker for active vitamin D levels.

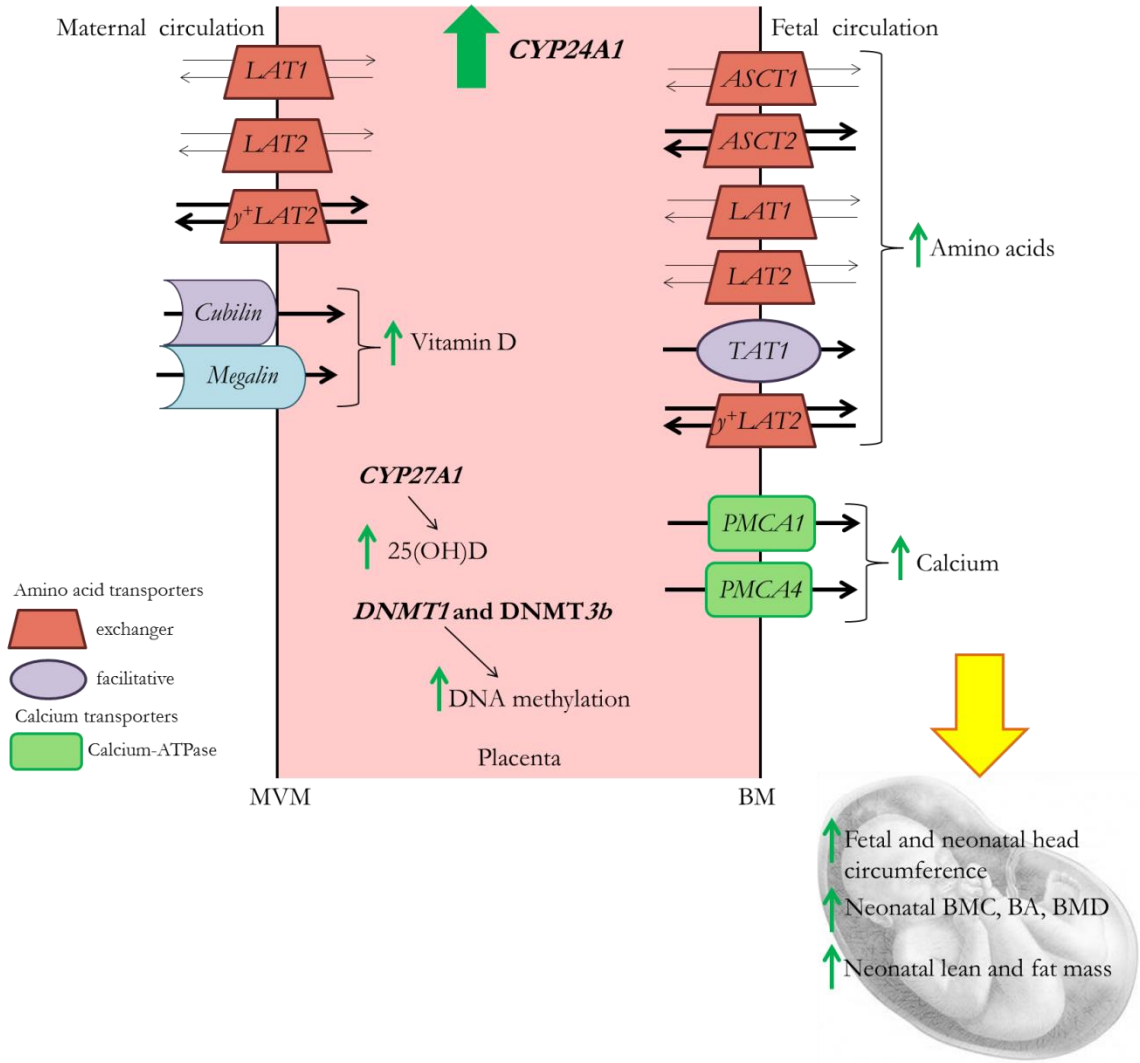

Figure 7.5: Associations between placental *CYP24A1* mRNA expression with placental gene expression and fetal growth. Transporters with bold arrows showed positive relationships with *CYP24A1*, while those with thin arrows showed negative relationships. *CYP24A1* mRNA expression was positively associated with a number of placental amino acid and calcium transporters, which would presumably result in increased transfer of calcium and amino acids to the fetus. *CYP24A1* also showed a positive association with *CYP27A1* mRNA expression which would likely result in increased placental production of 25(OH)D. mRNA expression of *DNMT1* and *DNMT3b* was also positively associated with *CYP24A1*, which would result in increased methylation of placental DNA. The culmination of these effects could explain the enhanced fetal and neonatal anthropometric measurements associated with placental *CYP24A1* expression.

In vitro cell culture studies with HEK293 cells revealed no changes in mRNA levels of amino acid and calcium transport or vitamin D-related genes following treatment with inactive or active vitamin D. However, *ex vivo* work using placental villous fragments did show increased *TAT1* mRNA expression in response to 1,25(OH)$_2$D and increased *PMCA1* mRNA following treatment with 1,25(OH)$_2$D and albumin. These associations follow the pattern of the positive associations

observed with *CYP24A1* and expression of these genes in SWS and MAVIDOS placenta samples, and support the notion that the associations with *CYP24A1* observed in the human cohort studies are vitamin D-mediated effects. Furthermore, *PMCA1* mRNA expression has previously been shown to be up-regulated in rat and human duodenum and rat osteoclast cells following exposure to 1,25$(OH)_2$D (Brown *et al.*, 2002; Balesaria *et al.*, 2009).

RNA sequencing on placental villous fragments cultured with 25(OH)D, 25(OH)D and albumin or vehicle and albumin control revealed additional changes in mRNA expression of some vitamin D genes as well as calcium and amino acid transporters. Specifically, *CYP27A1* mRNA was increased 1.8 fold in response to 25(OH)D alone in placental villous fragments. In SWS placental samples this gene was positively associated with maternal 11 week serum 25(OH)D and with placental mRNA expression of *CYP24A1*. The combination of these data suggest that *CYP27A1* is regulated positively by vitamin D. In terms of amino acid transporters, none other than *xCT* showed an up-regulation over the 2 fold threshold in response to 25(OH)D or 25(OH)D and albumin in RNA sequencing data. However, some were significantly up-regulated at a lower level, for example *ASCT1* showed a 1.4 fold up-regulation in expression in response to 25(OH)D and 25(OH)D with albumin compared to control treated villous fragments. In addition, this gene was positively associated with maternal 34 week serum 25(OH)D in SWS placentas. This suggests a positive effect of vitamin D on expression of this amino acid transporter may occur within the placenta. Vitamin D has previously been linked to up-regulation of amino acid transport. A synergistic effect of 1,25$(OH)_2$D and retinoic acid on TauT promoter activity and uptake activity has been described in a porcine kidney cell line (Chesney and Han, 2013), while vitamin D deficient rats showed reduced renal uptake of proline and taurine (Dabbagh *et al.*, 1990). This supports the notion that vitamin D can regulate mRNA expression of amino acid transporters.

In addition to amino acid transport, vitamin D may also impact placental calcium transport. RNA sequencing revealed that *PMCA1* mRNA expression was increased 1.4 fold in 25(OH)D and albumin treated placental fragments compared to those treated with 25(OH)D alone. The same pattern was observed by qrt-PCR for *PMCA1* in placental fragments in response to 1,25$(OH)_2$D with albumin compared to 1,25$(OH)_2$D alone. Furthermore, in SWS placentas *PMCA1* mRNA expression was positively associated with *CYP24A1* mRNA expression. These findings suggest that *PMCA1* within the placenta is regulated by vitamin D, and the increased expression observed in placental fragments incubated with albumin could be a result of the increased transport of vitamin D into the placenta. This idea is supported by the fact that *PMCA1* mRNA expression was increased in human duodenal biopsies following exposure to 1,25$(OH)_2$D (Balesaria *et al.*, 2009). *PMCA1* mRNA expression was not increased in rat cochlea cells in response to 1,25$(OH)_2$D (Yamauchi *et al.*, 2010), which may represent species- or tissue-specific responses to active vitamin

D. In addition, mRNA expression of *CaT1*, a calcium influx transporter, showed a positive association with *CYP24A1* mRNA expression in MAVIDOS placenta samples and was up-regulated 3.7 fold in placental villous fragments exposed to 25(OH)D and albumin compared to albumin alone. However, in placental villous fragments exposed to 25(OH)D, mRNA expression of *CaT1* was down-regulated 0.3 fold compared to those exposed to control buffer. The contrasting effects of 25(OH)D alone and with albumin could represent an albumin-mediated effect (see section 5.4.3). However, the positive association with the vitamin D responsive gene, *CYP24A1*, in MAVIDOS placentas suggests that this relationship may instead be mediated by the increased vitamin D uptake. In support of this, *CaT1* has previously been shown to be up-regulated by $1,25(OH)_2D$ exposure in *ex vivo* studies on human duodenal biopsies (Balesaria *et al.*, 2009). The combination of RNA sequencing and qrt-PCR data with mRNA expression associations from the SWS and MAVIDOS human cohorts suggests that vitamin D may regulate expression of specific nutrient transporters within the placenta.

While maternal 25(OH)D was not associated with expression of many genes in the human cohort studies, placental villous fragment culture with 25(OH)D did result in changes to placental mRNA expression. These differences could result from a number of possibilities. Firstly, there was a delay between maternal blood sample at 34 weeks of gestation and the collection of placenta samples at delivery. The average length of human gestation is 40 weeks, therefore this would result in a 6 week lag between vitamin D measures and placental sampling. 25(OH)D is considered the more stable form of vitamin D compared to $1,25(OH)_2D$ and serum levels should remain relatively stable over time which it why it is used routinely to assess vitamin D status. However, fluctuations in circulating 25(OH)D could have resulted in the lack of associations observed in the human cohorts compared to those in *ex vivo* placental studies. Secondly, the maternal vitamin D supplementation in MAVIDOS was a long-term exposure to a vitamin D supplement, compared to the 8 hr exposure in placental fragment studies. In addition, serum levels measured in SWS and MAVIDOS are likely to represent long-term vitamin D levels. Over the longer time period it is possible that the placenta may have adapted in response to the chronic increased levels of vitamin D. Cultured human syncytiotrophoblast has previously been shown to exhibit altered responses to $1,25(OH)_2D$ depending on the duration of exposure (Barrera *et al.*, 2008). Thirdly, the placental fragment studies used a large dose of 25(OH)D as we needed to initiate changes to mRNA transcription in order to measure *CYP24A1* mRNA levels and assess vitamin D uptake. It is possible that due to the larger exposure to vitamin D this would have resulted in an up-regulation of CYP27B1 activity, resulting in activation of a larger portion of 25(OH)D than usual. *CYP27B1* mRNA levels were increased in 25(OH)D and albumin treated placental villous fragments compared to 25(OH)D treated fragments, supporting the notion that the increased exposure to 25(OH)D may have driven increased conversion to $1,25(OH)_2D$.

Another factor which may have resulted in only a few associations being detected between maternal vitamin D and placental gene expression in the human cohort studies is the analysis of male and female placentas together. A number of studies have shown that in response to factors such as maternal undernutrition, changes in placental gene expression differ depending on the sex of the fetus (Rosenfeld, 2015). In the two human cohort studies used in this thesis, the data were adjusted for sex of the offspring, but as discussed in Section 3.4.8 it would be interesting to separate the two cohorts by offspring sex and then look at associations between maternal vitamin D levels and placental gene expression. Male placentas are thought to be more efficient, while female placentas are thought to have a greater capacity to deal with adverse stimuli, such as nutrient restriction (Eriksson *et al.*, 2010). In light of this, it is interesting to speculate that female placentas may be more responsive to factors such as maternal vitamin D levels, and therefore more associations between maternal vitamin D levels and placental nutrient transporter gene expression may be observed in female placentas compared to male placentas.

- **Vitamin D may affect expression of specific nutrient transporters within the placenta, as *ex vivo* studies using placental villous fragment culture revealed altered expression of specific genes involved in nutrient and calcium transport. In addition, placental expression of *CYP24A1,* a known vitamin D responsive gene, was positively associated with mRNA levels of specific amino acid and calcium transporters within the placenta, suggesting that the placentas exposure to active vitamin D may result in enhanced nutrient transport to the fetus.**

7.4.2. Potential mechanisms of action of vitamin D on placental mRNA expression

Vitamin D could affect mRNA expression through a number of mechanisms. Vitamin D could regulate mRNA expression directly via the action of VDR/RXRα binding to VDREs within the promoter region of genes. Analysis using MatInspector revealed a potential VDRE within the *TAT1* promoter region, so this could explain the mechanism of *TAT1* mRNA induction with vitamin D. However, further mechanistic studies are required to confirm whether the predicted site is indeed a VDRE. Vitamin D has also been shown to affect mRNA expression via post-transcriptional effects on mRNA half-life. Specifically, vitamin D was shown to increase mRNA expression of the *epidermal growth factor receptor* in an osteoblast cell line through prolonging the half-life of the mRNA rather than through promotion of transcription (Gonzalez *et al.*, 2002).

Alternatively, vitamin D may regulate mRNA expression indirectly via epigenetic mechanisms. In both human cohorts, *DNMT1* mRNA expression within the placenta was positively associated with *CYP24A1* mRNA expression. *DNMT3b* expression has only been investigated in SWS placentas to

date, but this also showed a positive association with *CYP24A1* mRNA. Therefore for some genes, changes in mRNA expression may be a result of indirect mechanisms via vitamin D-mediated alteration in methylation status. *In vitro* studies where HEK293 and BeWo cells were exposed to the DNMT inhibitor, AZA, resulted in increased expression of a number of genes including *ASCT1*, *ASCT2*, *LAT1* and *LAT2*, and reduced expression of *TAT1*, *y*$^+$*LAT2* and *megalin*. In addition, *DNMT1* expression was positively associated with mRNA expression of *TAT1*, *y*$^+$*LAT2*, cubilin, *megalin* and *PMCA1* in SWS placentas. *DNMT3b* mRNA expression was associated positively with *y*$^+$*LAT2*, *cubilin*, *megalin* and *PMCA1* and *ASCT2*. DNA methylation is generally associated with transcriptional repression, therefore the positive associations observed with DNMT expression and mRNA expression in SWS placentas are interesting. These could be mediated by methylation occurring at CpGs within negative response elements. Potential links between vitamin D, DNMT, and DNA methylation are displayed in Figure 7.6.

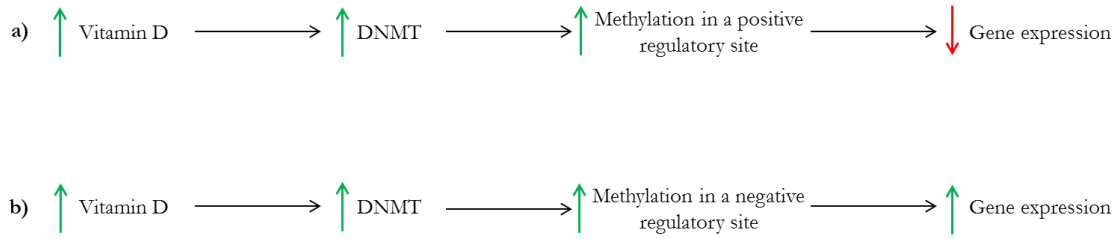

Figure 7.6: Differential effects of vitamin D-mediated changes in DNA methylation. In both a) and b) increased vitamin D levels are associated with increased expression of DNMTs as was observed in placentas from the SWS. In a) methylation within a positive regulatory region of the target genes promoter is catalysed by DNMTs, this would prevent the binding of transcription factors and co-activators and therefore prevent transcriptional activation resulting in a reduction in gene expression. Genes for which this occurs would be expected to show inverse relationships with both vitamin D status and DNMT expression. However, no genes in SWS placentas showed negative relationships with DNMT expression. In b) the increased DNMT expression catalyses DNA methylation within a negative regulatory site of the target genes promoter region. This prevents the binding of negative regulatory factors, such as co-repressors, and would therefore result in increased expression of the target gene. Genes that are regulated in this manner would be expected to show positive relationships with 25(OH)D levels and DNMT expression. *y*$^+$*LAT1* in SWS placentas showed a positive association with maternal 34 week 25(OH)D as well as a positive association with placental mRNA expression of *DNMT3b*.

Additional epigenetic mechanisms for vitamin D-mediated regulation of mRNA expression could occur via histone modifications or miRNAs. Ligand bound VDR interacts with histone acetyl transferases leading to the formation of an open chromatin state. This open chromatin state means the DNA is accessible to transcription factors and is available for mRNA transcription (Fetahu *et al.*, 2014). In addition, vitamin D has also been shown to induce expression of miRNAs (Alvarez-Diaz *et al.*, 2012). miRNAs are small noncoding RNA molecules that are generally associated with down-regulation of protein expression, through causing mRNA cleavage or translational repression. The mechanism of miRNA action is determined by the degree of sequence complementarity between the miRNA and mRNA sequences. If there is a high degree of complementarity between the miRNA and its mRNA target cleavage of the mRNA is induced, while if the degree of

complementarity between the two RNA molecules is lower, translation repression occurs resulting in down-regulation of protein expression without a change in mRNA levels (Ambros, 2001; Bartel, 2004). As the data in this thesis is focussed on mRNA transcript levels, we will only observe miRNA-mediated effects with high complementarity between the miRNA and mRNA molecules.

7.4.3. Summary

The third aim of this thesis was to investigate effects of vitamin D on placental mRNA expression. Data from human cohorts revealed few associations between maternal 25(OH)D and placental mRNA expression, while maternal vitamin D supplementation also had few impacts on mRNA expression. However, *CYP24A1* mRNA expression within the placenta showed associations with expression of a number of placental genes. Furthermore, expression of some of these genes was also altered in placental villous fragments upon exposure to $1,25(OH)_2D$. This suggests that the placental exposure to $1,25(OH)_2D$ may be more important in determining vitamin D-mediated effects on mRNA expression. Vitamin D could affect mRNA expression via direct actions on the VDRE promoting transcription, or via epigenetic mechanisms. We now need to investigate what mechanisms are responsible for the observed effects of vitamin D on placental mRNA expression.

In conclusion, this thesis has revealed that both 25(OH)D and $1,25(OH)_2D$ are transported into the placenta, and that this transport occurs via a specific process, possibly involving receptor-mediated endocytosis. Placental uptake of 25(OH)D was increased by the presence of albumin as a carrier protein, suggesting that albumin and possibly DBP bound vitamin D are taken up into the placenta. Furthermore, the placenta is able to metabolise 25(OH)D into the active $1,25(OH)_2D$, and respond to $1,25(OH)_2D$ through increased mRNA expression of the vitamin D responsive gene, *CYP24A1*. The placentas ability to metabolise and respond to vitamin D may be important for fetal growth and exposure of the placenta to vitamin D results in changes in mRNA expression of specific amino acid and calcium transporters. These changes in nutrient transporter expression may also mediate effects of vitamin D on fetal growth.

7.5. Limitations

For the human cohort studies, data are limited by the sample numbers. For MAVIDOS there were ~30 fewer samples compared to SWS which would have resulted in less power to detect associations in MAVIDOS samples compared to SWS. This is evidenced by retrospective power calculations on the two data sets which showed that while both data sets had adequate power to detect associations with an r value of 0.4, MAVIDOS was underpowered to detect associations with an r value of 0.3. In the SWS cohort associations between maternal serum 25(OH)D levels at 34

weeks of gestation and expression of specific placental genes were observed. These genes did not show the same associations with maternal 34 week 25(OH)D levels in MAVIDOS placentas. The differences could be a reflection of the lower sample numbers in MAVIDOS. It was also thought that the differences could reflect discrepancies in the range of vitamin D levels in the two cohorts, however, the range of vitamin D levels did not differ widely between the two studies. Although it is possible that as supplementation will have resulted in increased vitamin D levels in half of the MAVIDOS population, this rise in vitamin D status will have resulted in a smaller portion of the study population with vitamin D levels in the low range. This could have resulted in a reduced ability to detect associations with lower vitamin D levels. In addition, the exploratory nature and possibility of chance findings in relation to the human cohort studies needs to be acknowledged.

Further contrasts between the two human cohorts are also apparent. Maternal serum measures for both cohorts are slightly different. In SWS early pregnancy serum measures were taken at 11 weeks of gestation, while in MAVIDOS they were slightly later, at 14 weeks of gestation. The maternal blood supply to the placenta is established around 12 weeks of gestation (Johnson and Everitt, 2007), therefore the slight inconsistency between the timing of samples occurs across a particularly important part of gestation in terms of placental physiology. In addition, different metabolites have been measured in the two cohorts. In SWS we have measures of maternal 25(OH)D and DBP, while in MAVIDOS we have measures of maternal 25(OH)D, albumin and calcium. This means it was not possible to compare associations with some maternal metabolites across the two cohorts. A particular limitation of the human cohort studies is the lack of maternal serum $1,25(OH)_2D$ measurement. To gain a more detailed picture of vitamin D status, maternal serum measures of 25(OH)D, $1,25(OH)_2D$, albumin and DBP are required in both of the cohorts.

A slightly different range of genes were investigated in the two cohorts. *PMCA4* mRNA expression was not investigated in the SWS cohort due to issues with amplification of NECs. SWS RNA was extracted with a method that did not include a DNase step, whereas the extraction method for MAVIDOS RNA included a DNase step and thus *PMCA4* could accurately be measured. Some genes, such as *LPL*, that did not show associations with maternal 25(OH)D, DBP or placental *CYP24A1* in SWS placentas, were not pursued further in MAVIDOS. In addition, due to time constraints, some genes were not investigated in MAVIDOS, such as *DNMT3b*. *DNMT1* was studied in MAVIDOS as it is involved in maintenance methylation and therefore is more likely to play a role in methylation status of the placenta at term. However, if time had permitted it would have been interesting to also measure *DNMT3b*.

This investigation revealed problems with finding a suitable cell culture model to study placental vitamin D transport and metabolism. Two potential models, BeWo and HEK293 cells, were

investigated and both models showed differences in expression levels of important vitamin D or amino acid transporter genes. Studies with the HEK293 cell line were pursued slightly further, however, this model did not metabolise 25(OH)D into $1,25(OH)_2D$ as occurs within the placenta. Initially, a cell line based model was pursued as this was thought to provide the better option in terms of reproducibility and access to tissue. However, as nether cell line was appropriate this led to the use of placental villous fragment culture. While this provided a much better system to study placental transport and metabolism of vitamin D, this model introduced the added variation that occurs as a result of collecting tissue from different individuals. In particular, this variation was observed with the placental uptake of $1,25(OH)_2D$ which was aided by albumin in some samples and was not in other. Furthermore, experimental replicates were limited by the availability of tissue.

Further limitations of *ex vivo* placental villous fragment culture include the fact that $1,25(OH)_2D$ uptake was focussed on more than 25(OH)D. $1,25(OH)_2D$ uptake was investigated in the presence of both albumin and DBP, while 25(OH)D was only investigated with albumin. Therefore, there are still questions to be answered regarding DBP-mediated uptake of 25(OH)D. In addition, investigation into the mechanism(s) of uptake requires further investigation with more specific endocytic inhibitors, as some of the inhibitors used may not have been specific enough. Positive controls are also required to prove efficacy of the specific endocytic inhibitors.

7.6. Future directions

Future directions for the two human cohort studies include the measurement of $1,25(OH)_2D$ in maternal serum, as well as measures of both 25(OH)D and $1,25(OH)_2D$ in cord serum samples. These measures would enable estimation of maternofetal transfer of 25(OH)D and $1,25(OH)_2D$. These measures may provide support for the hypothesis that 25(OH)D is largely transferred to the fetus while $1,25(OH)_2D$ is not. In addition placental expression of *CYP24A1* could be tested for associations with maternal and cord serum $1,25(OH)_2D$. This would provide support for whether or not placental *CYP24A1* is a marker for placental exposure to active vitamin D.

Placental perfusion studies would provide a great tool to more thoroughly investigate the transport of vitamin D across the human placenta. Perfusion of the placenta with each type of vitamin D separately would allow measurement of the percentage of each that reaches the fetal circulation. This would build upon the current placental villous fragment studies which show entry into the placenta. Furthermore, this could be carried out for each type of vitamin D in the presence and absence of albumin and DBP to assess the effect of the binding proteins on placental transfer of both 25(OH)D and $1,25(OH)_2D$. In addition, mass spectrometry could be used to assess the type

of vitamin D emerging into both the fetal and maternal circulations and would give a measure of 25(OH)D to $1,25(OH)_2D$ conversion within the placenta.

This study has shown that 25(OH)D entry into the placenta is aided largely by albumin. It is still unclear whether DBP would have a similar effect, therefore repeating placental villous fragment experiments with 25(OH)D and DBP would answer this question. Furthermore, additional studies investigating the mechanism of entry of 25(OH)D and $1,25(OH)_2D$, both in the presence and absence of carrier proteins are now required. It is possible that different uptake mechanisms are used by the different types of vitamin D. Moreover the uptake mechanisms may also differ depending on the presence or absence and type of carrier protein. These questions could be answered using specific endocytic blockers, for example, RAP for inhibition of megalin (Nykjaer *et al.*, 2001).

Effects of vitamin D on expression of specific placental genes have been demonstrated. It is currently unclear whether these effects are directly mediated through VDREs within the genes promoter regions or whether the effects are mediated indirectly via epigenetic mechanisms. The use of gene reporter constructs could identify whether alterations in expression of specific genes are a direct result of vitamin D. The reporter constructs consist of a luciferase gene under control of an experimental promoter. For example, the promoter region of *NCX2* a gene that was up-regulated by vitamin D. This reporter construct would then be transfected into cells and the response to vitamin D examined. If vitamin D acted directly upon the promoter region, transcription of luciferase would be initiated which results in the measurable output of light. This technique would reveal whether vitamin D acts directly upon a genes promoter, however, only one gene could be investigated at a time. Chromatin immunoprecipitation and DNA sequencing could be conducted on placental villous fragment samples to give a broader picture of the genes regulated by vitamin D. This would reveal the sequences of DNA which are directly associated with VDR within the placenta. Additionally, a methylation array would reveal whether the addition of vitamin D to placental villous fragments resulted in widespread changes in DNA methylation of the samples, and would pinpoint the regions of the DNA in which methylation changes occurred.

7.7. Implications

In summary, this study has shown that vitamin D is likely to be transported into the placenta via specific mechanisms rather than through passive diffusion. Differential mechanisms of transport may be in operation for 25(OH)D and $1,25(OH)_2D$, with 25(OH)D transport relying largely on carrier proteins. In addition, active vitamin D levels within the placenta may impact expression of genes involved in DNA methylation, and amino acid and calcium transport. These vitamin D-

mediated effects on placental mRNA expression could provide an explanation for positive associations of placental *CYP24A1* expression, a potential biomarker for active vitamin D levels, with fetal and neonatal size in SWS placentas. Increased active vitamin D levels within the placenta may drive increased transport of amino acids and calcium to the fetus therefore promoting fetal growth. This potential effect on fetal growth is important not only for the immediate health of the infant, but also has implications for the lifelong health of the individual. The DOHaD theory states that the *in utero* environment programs specific changes to the fetus which can have a long-term impact on the health of the individual (Barker, 1990; Gluckman and Hanson, 2004). In light of the data presented here, the increased calcium transport which is likely to occur through the up-regulation of placental calcium transporter expression, may result in enhanced bone growth and development. The generation of stronger bones at this early point in gestation may then set the individual onto a path where a higher peak bone mass is attained and the risk of osteoporosis in later life is minimised (Figure 7.7). In addition, smaller size at birth has been linked with increased risk of other non-communicable diseases, including cardiovascular disease and type 2 diabetes, therefore the effects of low maternal vitamin D and altered placental response to vitamin D may not just be confined to bone development.

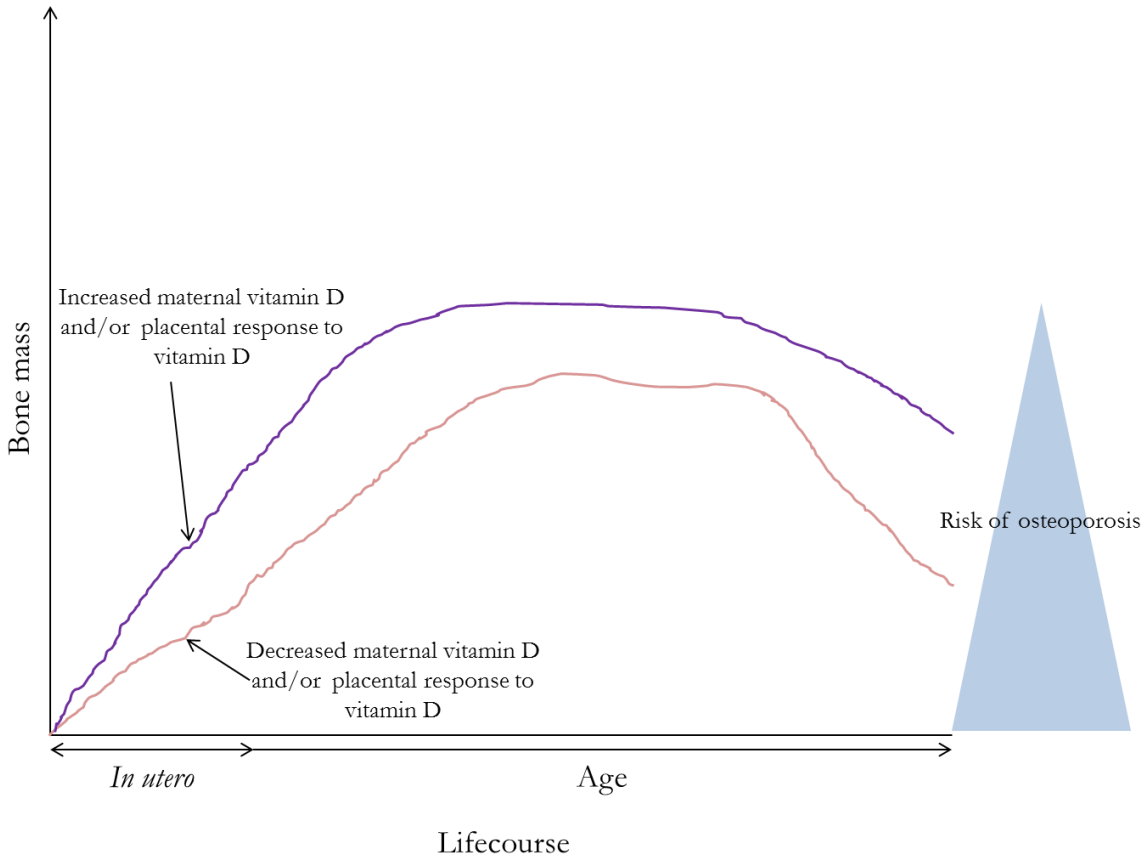

Figure 7.7: Placental exposure and response to vitamin D may impact bone development *in utero*. Increased exposure and ability to respond to vitamin D may increase bone mass attained *in utero*. This increase could then put the individual on a pathway for higher bone mass throughout the lifecourse, reducing the risk of developing osteoporosis in later life.

Placental exposure to vitamin D may also impact DNA methylation within the placenta via effects on DNMT expression. This means that vitamin D could have wide ranging impacts on placental mRNA expression and function. In addition, placental expression of genes such as *megalin* and *cubilin*, showed associations with measures of infant size. This suggests that not only is vitamin D important for fetal growth, but the placentas ability to transport vitamin D is also significant. The combination of maternal vitamin D levels and the placentas response to vitamin D are likely to determine the impact of vitamin D on fetal growth (Figure 7.8). This may explain why associations with maternal vitamin D levels and measures of fetal growth and bone development are not consistently observed (Harvey *et al.*, 2014a). This also has implications in terms of vitamin D supplementation during pregnancy. While vitamin D supplementation during pregnancy consistently shows a positive effect on maternal serum 25(OH)D levels, the effects on fetal growth are again inconsistent (Harvey *et al.*, 2014a). The results presented here suggest that to enhance efficacy of vitamin D supplementation during pregnancy, factors which may impact the placentas response to vitamin D should also be considered. For example, in our SWS data set, maternal size before and during pregnancy showed associations with placental expression of vitamin D metabolising enzymes, therefore the placental response to vitamin D may be partly mediated through maternal size (Figure 7.8).

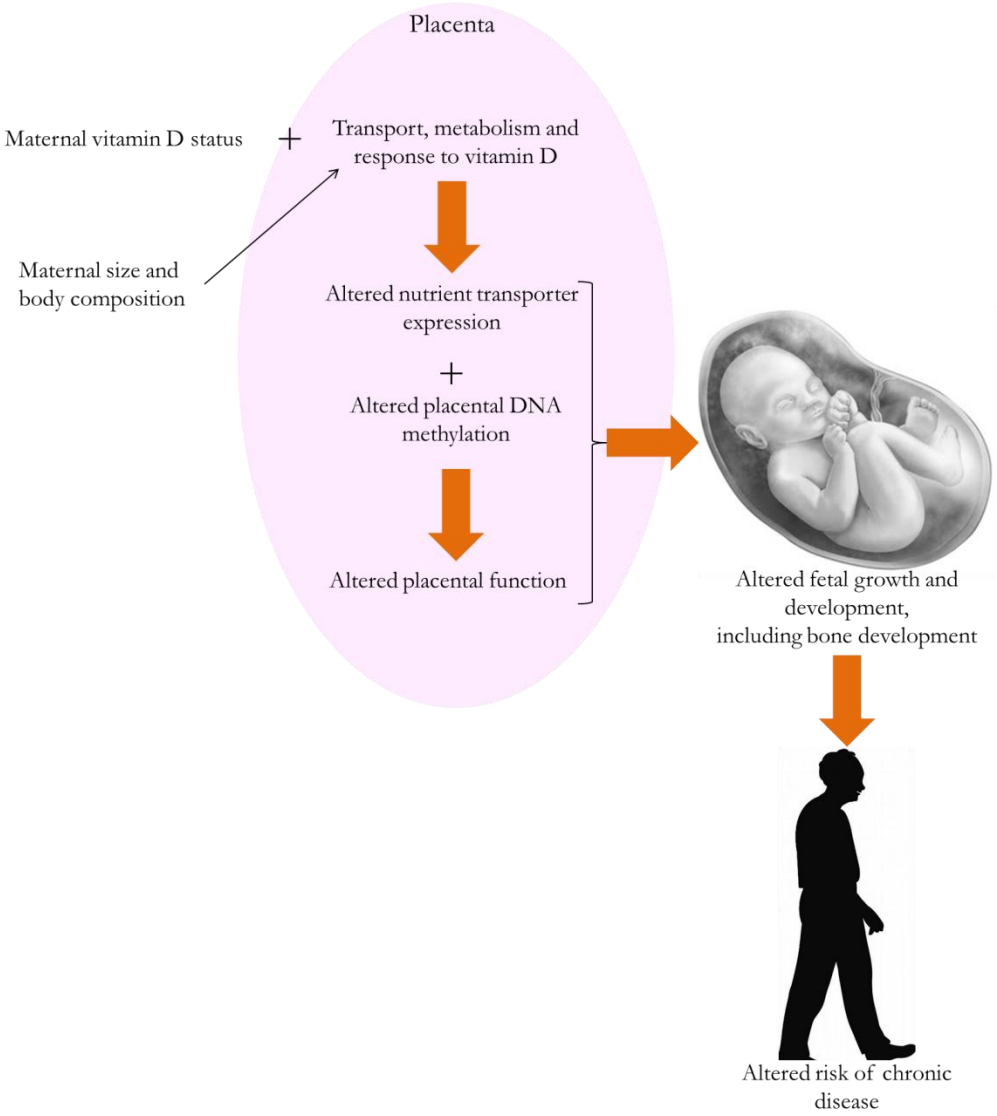

Figure 7.8: Potential impacts of maternal vitamin D status and placental handling of vitamin D on fetal growth and subsequent adult health. The combination of maternal vitamin D status as well as the placentas ability to transport, metabolise and respond to the available vitamin D could have effects on placental expression of nutrient transporters as well as resulting in altered DNA methylation through differential expression of DNMTs within the placenta. The culmination of vitamin D-mediated effects may ultimately result in alterations in placental function which consequently result in modifications to fetal growth, for example, increased growth in the presence of up-regulation of amino acid and calcium transport. The alterations in fetal growth could ultimately result in an altered risk of chronic disease in later life. In general, poor infant growth, i.e. small size at birth is associated with an increased risk of disease such as cardiovascular disease, type 2 diabetes and osteoporosis in later life (Barker, 1990; Hales *et al.*, 1991; Cooper, 1997, 2001). In addition, maternal size and body composition may play a role in mediating these effects though associations with placental handling of vitamin D.

Appendices

Appendix 1: Gelatin coating alters protein assay results

Methods

To investigate whether gelatin coating of cell culture plates would affect protein assay results, 32 mm well plates were gelatin coated as described in section 2.3.2. Protein was extracted from the coated wells and a protein assay carried out as described in sections 2.7.2 and 2.7.3, respectively.

Results

Gelatin coating of cell culture plates resulted in protein concentrations ranging from 88.33 to 192.5 µg/ml (Table A.1), therefore for experiments requiring measurement of protein gelatin coating of plates was not used.

Table A.1: Gelatin coating of cell culture plates affects protein assay readings.

Gelatin coated well	Protein concentration (µg/ml)
1	88.33
2	89.17
3	133.33
4	114.17
5	106.67
6	105.83
7	112.50
8	131.67
9	127.50
10	160.00
11	152.50
12	192.50

Appendix 2: Syncytialisation of the BeWo cell line

Methods

BeWo cell culture

To induce syncytialisation BeWo cells were plated out at 5 x 10^5 in 32 mm well plates (section 2.3.2). After 24 h, media was replaced with media containing 20 μmol/l forskolin, with a final concentration of 0.2% DMSO. DMSO and media controls were also carried out. Media was replaced every 24 h and 1 ml of the media removed was centrifuged at 12,000 rpm for 5 min at 4°C and stored at -80°C. After 72 h, the final media sample was taken, and RNA was extracted from the wells as described below.

Measurement of hCG levels

hCG content of media removed during syncytialisation of BeWo cells was measured to determine whether syncytialisation had occurred. hCG levels in cell culture media were determined using an hCG solid phase ELISA as per manufacturer's instructions. The assay works on the sandwich principle, with microtiter wells coated with a monoclonal antibody against hCG. Samples are incubated in the wells along with Enzyme Conjugate which contains a monoclonal antibody against the alpha chain of hCG conjugated with HRP. After washing off unbound conjugate, substrate is added causing a colour change, which is proportional to the hCG concentration.

Table A.2: hCG concentrations in standards.

Standard	hCG concentration
1	5 mIU/ml
2	50 mIU/ml
3	200 mIU/ml
4	500 mIU/ml
5	1000 mIU/ml

Briefly, 25 μl of standards (Table A.2) and samples were added to wells of a microtiter plate in duplicate. 100 μl of Enzyme Conjugate was added to each well and mixed for 10 s. After 30 min incubation at RT, wells were rinsed five times with 400 μl ddH_2O and 100 μl of Substrate Solution was added per well. Following 10 min incubation at RT, the reaction was stopped by adding 50 μl Stop Solution and absorbance read at 450 nm on a Multiskan EX plate reader (Thermo Scientific,

Massachusetts, USA). Absorbance of the standards was plotted against hCG concentration (Figure A.1), and hCG concentration in the samples was determined using 4 Parameter Logistics.

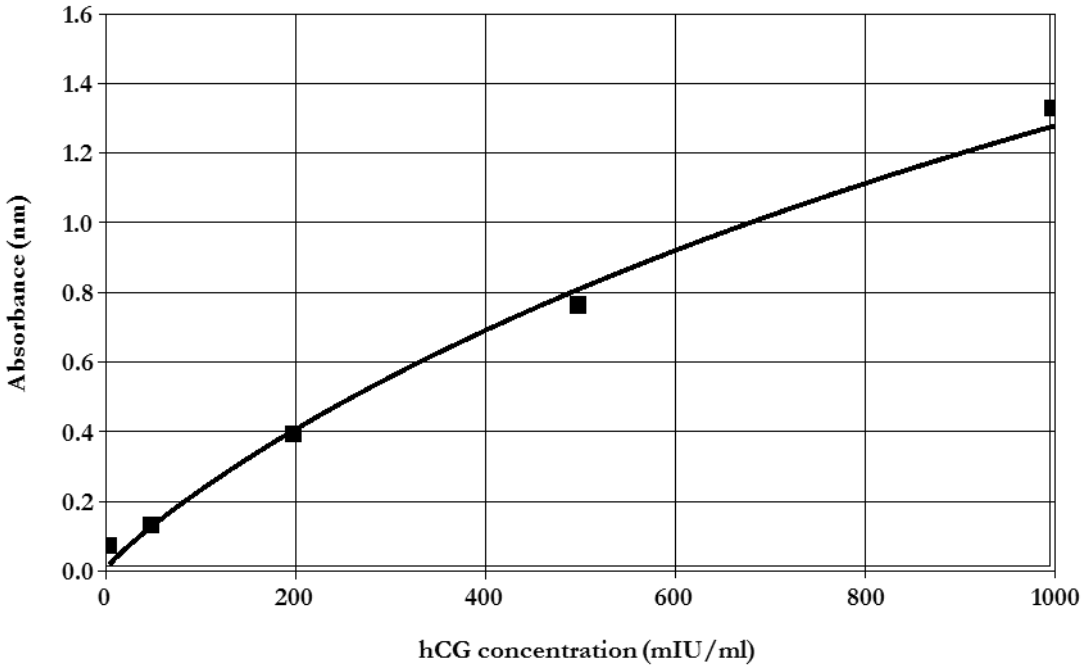

Figure A.1: An example standard curve plotted from hCG standards.

4 Parameter Logistics uses the equation: F (x) = ((A-D)/(1+((X/C)^B))) + D

Where:

X = concentration, F(x) = absorbance, A = absorbance for standard 1 concentration, B = slope factor, C = inflection point (the point on the curve where the curvature changes direction) and D = absorbance for standard 5 concentration. Concentration of the unknown samples was determined by rearranging the equation to give X for each sample once the standard curve has been plotted.

Immunocytochemistry

Immunocytochemistry was used to investigate syncytialisation of BeWo cells. Cells were plated on gelatin coated coverslips, and 24, 48 and 72 h after forskolin treatment, cells were stained for the desmosomes and nuclei to visualise whether any multi-nucleated syncytia were present. Cells were washed twice in PBS (all washes were for 5 min) and fixed in 4% PFA for 5 min. This was followed by three washes in filtered 100 nmol/l glycine solution in PBS to quench PFA autofluorescence. Cells were then permeabilised in 1% Triton X-100, which was followed by three more PBS washes. After blocking non-specific antibody binding with 3% BSA in PBS for 30 min, cells were incubated with a desmosome specific antibody; Mouse monoclonal anti-desmoplakin I+II in 1% BSA in PBS for 2 h. Following antibody incubation, cells were washed three times in PBS and then incubated with a complementary fluorescently labelled secondary antibody, polyclonal rabbit anti-mouse IgG

FITC for 1 h in the dark. Coverslips were then washed three times and DAPI stained as described in section 2.3.4, mounted on glass slides and visualised with Axiovert 200 fluorescent microscope.

mRNA expression

RNA was extracted from cells by TRI Reagent® as described in General methods 2.5.2, and 200 ng of RNA was converted to cDNA using the Promega method described in section 2.5.4. qrt-PCR (see 2.5.8) was used to compare mRNA expression of amino acid transporters in BeWo cells in response to forskolin treatment. For each qrt-PCR assay, samples (2.4 ng) were run alongside a standard curve, NECs and NTCs in triplicate. Cycling conditions were 95°C for 10 min; 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 s; and 50°C for 30 s. Data was collected at the 72°C step. Primers used are displayed in Table 4.2. Data were normalised to the HKG *UBC*. HKGs were measured with Perfect Probe primers and the cycling conditions were 95°C for 10 min; 50 cycles of 95°C for 15 s, 50°C for 30 s and 72°C for 15 s; and 50°C for 30 s. Data were collected at the 50°C step. Cp values were determined by the second derivative method and were converted to DNA concentration (ng/5 µl) using a standard curve (see General methods 2.5.8).

Statistical analysis

All data were analysed in SPSS, and graphs were created in Graph Pad Prism V6 or Excel. Unless otherwise stated, data are presented as mean + SEM. $p < 0.05$ was deemed statistically significant. Data were tested for normal distribution and log transformed or square rooted if non-normally distributed. All data were normally distributed before or after transformation. hCG production was analysed for the effect of time and treatment with two-way ANOVA. mRNA expression data were analysed with one way ANOVA plus Levene Statistic to test for homogeneity of variance. For data with a non-significant Levene Statistic, Tukey's post hoc test was used, while Games-Howell post-hoc test was used for data with a significant Levene Statistic.

Results

hCG production was not increased with forskolin treatment

BeWo syncytialisation was attempted with 20 µmol/l forskolin. To test whether syncytialisation was achieved hCG levels were measured in the media over a three day period. hCG production decreased with time in BeWo cells treated with 20 µmol/l forskolin (Figure A.2).

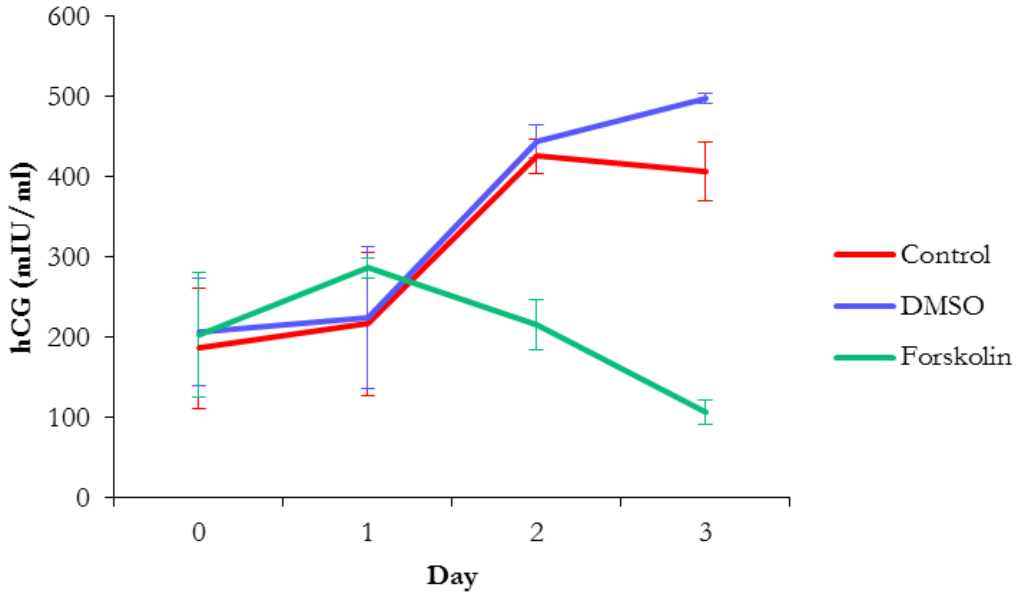

Figure A.2: hCG production (mIU/ml) decreased over 3 days with forskolin treatment. n = 4 for each condition. 2 way ANOVA revealed a significant effect of both time and treatment p = 0.003 for each factor.

Desmosome staining was unspecific

Staining of the desmosomes with anti-desmoplakin was also attempted to visualise the cellular junctions to see if fusion of the cells had occurred. Unfortunately, desmoplakin staining did not work so we could not use this as a second measure of syncytialisation. Desmosome staining should have appeared as punctate dots outlining the cell membrane, however, the staining of BeWo cells was not specific (Figure A.3).

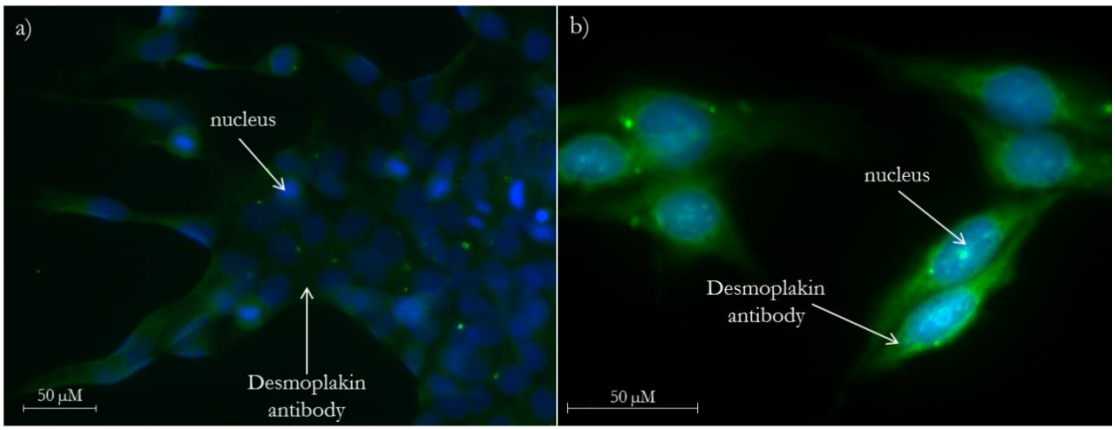

Figure A.3: Desmoplakin staining of BeWo cells was unsuccessful as staining was not specific. a) 20 x magnification and b) 40 x magnification images. Cells were stained 96 h after plating out. Green shows desmoplakin staining and blue shows DAPI staining of nuclei.

LAT4 expression was increased with forskolin treatment

qrt-PCR analysis on mRNA extracted from forskolin treated cells revealed that although the cells did not syncytialise treatment with forskolin resulted in a significant increase in *LAT4* mRNA expression compared to media control. Expression of *LAT3* and *TAT1* were unaffected by forskolin treatment (Figure A.4).

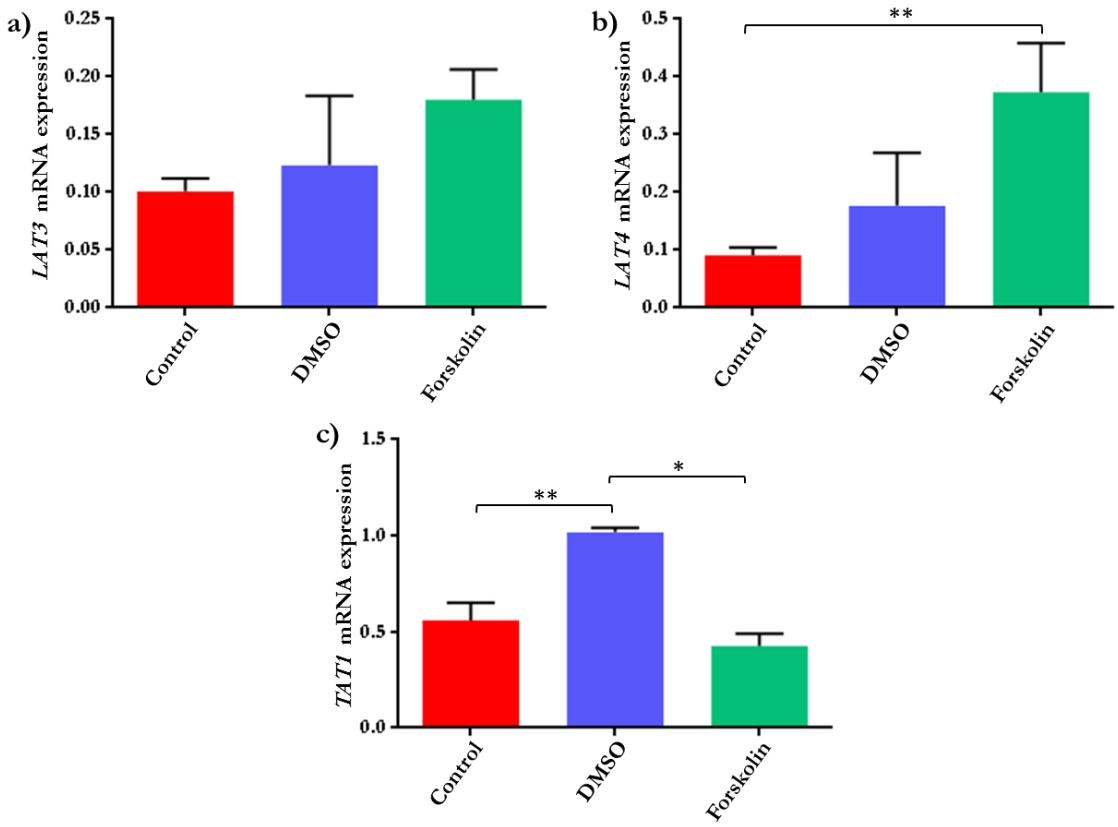

Figure A.4: Relative mRNA expression of *LAT4* was significantly increased following treatment of BeWo cells with 20 µmol/l forskolin. a) mRNA expression of *LAT3* was unaffected by forskolin treatment. b) mRNA expression of *LAT4* was significantly increased by forskolin treatment in comparison to media control. c) mRNA expression of *TAT1* was not affected by forskolin but was significantly increased by DMSO alone. n = 10 for media control and n = 3 for DMSO and forskolin treated. Data presented as mean + SEM. * $p < 0.05$, ** $p < 0.01$.

Appendix 3: Full data set for SWS placental mRNA expression measures

Below are tables displaying the full data set for SWS placental mRNA expression measures associated with measures of maternal lifestyle and anthropometry, fetal outcomes and other placental factors.

Table A.3: Associations between relative mRNA expression of placental vitamin D-related genes, *TAT1* VDRE promoter methylation and pre-pregnancy maternal anthropometry.

Gene		BMI	Fat (kg)	Sum of skin thickness (mm)	Subscapular/ triceps ratio skinfold thicknesses (mm)	Mid-upper arm circ (cm)	Arm muscle area (cm^2)	Calf circ (cm)	Height (cm)
Vitamin D genes									
Cubilin	r	0.09	0.11	0.06	0.03	0.11	0.15	0.16	0.13
	p	0.41	0.29	0.59	0.74	0.26	0.13	0.12	0.19
	n	96	95	95	95	96	96	95	96
CYP2J2	r	-0.10	-0.13	-0.07	0.16	-0.15	-0.11	-0.17	**-0.23**
	p	0.35	0.21	0.47	0.11	0.15	0.31	0.10	**0.03**
	n	96	96	95	95	96	96	95	**96**
CYP24A1	r	*0.17*	*0.19*	0.15	0.15	**0.24**	**0.33**	0.12	0.15
	p	*0.09*	*0.06*	0.13	0.13	**0.02**	**0.001**	0.26	0.15
	n	*96*	*96*	95	95	**96**	**96**	95	96
CYP27A1	r	0.04	0.06	0.05	0.13	0.01	0.02	0.05	0.04
	p	0.67	0.56	0.66	0.20	0.90	0.82	0.66	0.72
	n	95	95	94	94	95	95	94	95
CYP27B1	r	*-0.18*	-0.15	-0.17	0.12	*-0.18*	-0.07	-0.15	0.11
	p	*0.09*	0.16	0.12	0.28	*0.09*	0.53	0.16	0.30
	n	*89*	89	88	88	*89*	89	89	89
Legumain	r	0.10	0.12	0.11	0.13	0.12	0.11	0.11	0.01
	p	0.33	0.24	0.29	0.22	0.26	0.28	0.30	0.96
	n	96	96	95	95	96	96	95	96
Megalin	r	0.03	0.06	0.07	0.04	0.13	*0.18*	0.05	0.03
	p	0.80	0.55	0.50	0.72	0.21	*0.07*	0.64	0.74
	n	96	96	95	95	96	*96*	95	96
RXRα	r	0.13	0.03	-0.05	-0.04	0.05	0.09	0.07	-0.13
	p	0.21	0.78	0.66	0.68	0.63	0.36	0.53	0.19
	n	96	96	95	95	96	96	95	96
VDR	r	0.10	0.05	0.06	**0.23**	0.03	0.03	0.04	-0.09
	p	0.32	0.63	0.58	**0.02**	0.75	0.77	0.72	0.37
	n	96	96	95	**95**	96	96	95	96
Calcium transporter genes									
PMCA1	r	-0.05	-0.03	-0.01	0.04	-0.003	0.04	-0.02	-0.01
	p	0.65	0.81	0.91	0.67	0.98	0.68	0.87	0.91
	n	96	96	95	95	96	96	95	96
Lipid genes									
LPL	r	-0.12	-0.06	-0.14	-0.05	-0.05	0.02	0.02	0.16
	p	0.87	0.56	0.18	0.66	0.61	0.84	0.83	0.13
	n	94	94	93	93	94	94	93	94
Methylation genes									
DNMT1	r	-0.07	-0.07	-0.04	0.06	-0.03	0.06	-0.06	-0.10
	p	0.49	0.53	0.70	0.55	0.79	0.57	0.55	0.34
	n	96	96	95	95	96	96	95	96
DNMT3b	r	-0.01	-0.06	-0.09	0.07	0.03	0.16	-0.08	-0.10
	p	0.89	0.59	0.39	0.53	0.75	0.12	0.43	0.35
	n	94	94	93	93	94	94	93	94
***TAT1* VDRE promoter methylation**									
CpG -1062	r	**-0.25**	**-0.24**	*-0.19*	-0.02	**-0.25**	*-0.18*	**-0.25**	-0.08
	p	**0.01**	**0.02**	*0.07*	0.82	**0.02**	*0.09*	**0.02**	0.41
	n	**95**	**95**	*94*	94	**95**	*95*	**94**	95
CpG -1041	r	0.06	0.02	-0.01	*0.18*	0.01	0.15	0.07	0.08
	p	0.53	0.84	0.98	*0.08*	0.92	0.15	0.50	0.45
	n	95	95	94	*94*	95	95	94	95

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Table A.4: Associations between relative mRNA expression of placental vitamin D-related genes and pre-pregnancy maternal lifestyle.

Gene		Hours standing/walking per day	Frequency of strenuous exercise	Dietary prudence	High energy diet
Vitamin D genes					
Cubilin	r	-0.15	-0.11	0.05	-0.07
	p	0.14	0.27	0.60	0.49
	n	97	97	97	97
CYP2J2	r	0.08	0.01	0.03	0.10
	p	0.44	0.91	0.75	0.34
	n	97	97	97	97
CYP24A1	r	0.01	-0.01	-0.01	**-0.21**
	p	0.90	0.90	0.89	**0.04**
	n	97	97	97	**97**
CYP27A1	r	0.10	0.11	**0.25**	0.06
	p	0.35	0.30	**0.02**	0.54
	n	96	96	**96**	96
CYP27B1	r	0.03	-0.09	0.07	-0.10
	p	0.79	0.38	0.51	0.37
	n	90	90	90	90
Legumain	r	-0.03	*-0.17*	0.05	-0.01
	p	0.74	*0.09*	0.65	0.94
	n	97	*97*	97	97
Megalin	r	0.07	-0.002	-0.02	0.01
	p	0.47	0.99	0.88	0.95
	n	97	97	97	97
RXRα	r	0.09	-0.10	-0.01	-0.14
	p	0.36	0.32	0.95	0.17
	n	97	97	97	97
VDR	r	0.08	-0.08	-0.02	0.13
	p	0.41	0.41	0.83	0.19
	n	97	97	97	97
Calcium transporter genes					
PMCA1	r	0.01	0.12	-0.05	0.002
	p	0.91	0.24	0.65	0.99
	n	97	97	97	97
Lipid genes					
LPL	r	0.06	-0.07	0.06	0.04
	p	0.56	0.48	0.56	0.72
	n	95	95	95	95
Methylation genes					
DNMT1	r	-0.02	-0.02	-0.02	-0.001
	p	0.86	0.82	0.86	0.99
	n	97	97	97	97
DNMT3b	r	-0.04	0.02	0.08	-0.06
	p	0.72	0.81	0.46	0.53
	n	95	95	95	95
***TAT1* VDRE promoter methylation**					
CpG -1062	r	0.12	0.13	-0.05	0.16
	p	0.23	0.22	0.65	0.12
	n	96	96	96	96
CpG -1041	r	0.05	-0.07	-0.04	0.08
	p	0.66	0.53	0.68	0.45
	n	96	96	96	96

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics

Table A.5: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE methylation and maternal anthropometry during pregnancy.

		11 weeks of gestation				34 weeks of gestation		Delivery
Gene		**Mid-upper arm circ (cm)**	**Arm muscle area (cm²)**	**Dietary prudence**	**High energy diet**	**Mid-upper arm circ (cm)**	**Arm muscle area (cm²)**	**Age at delivery (years)**
Vitamin D genes								
Cubilin	r	0.10	0.03	0.08	0.09	*0.19*	0.14	0.13
	p	0.42	0.80	0.48	0.44	*0.07*	0.18	0.20
	n	72	72	75	75	*90*	90	97
CYP2J2	r	-0.13	-0.09	0.09	0.11	-0.12	-0.09	*0.17*
	p	0.28	0.43	0.44	0.34	0.25	0.38	*0.09*
	n	72	72	75	75	90	90	*97*
CYP24A1	r	*0.21*	**0.33**	0.02	0.04	**0.24**	**0.26**	0.03
	p	*0.08*	**0.004**	0.90	0.76	**0.02**	**0.01**	0.77
	n	*72*	**72**	75	75	**90**	**90**	97
CYP27A1	r	0.02	0.05	0.16	0.12	0.07	0.13	**0.26**
	p	0.84	0.70	0.17	0.30	0.50	0.22	**0.01**
	n	71	71	74	74	89	89	**96**
CYP27B1	r	-0.19	-0.19	0.11	-0.01	*-0.19*	-0.07	0.11
	p	0.12	0.11	0.35	0.95	*0.08*	0.53	0.28
	n	68	68	71	71	*68*	84	90
Legumain	r	0.05	0.06	0.12	0.12	0.11	0.08	0.04
	p	0.65	0.61	0.32	0.30	0.32	0.45	0.67
	n	72	72	75	75	90	90	97
Megalin	r	-0.01	0.08	-0.04	0.14	0.09	0.09	0.08
	p	0.93	0.50	0.72	0.24	0.37	0.40	0.45
	n	72	72	75	75	90	90	97
RXRα	r	0.09	0.12	0.07	-0.06	0.03	0.05	0.17
	p	0.44	0.31	0.53	0.60	0.79	0.65	0.10
	n	72	72	75	75	90	90	97
VDR	r	0.09	0.12	0.02	0.03	0.04	0.11	0.02
	p	0.47	0.32	0.90	0.83	0.70	0.30	0.84
	n	72	72	75	75	90	90	97
Calcium transporter genes								
PMCA1	r	-0.04	0.04	-0.04	0.15	0.03	0.003	0.05
	p	0.73	0.76	0.74	0.19	0.82	0.98	0.61
	n	72	72	75	75	90	90	97
Lipid genes								
LPL	r	-0.03	-0.06	0.06	0.04	0.05	0.03	0.09
	p	0.80	0.64	0.63	0.75	0.63	0.77	0.40
	n	70	70	73	73	88	88	95
Methylation genes								
DNMT1	r	-0.06	0.02	-0.15	0.12	0.000	-0.003	0.05
	p	0.59	0.88	0.19	0.29	0.99	0.98	0.65
	n	72	72	75	75	90	90	97
DNMT3b	r	0.08	0.07	0.07	0.18	0.07	0.07	*0.17*
	p	0.51	0.58	0.57	0.13	0.54	0.53	*0.09*
	n	70	70	73	73	88	88	*95*
***TAT1* VDRE promoter methylation**								
CpG -1062	r	-0.13	-0.13	-0.04	*0.20*	*-0.18*	**-0.23**	0.05
	p	0.27	0.26	0.77	*0.09*	*0.09*	**0.03**	0.64
	n	70	70	73	*73*	*89*	**89**	96
CpG -1041	r	0.02	0.05	-0.06	0.02	0.05	0.05	-0.04
	p	0.90	0.69	0.63	0.85	0.62	0.62	0.71
	n	70	70	73	73	89	89	96

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Table A.6: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE methylation and fetal abdominal circumference measurements throughout gestation.

Gene		11 week Royston abdominal circ z-score	19 week Royston abdominal circz-score	34 week Royston abdominal circ z-score	11-19 week conditional Royston abdominal circ z-score	19-34 week conditional Royston abdominal circ z-score
Vitamin D genes						
Cubilin	r	0.05	-0.07	-0.13	0.23	-0.10
	p	0.77	0.61	0.36	0.20	0.46
	n	30	53	54	30	53
CYP2J2	r	-0.11	0.10	0.20	0.20	0.14
	p	0.55	0.48	0.15	0.27	0.29
	n	30	53	54	30	53
CYP24A1	r	-0.11	*-0.23*	-0.21	-0.05	-0.94
	p	0.54	*0.09*	0.12	0.79	0.50
	n	30	*53*	54	30	53
CYP27A1	r	-0.12	-0.01	0.03	0.24	0.01
	p	0.51	0.97	0.83	0.21	0.95
	n	29	52	53	29	52
CYP27B1	r	0.09	0.06	-0.05	0.19	-0.18
	p	0.65	0.69	0.75	0.30	0.20
	n	29	52	53	29	52
Legumain	r	0.15	0.21	0.21	*0.35*	0.05
	p	0.40	0.13	0.12	*0.05*	0.73
	n	30	53	54	*30*	53
Megalin	r	*0.31*	-0.02	0.05	0.15	0.07
	p	*0.09*	0.84	0.73	0.40	0.64
	n	*30*	53	54	30	53
RXRα	r	-0.06	0.05	-0.07	0.21	-0.08
	p	0.73	0.71	0.61	0.26	0.55
	n	30	53	54	30	53
VDR	r	-0.25	-0.03	**-0.32**	-0.49	**-0.35**
	p	0.17	0.83	**0.02**	0.79	**0.01**
	n	30	53	**54**	30	**53**
Calcium transporter genes						
PMCA1	r	0.03	-0.19	0.04	-0.14	0.20
	p	0.89	0.17	0.76	0.46	0.14
	n	30	53	54	30	53
Lipid genes						
LPL	r	-0.26	-0.20	0.12	-0.15	*0.26*
	p	0.16	0.15	0.39	0.41	*0.05*
	n	30	53	54	30	*53*
Methylation genes						
DNMT1	r	0.26	0.04	0.07	0.19	0.05
	p	0.16	0.79	0.60	0.29	0.73
	n	30	53	54	30	53
DNMT3b	r	0.16	0.01	0.02	0.09	0.03
	p	0.37	0.95	0.90	0.64	0.85
	n	30	53	54	30	53
***TAT1* VDRE promoter methylation**						
CpG -1062	r	0.11	0.09	*0.25*	0.22	0.19
	p	0.57	0.54	*0.07*	0.24	0.17
	n	29	53	*54*	29	53
CpG -1041	r	**-0.38**	-0.20	-0.07	**-0.37**	0.08
	p	**0.04**	0.14	0.60	**0.04**	0.54
	n	**29**	53	54	**29**	53

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics
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Table A.7: Associations between relative mRNA expression of placental vitamin D-related genes, other genes, *TAT1* VDRE methylation and fetal measurements throughout gestation.

Gene		11 week Royston crown-heel length z-score	11 week Royston head circ z-score	19 week Royston head circ z-score	34 week Royston head circ z-score	11-19 week conditional Royston head circ z-score	19-34 week conditional Royston head circ z-score	19 week Royston femur length z-score	34 week Royston femur length z-score	19-34 week conditional Royston femur length z-score
Vitamin D genes										
Cubilin	r	0.03	-0.07	0.02	0.18	0.13	0.20	-0.06	*0.23*	**0.27**
	p	0.82	0.69	0.86	0.18	0.47	0.15	0.67	*0.08*	**0.046**
	n	47	32	53	54	32	53	53	*54*	**53**
CYP2J2	r	0.18	0.13	0.10	0.16	-0.001	0.15	-0.01	-0.05	-0.04
	p	0.23	0.47	0.48	0.24	0.99	0.29	0.95	0.72	0.75
	n	47	32	53	54	32	53	53	54	53
CYP24A1	r	-0.16	0.02	-0.08	**0.31**	0.14	**0.44**	-0.13	0.15	*0.23*
	p	0.28	0.92	0.57	**0.02**	0.45	**0.001**	0.33	0.27	*0.09*
	n	47	32	53	**54**	32	**53**	53	54	*53*
CYP27A1	r	-0.08	-0.02	-0.02	0.18	0.03	*0.23*	0.03	-0.01	-0.03
	p	0.59	0.93	0.87	0.18	0.85	*0.09*	0.83	0.96	0.81
	n	46	32	52	53	31	*52*	52	53	52
CYP27B1	r	0.003	0.17	-0.03	-0.01	-0.15	-0.11	0.09	-0.19	*-0.26*
	p	0.99	0.35	0.83	0.97	0.41	0.45	0.45	0.17	*0.06*
	n	46	31	52	53	31	52	52	53	*52*
Legumain	r	0.04	0.22	0.13	**0.29**	-0.15	*0.26*	0.18	**0.30**	0.19
	p	0.80	0.21	0.35	**0.03**	0.38	*0.06*	0.19	**0.03**	0.16
	n	47	32	53	**54**	32	*53*	53	**54**	53
Megalin	r	**0.29**	0.21	0.13	0.22	0.13	0.19	-0.02	0.000	0.01
	p	**0.045**	0.24	0.35	0.10	0.45	0.17	0.91	0.99	0.96
	n	**47**	32	53	54	32	53	53	54	53
RXRα	r	-0.13	0.01	-0.07	-0.12	0.07	-0.07	-0.11	-0.08	-0.01
	p	0.38	0.96	0.63	0.36	0.69	0.61	0.43	0.58	0.94
	n	47	32	53	54	32	53	53	54	53
VDR	r	-0.18	-0.20	-0.08	**-0.33**	-0.03	**-0.42**	-0.05	**-0.28**	*-0.26*
	p	0.21	0.25	0.57	**0.01**	0.88	**0.001**	0.74	**0.04**	*0.05*
	n	47	32	53	**51**	32	**53**	53	**54**	*53*
Calcium transporter genes										
PMCA1	r	0.10	-0.02	-0.01	0.17	0.06	*0.27*	-0.11	-0.03	0.03
	p	0.49	0.91	0.95	0.20	0.73	*0.05*	0.43	0.82	0.82

	n	47	32	53	54	32	*53*	53	54	53
Lipid genes										
LPL	r	-0.02	-0.22	-0.09	0.09	0.17	0.17	-0.11	0.06	0.13
	p	0.91	0.21	0.51	0.53	0.34	0.21	0.44	0.64	0.35
	n	47	32	53	54	32	53	53	54	53
Methylation genes										
DNMT1	r	*0.24*	0.18	0.12	0.16	0.16	0.12	0.01	0.04	0.03
	p	*0.099*	0.32	0.39	0.23	0.38	0.38	0.92	0.78	0.83
	n	*47*	32	53	54	32	53	53	54	53
DNMT3b	r	0.10	0.15	0.08	0.17	0.21	0.19	0.04	-0.02	-0.04
	p	0.50	0.41	0.56	0.21	0.24	0.17	0.76	0.89	0.77
	n	47	32	53	54	32	53	53	54	53
***TAT1* VDRE promoter methylation**										
CpG -1062	r	0.10	-0.01	0.01	0.04	0.01	0.01	0.07	-0.06	-0.10
	p	0.48	0.98	0.95	0.75	0.95	0.96	0.62	0.68	0.47
	n	46	31	53	54	31	53	53	54	53
CpG -1041	r	-0.15	**-0.42**	-0.14	-0.14	-0.11	-0.08	-0.11	-0.13	-0.07
	p	0.33	**0.02**	0.32	0.27	0.55	0.59	0.41	0.36	0.61
	n	46	**31**	53	53	31	53	53	54	53

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Table A.8: Associations between relative mRNA expression of placental vitamin D-related genes and other genes and placental and neonatal measurements.

Gene		Placental weight (g)	Birth weight (kg)	Head circ (cm)	Abdominal circ (cm)	Crown-heel length (cm)	Subscapular skinfold thickness (mm)	Mid-upper arm circ (cm)	Lean mass (g)	Fat mass (g)	BMC (g)	% BMC
Vitamin D genes												
Cubilin	r	0.16	*0.18*	**0.22**	0.07	**0.21**	0.01	0.08	*0.19*	-0.03	0.02	-0.08
	p	0.12	*0.07*	**0.03**	0.50	**0.04**	0.89	0.44	*0.07*	0.80	0.83	0.46
	n	95	*96*	**96**	96	**96**	96	96	*96*	96	96	96
CYP2J2	r	-0.06	-0.09	0.06	-0.08	-0.02	-0.002	0.03	-0.06	0.06	-0.01	0.01
	p	0.55	0.37	0.57	0.41	0.85	0.98	0.75	0.57	0.57	0.90	0.90
	n	95	96	96	96	96	96	96	96	96	96	96
CYP24A1	r	0.15	*0.19*	**0.26**	0.08	0.01	0.16	0.10	0.08	0.08	0.09	0.07
	p	0.15	*0.06*	**0.01**	0.46	0.95	0.12	0.34	0.46	0.44	0.38	0.47
	n	95	*96*	**96**	96	96	96	96	96	96	96	96
CYP27A1	r	0.12	0.14	**0.21**	0.09	0.03	0.12	0.09	0.02	0.05	-0.08	-0.16
	p	0.23	0.16	**0.04**	0.38	0.75	0.24	0.37	0.88	0.61	0.46	0.13
	n	94	95	**95**	95	95	95	95	95	95	95	95
CYP27B1	r	-0.10	-0.11	0.01	0.06	*-0.20*	-0.14	-0.04	-0.02	-0.16	-0.05	0.01
	p	0.36	0.30	0.95	0.56	*0.06*	0.19	0.74	0.87	0.14	0.63	0.94
	n	88	89	89	89	*89*	89	89	89	89	89	89
Legumain	r	0.12	0.07	0.08	0.05	0.11	0.04	0.06	0.03	0.15	0.05	-0.003
	p	0.25	0.49	0.46	0.63	0.27	0.72	0.54	0.75	0.14	0.64	0.97
	n	95	96	96	96	96	96	96	96	96	96	96
Megalin	r	0.14	*0.20*	0.11	0.02	0.11	0.13	0.07	0.11	0.07	-0.04	*-0.17*
	p	0.16	*0.05*	0.30	0.81	0.29	0.19	0.49	0.29	0.49	0.73	*0.098*
	n	95	*96*	96	96	96	96	96	96	96	96	*96*
RXRα	r	-0.16	-0.13	*-0.18*	-0.14	-0.10	-0.16	*-0.17*	-0.10	*-0.19*	-0.10	0.02
	p	0.11	0.21	*0.08*	0.17	0.31	0.12	*0.09*	0.31	*0.07*	0.35	0.88
	n	95	96	*96*	96	96	96	*96*	96	*96*	96	96
VDR	r	**-0.22**	*-0.19*	-0.11	-0.10	**-0.22**	-0.13	-0.15	**-0.24**	**-0.22**	-0.14	-0.02
	p	**0.03**	*0.06*	0.27	0.35	**0.03**	0.19	0.13	**0.02**	**0.03**	0.17	0.82
	n	**95**	*96*	96	96	**96**	96	96	**96**	**96**	96	96
Calcium transporter genes												
PMCA1	r	**0.26**	**0.28**	**0.26**	0.07	0.08	**0.31**	**0.21**	0.14	**0.21**	0.06	-0.10
	p	**0.01**	**0.01**	**0.01**	0.52	0.44	**0.002**	**0.04**	0.18	**0.04**	0.53	0.35
	n	**95**	**96**	**96**	96	96	**96**	**96**	96	**96**	96	96
Lipid genes												
LPL	r	-0.08	-0.02	-0.01	0.01	0.05	-0.01	-0.04	0.09	0.02	-0.03	-0.09

	p	0.44	0.85	0.93	0.92	0.62	0.96	0.72	0.36	0.83	0.80	0.37
	n	93	94	94	94	94	94	94	94	94	94	94
Methylation genes												
DNMT1	r	0.17	0.03	0.02	-0.06	-0.01	0.03	-0.03	0.004	0.002	-0.08	-0.12
	p	0.10	0.81	0.84	0.57	0.96	0.77	0.75	0.97	0.98	0.44	0.25
	n	95	96	96	96	96	96	96	96	96	96	96
DNMT3b	r	0.07	0.12	0.09	0.03	-0.05	*0.18*	0.01	-0.01	0.08	-0.003	-0.04
	p	0.52	0.24	0.36	0.75	0.65	*0.09*	0.89	0.91	0.42	0.98	0.68
	n	93	94	94	94	94	*94*	94	94	94	94	94
***TAT1* VDRE promoter methylation**												
CpG -1062	r	0.03	0.10	0.04	0.15	0.09	0.04	0.13	0.10	0.10	0.03	-0.04
	p	0.77	0.32	0.69	0.14	0.39	0.70	0.19	0.32	0.32	0.75	0.71
	n	94	95	95	95	95	95	95	95	95	95	95
CpG -1041	r	0.03	-0.03	-0.01	0.05	-0.01	0.000	-0.03	-0.06	-0.05	-0.10	-0.11
	p	0.74	0.79	0.93	0.66	0.90	0.99	0.81	0.55	0.63	0.35	0.29
	n	94	95	95	95	95	95	95	95	95	95	95

Circ = circumference. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Table A.9: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE methylation and infant anthropometry at 4 and 6 years of age.

		4 years of age				6 years of age		
Gene		**Weight (kg)**	**Fat mass (kg)**	**Lean mass (kg)**	**Height (m)**	**Weight (kg)**	**Fat mass (kg)**	**Lean mass (kg)**
Vitamin D genes								
Cubilin	r	0.09	0.08	-0.01	0.15	0.13	0.14	0.08
	p	0.53	0.61	0.97	0.27	0.39	0.34	0.62
	n	51	41	41	51	47	43	43
CYP2J2	r	-0.91	0.06	-0.16	-0.11	0.07	*0.28*	-0.09
	p	0.52	0.73	0.31	0.45	0.62	*0.07*	0.55
	n	51	41	41	51	47	*43*	43
CYP24A1	r	0.10	0.07	0.16	0.01	0.02	**0.34**	-0.06
	p	0.48	0.67	0.30	0.96	0.89	**0.02**	0.68
	n	51	41	41	51	47	**43**	43
CYP27A1	r	0.02	-0.14	*0.30*	-0.10	-0.02	-0.07	-0.13
	p	0.90	0.37	*0.05*	0.47	0.89	0.64	0.40
	n	50	40	*40*	50	47	43	43
CYP27B1	r	-0.14	*-0.27*	0.06	-0.05	-0.14	**-0.31**	-0.05
	p	0.33	*0.08*	0.71	0.74	0.35	**0.04**	0.76
	n	50	*42*	42	50	46	**42**	42
Legumain	r	-0.03	0.04	0.01	-0.12	0.12	0.004	-0.10
	p	0.84	0.79	0.97	0.38	0.42	0.98	0.53
	n	51	41	41	51	47	43	43
Megalin	r	0.01	0.17	-0.10	-0.02	-0.14	0.09	-0.17
	p	0.94	0.29	0.52	0.88	0.34	0.55	0.28
	n	51	41	41	51	47	43	43
RXRα	r	*-0.27*	**-0.34**	-0.19	*-0.26*	-0.13	-0.09	-0.05
	p	*0.05*	**0.03**	0.23	*0.07*	0.39	0.55	0.73
	n	*51*	**41**	41	*51*	47	43	43
VDR	r	**-0.32**	**-0.35**	**-0.42**	-0.13	-0.17	-0.15	-0.12
	p	**0.02**	**0.02**	**0.01**	0.35	0.24	0.32	0.45
	n	**51**	**41**	**41**	51	47	43	43
Calcium transporter genes								
PMCA1	r	0.07	0.19	0.002	-0.02	0.04	0.18	-0.06
	p	0.63	0.23	0.99	0.91	0.78	0.24	0.70
	n	51	41	41	51	47	43	43
Lipid genes								
LPL	r	*0.23*	0.09	*0.27*	**0.29**	0.19	0.12	0.17
	p	*0.095*	0.56	*0.09*	**0.04**	0.19	0.44	0.27
	n	*50*	40	*40*	**50**	47	43	43
Methylation genes								
DNMT1	r	-0.03	0.10	-0.03	-0.04	-0.02	0.15	-0.11
	p	0.83	0.53	0.84	0.77	0.88	0.32	0.47
	n	51	41	41	51	47	43	43
DNMT3b	r	-0.09	*-0.30*	0.15	-0.02	0.08	-0.05	0.04
	p	0.52	*0.05*	0.33	0.91	0.57	0.77	0.81
	n	50	*40*	40	50	47	43	43
***TAT1* VDRE promoter methylation**								
CpG -1062	r	0.05	-0.01	0.10	0.10	-0.12	-0.12	0.09
	p	0.74	0.95	0.53	0.48	0.42	0.43	0.56
	n	51	41	41	51	47	44	44
CpG -1041	r	-0.20	*-0.27*	-0.15	-0.14	-0.10	-0.13	0.12
	p	0.16	*0.09*	0.33	0.33	0.48	0.38	0.44
	n	51	*41*	41	51	47	44	44

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics

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Table A.10: Associations between relative mRNA expression of placental vitamin D-related genes and other genes, *TAT1* VDRE methylation and infant bone measures at 4 years of age.

		4 years of age						6 years of age
Gene		**Total BA (cm^2)**	**Total BA without heads (cm^2)**	**Total BMC (g)**	**% BMC without heads**	**Total Prentice BMD (g)**	**Total Prentice BMD without heads (g)**	**BMC without heads (g)**
Vitamin D genes								
Cubilin	r	0.08	0.07	0.11	-0.21	0.01	-0.001	0.15
	p	0.61	0.67	0.50	0.17	0.96	0.99	0.30
	n	38	38	38	43	38	38	48
CYP2J2	r	-0.08	-0.02	-0.06	0.14	-0.03	0.06	0.02
	p	0.64	0.92	0.71	0.35	0.86	0.72	0.89
	n	38	38	38	43	38	38	48
CYP24A1	r	-0.04	-0.10	0.003	-0.06	0.03	-0.08	-0.002
	p	0.82	0.54	0.98	0.70	0.87	0.63	0.99
	n	38	38	38	43	38	38	48
CYP27A1	r	*-0.31*	*-0.28*	-0.17	**-0.42**	0.001	-0.11	-0.22
	p	*0.05*	*0.08*	0.30	**0.01**	0.99	0.51	0.12
	n	*37*	*37*	37	**42**	37	37	48
CYP27B1	r	-0.07	-0.01	-0.07	0.05	0.06	0.13	-0.01
	p	0.66	0.96	0.65	0.73	0.73	0.41	0.97
	n	39	39	39	42	39	39	46
Legumain	r	0.02	0.02	-0.01	-0.08	-0.10	0.20	-0.12
	p	0.89	0.91	0.96	0.62	0.52	0.21	0.39
	n	38	38	38	43	38	38	48
Megalin	r	-0.11	-0.05	-0.08	-0.22	-0.10	0.04	-0.22
	p	0.49	0.76	0.64	0.15	0.55	0.79	0.15
	n	38	38	38	43	38	38	43
RXRα	r	-0.24	-0.21	-0.21	0.18	0.07	0.14	-0.09
	p	0.14	0.19	0.19	0.24	0.68	0.40	0.53
	n	38	38	38	43	38	38	48
VDR	r	-0.14	-0.22	-0.05	0.14	**0.35**	0.02	-0.10
	p	0.40	0.18	0.75	0.36	**0.03**	0.91	0.51
	n	38	38	38	43	**38**	38	48
Calcium transporter genes								
PMCA1	r	*-0.27*	-0.20	-0.23	**-0.42**	-0.22	-0.22	-0.09
	p	*0.09*	0.21	0.16	**0.004**	0.18	0.17	0.54
	n	*38*	38	38	**43**	38	38	48
Lipid genes								

LPL	r	**0.37**	**0.35**	**0.45**	0.06	**0.35**	0.25	0.18
	p	**0.02**	**0.03**	**0.004**	0.69	**0.03**	0.13	0.22
	n	**37**	**37**	**37**	42	**37**	37	48
Methylation genes								
DNMT1	r	-0.10	-0.02	-0.11	-0.20	-0.17	-0.07	-0.12
	p	0.55	0.92	0.51	0.20	0.31	0.69	0.41
	n	38	38	38	43	38	38	48
DNMT3b	r	-0.20	-0.14	-0.19	-0.14	-0.07	-0.17	-0.10
	p	0.22	0.41	0.24	0.38	0.68	0.32	0.51
	n	37	37	37	42	37	37	48
***TAT1* VDRE promoter methylation**								
CpG -1062	r	-0.07	-0.11	0.03	-0.05	0.14	-0.01	0.03
	p	0.66	0.48	0.85	0.76	0.37	0.93	0.84
	n	39	39	39	43	39	39	48
CpG -1041	r	-0.21	-0.19	-0.15	*0.27*	0.22	0.14	-0.08
	p	0.19	0.24	0.36	*0.07*	0.18	0.40	0.58
	n	39	39	39	*43*	39	39	48

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Table A.11: Associations between relative mRNA expression of placental DNMT expression with placental gene expression.

		DNMT1	*DNMT3b*			*DNMT1*	*DNMT3b*
Amino acid transporter genes							
4F2hc	r	-0.05	-0.04	***LAT4***	r	-0.003	-0.001
	p	0.63	0.72		p	0.98	0.99
	n	100	98		n	100	98
ASCT1	r	0.16	0.11	***SNAT1***	r	-0.03	0.04
	p	0.11	0.30		p	0.75	0.69
	n	100	98		n	100	98
ASCT2	r	0.16	**0.25**	***SNAT2***	r	0.06	0.12
	p	0.10	**0.01**		p	0.58	0.25
	n	100	**98**		n	100	98
EAAT2	r	*0.17*	**0.29**	***SNAT4***	r	0.11	0.10
	p	*0.09*	**0.004**		p	0.27	0.32
	n	*100*	**98**		n	100	98
EAAT3	r	*0.17*	**0.26**	***TAT1***	r	**0.20**	0.09
	p	*0.09*	**0.01**		p	**0.049**	0.37
	n	*100*	**98**		n	**100**	98
LAT1	r	-0.12	-0.08	***y⁺LAT1***	r	*0.20*	**0.22**
	p	0.25	0.41		p	*0.05*	**0.03**
	n	100	98		n	*100*	**98**
LAT2	r	-0.15	-0.09	***y⁺LAT2***	r	**0.31**	**0.33**
	p	0.13	0.38		p	**0.002**	**0.001**
	n	99	97		n	**100**	**98**
LAT3	r	0.16	0.17				
	p	0.10	0.10				
	n	100	98				
Vitamin D genes							
Cubilin	r	**0.37**	**0.25**	***Legumain***	r	0.08	0.06
	p	**0.001**	**0.01**		p	0.44	0.55
	n	**100**	**98**		n	100	98
CYP2J2	r	0.11	-0.01	***Megalin***	r	**0.71**	**0.40**
	p	0.27	0.92		p	**0.001**	**0.001**
	n	100	98		n	**100**	**98**
CYP24A1	r	**0.22**	**0.29**	***RXRα***	r	0.15	**0.35**
	p	**0.03**	**0.004**		p	0.13	**0.001**
	n	**100**	**98**		n	100	**98**
CYP27A1	r	0.16	**0.28**	***VDR***	r	-0.002	0.06
	p	0.11	**0.01**		p	0.98	0.55
	n	99	**97**		n	100	98
CYP27B1	r	0.03	0.04				
	p	0.82	0.68				
	n	91	89				
Calcium transporter genes				**Lipid genes**			
PMCA1	r	**0.70**	**0.51**	***LPL***	r	-0.04	-0.03
	p	**0.001**	**0.001**		p	0.68	0.77
	n	**100**	**98**		n	98	98
Methylation genes							
DNMT1	r		**0.57**	***DNMT3b***	r	**0.57**	
	p		**0.001**		p	**0.001**	
	n		**98**		n	**98**	
***TAT1* VDRE promoter methylation**							
CpG -1062	r	-0.04	0.11	**CpG -1041**	r	0.02	0.16
	p	0.71	0.28		p	0.86	0.13
	n	97	95		n	97	95

Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Appendix 4: LAT4 protein expression in placenta, HEK293 and BeWo cells

LAT4 protein expression was detected in all three cell types, and expression was significantly higher in HEK293 compared to placenta (Figure A.5).

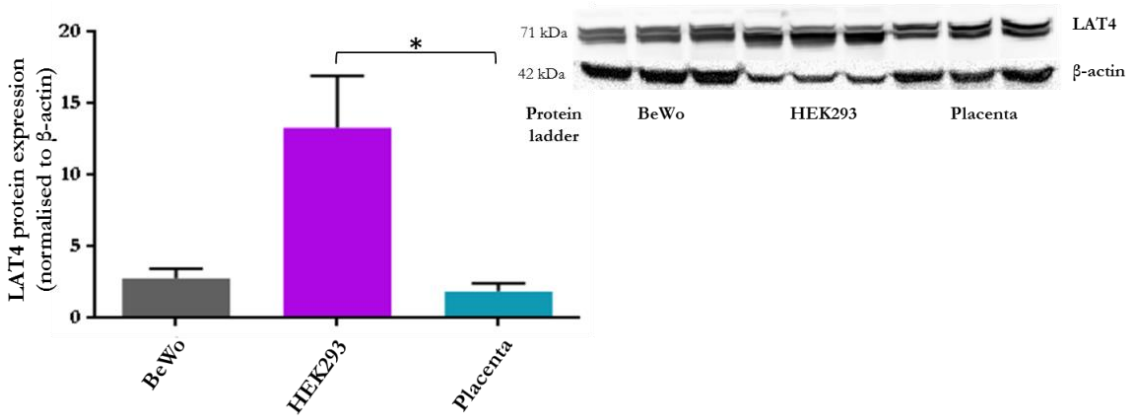

Figure A.5: Protein expression of LAT4 placenta, HEK293 and BeWo cells. LAT4 protein expression was significantly higher in HEK293 cells compared to placenta. BeWo n = 6, HEK293 n = 4, placenta n = 7. A representative western blot is shown. Data presented as mean + SEM. * $p < 0.05$.

Appendix 5: Transfection of *TAT1*-GFP into BeWo cells

Initial experiments were carried out to determine the best transfection reagent for use with BeWo cells. Transfection efficiencies were assessed at 24 and 48 h post-transfection for 3 transfection reagents; Lipofectamine®, FuGene® HD and Nanofectin. Transfection efficiency was determined as the ratio of GFP-expressing cells to DAPI stained nuclei. Transfection efficiencies for the 3 transfection reagents are displayed in Table A.12 and demonstrate that FuGene® HD had the better transfection efficiency of the three reagents tested.

Table A.12: Transfection efficiencies of Lipofectamine®, FuGene® HD and Nanofectin at 24 and 48 h post-transfection for transfection of GFP-*TAT1* into BeWo cells.

Transfection reagent	Transfection efficiency at 24 h	Transfection efficiency at 48 h
Lipofectamine®	5.8%	9.1%
FuGene® HD	21.2%	21.7%
Nanofectin		9.1%

There is no data for Nanofectin at 24 h post-transfection as cells were not healthy enough to count. Lipofectamine® at 24 h, FuGene® HD at 24 h and 48 h, and Nanofectin at 48 h were averaged from 2 coverslips, with 3 areas of each coverslip counted. Lipofectamine® at 48 h was averaged from 4 coverslips, with 3 areas from each coverslip counted.

The morphology of cells also differed with the various transfection reagents, as displayed in Figure A.6 and Figure A.7. Cells appeared healthier with FuGene® HD compared to Lipofectamine® and Nanofectin. Transfection controls were also used, where cells were cultured with transfection reagent and no expression vector. No GFP was seen in controls from any transfection reagent. Representative FuGene® HD controls are displayed.

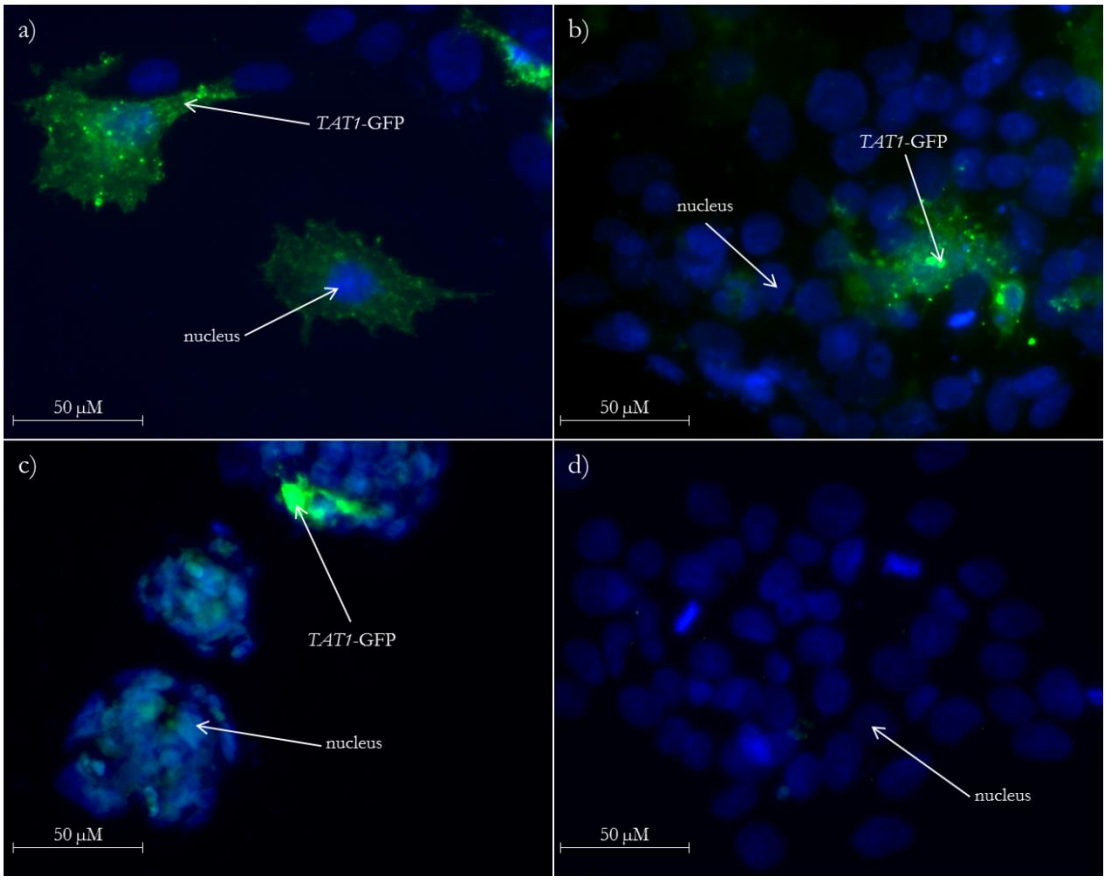

Figure A.6: Morphology of cells 24 h following transfection with GFP-*TAT1*. Representative images showing expression of GFP-*TAT1* with a) FuGene® HD, b) Liopfectamine®, c) Nanofectin and d) FuGene® HD control. Control cells were incubated with transfection reagent and no expression vector. GFP-*TAT1* is shown in green and DAPI stained nuclei in blue.

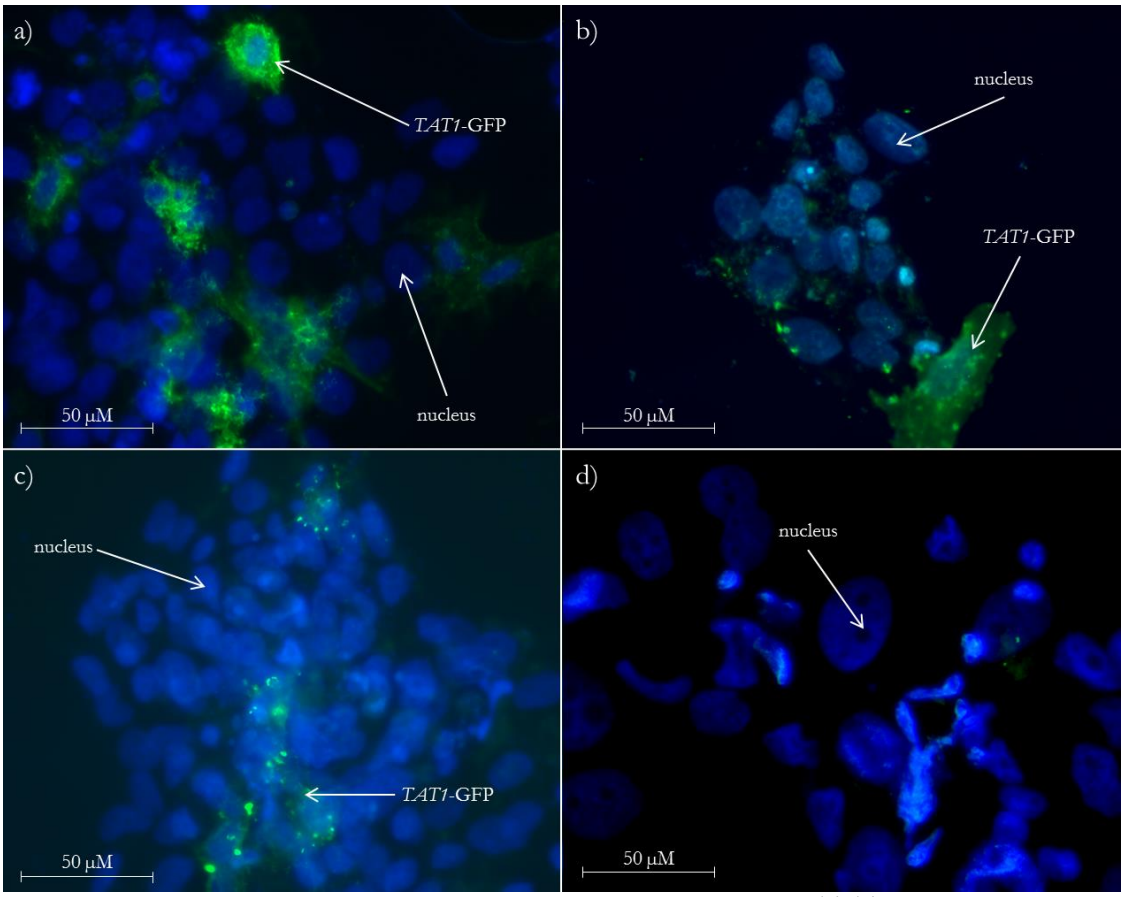

Figure A.7: Morphology of cells 48 h following transfection with GFP-*TAT1*. Representative images showing expression of GFP-*TAT1* with a) FuGene® HD, b) Liopfectamine®, c) Nanofectin and d) FuGene® HD control. Control cells were incubated with transfection reagent and no expression vector. GFP-*TAT1* is shown in green and DAPI stained nuclei in blue.

FuGene® HD was identified as the best option for transfecting BeWo cells with *TAT1*-GFP. The DNA reagent ratio was altered to increase transfection efficiency (Table A.13) however this did not greatly affect the efficiency.

Table A.13: Altering the reagent to DNA ratio did not considerably alter transfection efficiency at 24 and 48 h post-transfection.

Reagent to DNA ratio	24 h	48 h
3:1	13.0%	29.0%
3:2	20.3%	19.1%
1.5:2	9.6%	21.5%

All efficiencies were averaged from 2 coverslips, with 3 areas of each coverslip counted.

Appendix 6: Full data set for MAVIDOS placental mRNA expression measures

Below are tables displaying the full data set for MAVIDOS placental mRNA expression measures showing effects of vitamin D supplementation on mRNA expression.

Table A.14: Association between randomisation group and placental relative mRNA expression measures.

Gene		Crude	Adjusted for sex and gestational age
Vitamin D genes			
Cubilin	β	0.19	0.17
	95% CI	-0.15, 0.52	-0.17, 0.51
	p	0.28	0.32
CYP24A1	β	-0.09	-0.01
	95% CI	-0.52, 0.34	-0.43, 0.42
	p	0.68	0.97
CYP27B1	β	0.08	0.07
	95% CI	-0.11, 0.27	-0.12, 0.27
	p	0.40	0.45
Megalin	β	0.03	0.02
	95% CI	-0.24, 0.30	-0.26, 0.29
	p	0.85	0.92
RXRα	β	-0.10	-0.07
	95% CI	-0.26, 0.07	-0.22, 0.09
	p	0.25	0.39
VDR	β	0.10	0.08
	95% CI	-0.14, 0.34	-0.16, 0.32
	p	0.42	0.51
Amino acid transporter genes			
ASCT1	β	0.04	0.02
	95% CI	-0.13, 0.21	-0.16, 0.19
	p	0.64	0.84
ASCT2	β	-0.06	-0.05
	95% CI	-0.24, 0.11	-0.23, 0.13
	p	0.48	0.59
LAT1	β	-0.09	-0.07
	95% CI	-0.42, 0.24	-0.40, 0.27
	p	0.57	0.70
LAT3	β	0.07	0.06
	95% CI	-0.14, 0.28	-0.16, 0.28
	p	0.53	0.58
LAT4	β	-0.06	-0.06
	95% CI	-0.24, 0.11	-0.24, 0.12
	p	0.47	0.53
SNAT1	β	-0.04	-0.02
	95% CI	-0.18, 0.09	-0.15, 0.12
	p	0.52	0.82
TAT1	β	-0.17	-0.15
	95% CI	-0.38, 0.04	-0.36, 0.07
	p	0.11	0.18
Calcium transporter genes			

CAT1	β	0.11	0.10
	95% CI	-0.12, 0.34	-0.13, 0.34
	p	0.34	0.39
NCX1	β	-0.04	-0.01
	95% CI	-0.21, 0.14	-0.19, 0.17
	p	0.70	0.87
PMCA1	β	-0.07	-0.06
	95% CI	-0.29, 0.15	-0.28, 0.17
	p	0.52	0.63
PMCA4	β	-0.10	-0.10
	95% CI	-0.31, 0.10	-0.32, 0.11
	p	0.32	0.33
Other genes			
DNMT1	β	-0.05	-0.03
	95% CI	-0.22, 0.12	-0.20, 0.14
	p	0.54	0.74
PTHrP	β	-0.17	-0.12
	95% CI	-0.58, 0.25	-0.52, 0.28
	p	0.44	0.55
TXNIP	β	0.04	0.02
	95% CI	-0.11, 0.20	-0.13, 0.18
	p	0.58	0.77

Unadjusted outcomes and outcomes adjusted for sex and gestational age.

Table A.15: Association between randomisation group and placental relative mRNA expression measures separated by season of birth.

Gene		Spring	Summer	Autumn	Winter
			Vitamin D genes		
Cubilin	β	**0.65**	0.24	-0.98	0.23
	95% CI	**0.07, 1.23**	-0.30, 0.78	-2.23, 0.27	-0.51, 0.97
	p	**0.03**	0.37	0.11	0.51
CYP24A1	β	0.08	-0.11	-0.07	0.03
	95% CI	-0.73, 0.90	-0.81, 0.58	-1.88, 1.74	-0.88, 0.94
	p	0.83	0.73	0.93	0.95
CYP27B1	β	0.09	0.08	-0.37	0.22
	95% CI	-0.31, 0.49	-0.24, 0.40	-0.88, 0.13	-0.21, 0.66
	p	0.64	0.62	0.13	0.30
Megalin	β	0.31	-0.02	-0.11	-0.25
	95% CI	-0.33, 0.95	-0.44, 0.40	-1.13, 0.91	-0.81, 0.31
	p	0.32	0.93	0.81	0.35
RXRα	β	-0.11	-0.21	0.18	0.05
	95% CI	-0.43, 0.22	-0.48, 0.05	-0.40, 0.76	-0.23, 0.33
	p	0.50	0.11	0.50	0.70
VDR	β	0.06	-0.10	0.36	0.21
	95% CI	-0.52, 0.64	-0.41, 0.20	-0.44, 1.15	-0.31, 0.73
	p	0.83	0.49	0.33	0.41
			Amino acid transporter genes		
ASCT1	β	-0.03	0.13	-0.22	0.01
	95% CI	-0.42, 0.36	-0.11, 0.38	-0.95, 0.50	-0.34, 0.35
	p	0.88	0.27	0.50	0.97
ASCT2	β	0.04	**-0.17**	-0.14	0.09
	95% CI	-0.40, 0.47	**-0.34, -0.01**	-0.97, 0.68	-0.31, 0.50
	p	0.87	**0.04**	0.70	0.63
LAT1	β	-0.01	-0.51	0.75	0.14
	95% CI	-0.49, 0.46	-1.16, 0.13	-0.28, 1.78	-0.60, 0.87
	p	0.96	0.11	0.13	0.70
LAT3	β	0.02	0.20	-0.60	0.25
	95% CI	-0.42, 0.46	-0.10, 0.50	-1.58, 0.39	-0.16, 0.67
	p	0.93	0.18	0.20	0.21
LAT4	β	-0.01	-0.12	0.36	-0.16
	95% CI	-0.30, 0.28	-0.41, 0.16	-0.33, 1.06	-0.58, 0.26
	p	0.93	0.39	0.26	0.44
SNAT1	β	-0.09	0.07	0.20	*-0.19*
	95% CI	-0.35, 0.17	-0.16, 0.31	-0.35, 0.75	*-0.40, 0.02*
	p	0.46	0.53	0.42	*0.07*
TAT1	β	-0.11	-0.15	-0.41	-0.03
	95% CI	-0.49, 0.26	-0.48, 0.18	-1.43, 0.61	-0.48, 0.43
	p	0.53	0.35	0.38	0.90
			Calcium transporter genes		
CAT1	β	0.08	-0.04	1.05	-0.10
	95% CI	-0.29, 0.45	-0.36, 0.28	-0.26, 2.36	-0.36, 0.16
	p	0.64	0.80	0.10	0.42
NCX1	β	0.04	0.02	-0.43	0.11
	95% CI	-0.34, 0.42	-0.21, 0.25	-1.32, 0.46	-0.24, 0.46
	p	0.84	0.85	0.29	0.51
PMCA1	β	-0.09	-0.05	-0.18	0.10
	95% CI	-0.51, 0.33	-0.46, 0.35	-1.30, 0.94	-0.19, 0.39
	p	0.65	0.79	0.72	0.47
PMCA4	β	0.04	-0.15	-0.34	-0.05
	95% CI	-0.44, 0.52	-0.41, 0.12	-1.47, 0.79	-0.42, 0.32
	p	0.86	0.26	0.51	0.78
			Other genes		
DNMT1	β	0.06	-0.10	-0.01	-0.02
	95% CI	-0.32, 0.45	-0.38, 0.18	-0.68, 0.67	-0.32, 0.28
	p	0.74	0.46	0.99	0.88
PTHrP	β	0.33	-0.41	-0.28	-0.08
	95% CI	-0.39, 1.06	-1.13, 0.31	-1.97, 1.40	-0.87, 0.71
	p	0.34	0.25	0.71	0.84
TXNIP	β	-0.02	0.11	-0.13	0.01
	95% CI	-0.34, 0.30	-0.16, 0.39	-0.75, 0.48	-0.29, 0.32

p	0.91	0.41	0.63	0.93

All data were adjusted for sex and gestational age. Significant results ($p < 0.05$) are highlighted in bold, and trends ($p < 0.1$) are in italics.

Appendix 7: Abstracts and awards

Abstracts

- The expression of facilitated amino acid transporters in the human choriocarcinoma BeWo cell line. Simner, C.L., Harvey, N.C., Lillycrop, K.A., Lewis, R.M., and Cleal, J.K. (International Union of Physiological Sciences, 2013).
- Methylation levels of the placental facilitated transporter *TAT1* are related to maternal body composition prior to pregnancy. Simner, C.L., Lewis, R.M., Inskip, H.M., Cooper, C., Hanson, M.A., Godfrey, K.M., Harvey, N.C., Lillycrop, K.A., Cleal, J.K., and the SWS study group. (Developmental Origins of Health and Disease, 8th World Congress, 2013).
- Maternal vitamin D and vitamin D-binding protein: relationship with mRNA expression and epigenetic regulation of placental facilitated amino acid transporters. Cleal, J.K., Barton, S.J., Simner, C.L., Lillycrop, K.A., Inskip, H.M., Cooper, C., Hanson, M.A., Godfrey, K.M., Harvey, N.C., Lewis, R.M., and the SWS study group. (Developmental Origins of Health and Disease, 8th World Congress, 2013).
- Placental amino acid transport may be regulated by maternal vitamin D and vitamin D-binding protein: results from the Southampton Women's Survey. Simner, C.L., Barton, S.J., Lillycrop, K.A., Inskip, H.M., Cooper, C., Hanson, M.A., Godfrey, K.M., Harvey, N.C., Lewis, R.M., Cleal, J.K., and the SWS study group. (Vitamin D and Human Health. From the gamete to the grave, 2014).
- Expression of specific placental amino acid transporters is regulated by DNA methylation. Simner, CL., Lillycrop, K.A., Harvey, N.C., Lewis, R.M., and Cleal, J.K. (Physiology 2014).

Awards

- International Union of Physiological Sciences registration award from the DOHaD Society (2013).
- Primer Design Gold Sponsorship (2013).

Appendix 8: Publications

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Placental amino acid transport may be regulated by maternal vitamin D and vitamin D-binding protein: results from the Southampton Women's Survey

J. K. Cleal[1]*†, P. E. Day[1]†, C. L. Simner[1], S. J. Barton[2], P. A. Mahon[2], H. M. Inskip[2], K. M. Godfrey[1,2,3], M. A. Hanson[1,3], C. Cooper[2,3,4], R. M. Lewis[1], N. C. Harvey[2,3] and the SWS Study Group [2]

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Abstract

Both maternal 25-hydroxyvitamin D (25(OH)D) concentrations during pregnancy and placental amino acid transporter gene expression have been associated with development of the offspring in terms of body composition and bone structure. Several amino acid transporter genes have vitamin D response elements in their promoters suggesting the possible linkage of these two mechanisms. We aimed to establish whether maternal 25(OH)D and vitamin D-binding protein (VDBP) levels relate to expression of placental amino acid transporters. RNA was extracted from 102 placental samples collected in the Southampton Women's Survey, and gene expression was analysed using quantitative real-time PCR. Gene expression data were normalised to the geometric mean of three housekeeping genes, and related to maternal factors and childhood body composition. Maternal serum 25(OH)D and VDBP levels were measured by radioimmunoassay. Maternal 25(OH)D and VDBP levels were positively associated with placental expression of specific genes involved in amino acid transport. Maternal 25(OH)D and VDBP concentrations were correlated with the expression of specific placental amino acid transporters, and thus may be involved in the regulation of amino acid transfer to the fetus. The positive correlation of VDBP levels and placental transporter expression suggests that delivery of vitamin D to the placenta may be important. This exploratory study identifies placental amino acid transporters which may be altered in response to modifiable maternal factors and provides a basis for further studies.

Key words: **Vitamin D: Amino acid transporters: Placenta**

Vitamin D insufficiency is common in women of childbearing age and is associated with reduced foetal growth and poor postnatal health[1,2]. The biologically inactive 25-hydroxyvitamin D (25(OH)D) is used to monitor vitamin D status, as this is the major circulating form[3]. In the Southampton Women's Survey (SWS), a prospective longitudinal study of maternal nutrition and lifestyle before and during pregnancy, it was found that lower maternal 25(OH)D was associated with morphological changes in the foetal femur[4], lower neonatal fat mass and greater fat mass and lower grip strength in childhood[5,6]. Reduced 25(OH)D during late pregnancy was also associated with reduced bone mineral content in children at 9 years of age in another Southampton cohort study[2].

The mechanisms underlying these associations are not fully understood, but are likely to involve the placenta, the sole conduit for nutrients from mother to fetus. We previously reported that placental mRNA expression of the vitamin D sensitive Ca transporter plasma membrane Ca ATPase 3 *(PMCA3)* and the imprinted gene Pleckstrin homology-like domain family A member 2 *(PHLDA2)* is associated with offspring bone mass development and composition[7,8]. Other than Ca transport, a key element for foetal bone development is placental amino acid transport. Placental amino acid transfer is

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; DXA, dual-energy X-ray absorptiometry; SWS, Southampton Women's Survey; VDBP, vitamin D binding protein; VDRE, vitamin D response element.

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† These authors contributed equally.

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vital for foetal growth[9], and animal studies suggest that decreased amino acid transport precedes foetal growth restriction[10]. Amino acid transfer to the fetus involves amino acid transport across the microvillous and basal membranes of the placental syncytiotrophoblast[11] and potentially metabolic interconversion within the placenta[12]. Placental amino acid transfer is thought to be regulated by maternal nutritional and hormonal factors[13–15].

There are three classes of amino acid transporter in the human placenta; accumulative transporters, amino acid exchangers[16,17] and facilitated transporters[18] (Fig. 1). Accumulative transporters mediate net uptake of specific amino acids across the microvillous membrane (e.g. SNAT), and also play roles on the basal membranes such as uptake of foetal glutamate for placental glutamine synthesis (e.g. EAAT)[12]. The exchangers including LAT, y^+LAT and ASCT use the gradients built up by accumulative transporters to drive uptake and transfer of other amino acids including many essential amino acids[18]. The facilitated transporters TAT1, LAT3 and LAT4 are essential for net amino acid transport to the fetus, and their gene expression in human placenta is associated with measures of foetal growth[18]. The factors that regulate these changes in gene expression are not understood. However, as these and several other amino acid transporters have vitamin D response elements (VDRE) in their promoter regions, they could theoretically be regulated at the transcriptional level by maternal vitamin D. Specifically, the biologically active 1,25 dihydroxyvitamin D regulates transcription of specific genes by binding the vitamin D receptor and interacting with VDRE in their promoter regions[19,20].

We therefore investigated whether maternal 25(OH)D and vitamin D binding protein (VDBP) concentrations during pregnancy are related to gene expression of the amino acid transporters, essential for placental amino acid transfer. We used samples collected from a population based cohort, the SWS.

Methods

The study was conducted according to the guidelines in the Declaration of Helsinki, and the Southampton and South West Hampshire Research Ethics Committee approved all procedures (276/97, 307/97, 089/99, 153/99, 005/03/t, 06/Q1702/104). Written informed consent was obtained from all participating women and by parents or guardians with parental responsibility on behalf of their children.

Maternal measurements

We used data and samples from the SWS, a cohort study of 3158 pregnancies with information collected from the mothers before conception[21]. Non-pregnant women aged 20–34 years were recruited via their general practitioners; assessments of lifestyle, diet and anthropometry were performed by trained research nurses at study entry and then in early (11 weeks) and late (34 weeks) gestation among those women who became pregnant. Subscapular skinfold thicknesses were measured to the nearest 0·1 mm in triplicate using Harpenden skinfold callipers (Baty International)[22].

At 34 weeks of gestation, a maternal venous blood sample was obtained and an aliquot of maternal serum was frozen at −80°C. Serum 25(OH)D and VDBP concentrations were analysed by RIA (DiaSorin). The 25(OH)D assay measures both 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3. The detection range for this 25(OH)D assay is 3·8–250 nmol/l. The assays met the requirements of the UK National Vitamin D External Quality Assurance Scheme, and intra- and inter-assay CV were <10%.

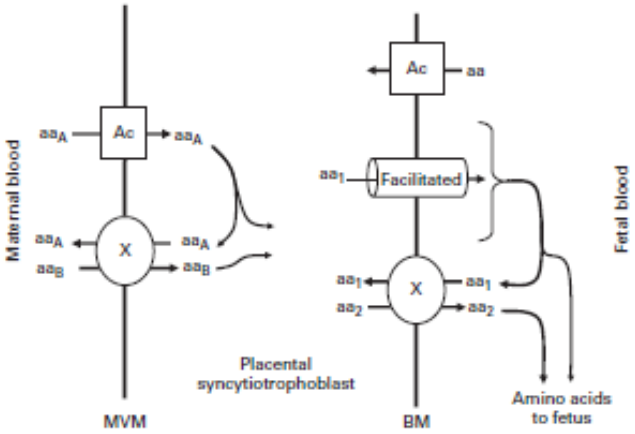

Fig. 1. Transport of amino acids across the placental syncytiotrophoblast. Amino acids are transported across the microvillous membrane (MVM) into the placental syncytiotrophoblast by active accumulative transporters (Ac; e.g. SNAT) and exchangers (X; e.g. ASCT). Amino acids transported by accumulative transporters (aa_A) are then exchanged back for those only transported by exchangers (aa_B). Amino acids are transported out of the placenta across the basal membrane (BM) by facilitated transporters (TAT1, LAT3 and LAT4) and exchangers (X). The facilitated transporters transport specific amino acids (aa_1) down their concentration gradient to the fetus. In order to transport other amino acids (aa_2) to the fetus, aa_1 must be exchanged for aa_2 via exchangers (X).

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Placental samples

Placentas were collected from term pregnancies within 30 min of delivery, and no clinical conditions such as pre-eclampsia or gestational diabetes. Placental weight was measured after removing blood clots, cutting the umbilical cord flush with its insertion into the placenta, trimming away surrounding membranes and removing the amnion from the basal plate. To ensure that the samples collected were representative of the placentas as a whole, five villous tissue samples were selected using a stratified random sampling method, and stored at −80°C. For the present study, a cohort of 102 placentas was selected from 300 collected in total, based on availability of neonatal dual-energy X-ray absorptiometry (DXA) data.

RNA extraction and complementary DNA synthesis

For each placenta five snap frozen samples were pooled and powdered in a frozen tissue press. Total RNA was extracted from 30 mg powdered placental tissue using the RNeasy fibrous tissue RNA isolation mini kit (Qiagen) according to the manufacturer's instructions. The integrity of total RNA was confirmed by agarose gel electrophoresis.

Total RNA (0·2 μg) was reverse transcribed with 0·5 μg random hexamer primer, 200 units Moloney murine leukaemia virus reverse transcriptase, 25 units recombinant RNasin ribonuclease inhibitor and 0·5 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate in a final reaction volume of 25 μl in 1× Moloney murine leukaemia virus reaction buffer (Promega). All 102 samples were produced in one batch to reduce variation.

Probe and primer design

Intron spanning oligonucleotide probes and primers were designed using the Roche ProbeFinder version 2.45 for human. Probes were supplied by Roche from the human universal probe library and primers were synthesised by Eurogentec. Control genes were selected using the geNorm™ human Housekeeping Gene Selection Kit (Primer Design Limited).

Target genes

The genes measured in the present study along with primer and probe details are listed in Table 1. And mRNA levels were measured using quantitative real-time PCR using a Roche LightCycler 480. For Roche universal probe library probes the cycle parameters were 95°C for 10 min, followed by forty cycles of 95°C for 15 s and 60°C for 1 min. For the primer design Perfect Probes, the cycle parameters were 95°C for 10 min, followed by forty cycles of 95°C for 10 s and 60 and 72°C for 15 s. Intra-assay CV's for each gene were 5–8 %. Each of the 102 samples was run on the same plate in triplicate. All mRNA levels are presented relative to the geometric mean of the three control genes, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), ubiquitin C (*UBC*) and topoisomerase (*TOP1*)[23].

Postnatal measurements

At birth (n 102) and 4 years of age (n 42–46) a whole-body DXA scan was obtained using a Hologic Discovery instrument (Hologic, Inc.) in paediatric scan mode (Apex 3.1 software), yielding fat mass, lean mass and bone mineral content. The CV for body composition analysis with the DXA instrument was 1·4–1·9 %.

Statistics

Maternal and placental mRNA data that were not normally distributed were transformed logarithmically. Previous data showed that gene expression of the control genes and many of the target genes was higher in male than in female placentas[24]. Adjustment was therefore made for sex in the correlation analysis between mRNA and all other variables. Pearson's correlation coefficient (r_p) was used to determine partial correlations adjusted for sex and gestational age between placental mRNA levels, neonatal body composition and maternal factors (IBM SPSS Statistics 20). The partial correlation between placental gene expression and maternal vitamin D measures was also adjusted for potential confounding factors: maternal sum of skinfold thickness, walking speed, parity and smoking during pregnancy. A value of $P<0·05$ was accepted as statistically significant, and, given the observational nature of the study together with the substantial co-linearity among both predictors and outcomes, testing for multiple comparisons was felt to be inappropriate[25].

Results

Characterisation of the subjects from the Southampton Women's Survey cohort

The mean age of the 102 mothers at the birth of their children was 30·9 (SD 3·9) years; 37·9 % were primiparous. 97 % of the women were of white European ethnicity. The median gestational age was 39·6 (inter-quartile range 38·8–40·7) weeks. The mean placental/foetal weight ratio was 0·13 (SD 0·02). Of the 102 placentas from SWS pregnancies studied here, fifty-three of the infants were male, forty-nine were female. The mean birth weight for males was 3547 (SD 417) g with 95% between the 33rd and 51st centile based on UK growth charts. The mean birth weight for females was 3455 (SD 489) g with 95% between the 36th and 59th centile.

Maternal plasma vitamin D and placental gene expression

The 34-week plasma 25(OH)D levels were measured for ninety-one of the 102 women and VDBP levels for eighty-five of the 102 women. The mean 25(OH)D levels were 71·7 (SD 32·1) nmol/l with a range of 20–158 nmol/l. The mean VDBP levels were 5622 (SD 806) mg/l with a range of 4160– 8570 mg/l. Of the women, 28·6 % were taking vitamin D

Table 1. Information on genes, primers and probes

Transporter	Gene	Gene ID	Genebank accession no.	Primers	Roche universal probe library no.
ASCT1	*SLC1A4*	6509	NM_003038.2	F: 5′-ttggcacagcatttgctac-3′ R: 5′-gcacttcatcatagagggaagg-3′	78
ASCT2	*SLC1A5*	6510	NM_005628.2 NM_001145144.1	F: 5′-gaggaatatcacggaacca-3′ R: 5′-aggatgttcatccactcca-3′	43
EAAT1	*SLC1A3*	6507	NM_004172.4	F: 5′-ttgaactgaacttcggacaaatta-3′ R: 5′-attccagctgcccaaatact-3′	76
EAAT2	*SLC1A2*	6506	NM_004171.3	F: 5′-aaaatgctcatttctcctctaato-3′ R: 5′-gccactagccttagcatcca-3′	78
EAAT3	*SLC1A1*	6505	NM_004170.4	F: 5′-agttgaatgaccttggacttgg-3′ R: 5′-gcagatgtggccgtgatac-3′	9
EAAT4	*SLC1A6*	6511	NM_005071.1	F: 5′-tgaagatgctggtgttacct-3′ R: 5′-gttgtccagggatgcaata-3′	19
EAAT5	*SLC1A7*	6512	NM_006671.4	F: 5′-cgccaaggtcaacaactta-3′ R: 5′-gctgcagtggctgtgatact-5′	9
LAT1	*SLC7A5*	8140	NM_003486.5	F: 5′-gtggaaaaaaaagaccaagt-3′ R: 5′-gcatgagcttctgaccagg-3′	25
LAT2	*SLC7A8*	23428	NM_182728.1 NM_012244.2	F: 5′-ttgccaatgtcgattatgtc-3′ R: 5′-ggagcttctctccaaaagtcac-3′	17
LAT3	*SLC43A1*	8501	NM_003627.5 NM_001198810.1	F: 5′-gccctatgattggctctta-3′ R: 5′-ccggatcgtagatcagc-3′	29
LAT4	*SLC42A2*	124935	NM_001284498.1 NM_152346.2	F: 5′-acaagtatggccgaggaa-3′ R: 5′-gcaatcagcaagaaggaaa-3′	3
SNAT1	*SLC38A1*	81539	NM_030674.3 NM_001077484.1	F: 5′-atttgggactagccttg-3′ R: 5′-agcaatgtcactgaagtaaaaagt-3′	47
SNAT2	*SLC38A2*	54407	NM_018976.3	F: 5′-cctatgaaatctgtacaaaagattgg-3′ R: 5′-ttgtgtaccaatccaaaacaa-3′	9
SNAT4	*SLC38A4*	55089	NM_018018.4 NM_001143824.1	F: 5′-tgttctggtcatccttgtgc-3′ R: 5′-aaaactgctggaagaataaaaatcag-3′	29
TAT1	*SLC16A10*	117247	NM_018593.4	F: 5′-ggtgtgaagaaggtttatctacagg-3′ R: 5′-agggccccaaagatgcta-3′	6
y⁺LAT1	*SLC7A7*	9056	NM_001126105.1 NM_001126106.1	F: 5′-acactgcagtgagaacctg-3′ R: 5′-aggagaggaaaaccttaacc-3′	72
y⁺LAT2	*SLC7A6*	9057	NM_001076785.1 NM_003983.4	F: 5′-gctgtgatcccccataact-3′ R: 5′-ggcacagttcaaaaatgtcag-3′	66
4F2HC	*SLC3A2*	6520	NM_001012661.1	F: 5′-tggttdccactcaggttga-3′ R: 5′-cagcaaaactcaagagaat-3′	49

SLC, solute carrier; F, forward; R, reverse; 4F2HC, type-II membrane glycoprotein heavy chain.

supplements of 10 μg/d (400IU/d). The mean vitamin D intake (from FFQ and data on supplements) from the ninety-eight available (out of 102) women's diets is 3·5 μg/d (ranging from 1·3 to 9·0 μg/d).

Of the genes investigated mRNA for *EAAT1*, *EAAT4* and *EAAT5* were not detected in human placenta.

In this subset of SWS woman, there was a positive correlation between maternal 34-week plasma 25(OH)D levels and the mRNA expression of *LAT3* (Fig. 2), *ASCT1* and *y⁺LAT1*, and a negative correlation with *SNAT1* (Table 2). Maternal VDBP levels correlated positively with mRNA expression of *TAT1*, *LAT3*, *LAT4*, *SNAT1*, *SNAT2*, *y⁺LAT2*, type-II membrane glycoprotein heavy chain *(4F2HC)*, and *EAAT3*, and there was a trend with *LAT1* (Table 2).

When the correlation was also adjusted for maternal confounding factors (maternal sum of skinfold thickness, walking speed, parity and smoking during pregnancy) all correlations were still present, except for the relationships between 25(OH)D and *ASCT1*, and VDBP and *TAT1*, which were no longer statistically significant at the $P<0·05$ level (Table 2). The adjusted data also showed a positive association between VDBP and *LAT1* mRNA (Table 2).

Neonatal body composition

At birth, there were no significant associations between placental amino acid transporter gene expression and neonatal lean mass, fat mass, or bone mineral content (data not shown).

At 4 years of age total lean mass was positively associated with *LAT3* (Fig. 2), *y⁺LAT1* and *TAT1* mRNA expression (Table 3). Bone mineral density was positively associated with *LAT4* mRNA and negatively associated with *ASCT2* and *EAAT3* mRNA expression (Table 3). *EAAT3* mRNA expression levels (n 42) were also negatively associated with bone mineral content (r_p −0·46, $P=0·003$) and total bone area (cm^2 without heads; r_p −0·43, $P=0·01$). *SNAT4* (r_p −0·40, $P=0·01$) and *y⁺LAT2* (r_p −0·32, $P=0·04$) expression levels were negatively associated with total bone area.

Discussion

Many genes related to placental function may be regulated directly or indirectly by vitamin D. The present study aimed to establish whether there are relationships between maternal vitamin D levels and changes in gene expression in placentas

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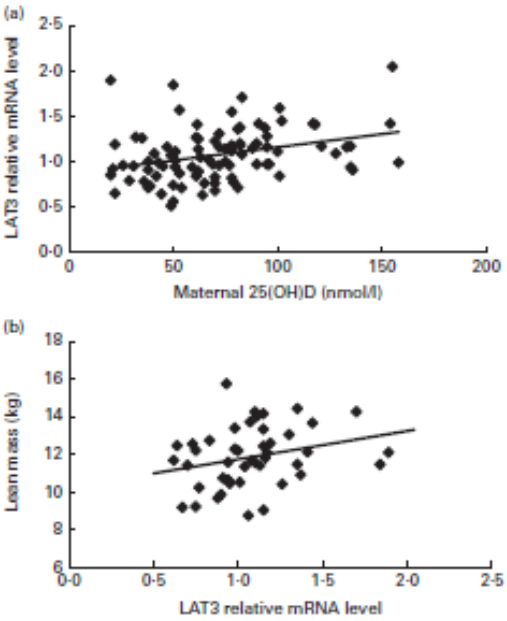

Fig. 2. *LAT3* mRNA expression is associated with postnatal body composition. *LAT3* relative mRNA expression in human placenta is positively correlated with maternal 25-hydroxyvitamin D (25(OH)D) (r_s 0·31, P=0·003, n 102) (a) and lean mass at 4 years of age (r_s 0·38, P=0·01, n 48) (b).

from the SWS. Maternal 25(OH)D and VDBP levels were positively associated with placental expression of genes involved in amino acid transport. This suggests that maternal vitamin D status may regulate the expression of placental amino acid transporters, and potentially influence the transfer of amino acids to the fetus and subsequent foetal growth. The observations that VDBP was associated with the expression of twice as many genes as vitamin D suggests that delivery of vitamin D to the placenta may be a crucial determinant of vitamin D activity. The associations seen may however, involve a more complex relationship between maternal vitamin D status and maternal body composition.

Vitamin D

Placental amino acid transport is important for foetal growth and development, so understanding how the amino acid transporters are regulated in the placenta will help us understand the mechanisms underlying foetal growth restriction and the associated postnatal phenotype. Maternal vitamin D status has also been shown to associate with both foetal and neonatal growth, and, taken with the fact that it modulates gene transcription; this suggests there may be an interaction between vitamin D and placental amino acid transport. This interaction could be a direct effect of vitamin D, and its receptor acting directly on the placental amino acid transporter genes at a VDRE or an indirect effect mediated via vitamin D's activation of another gene. Both the *LAT3* and *ASCT1* genes have been shown to have VDRE in their promoter region[26], which could underlie the association between their mRNA expression and maternal 25(OH)D levels. Vitamin D can also down-regulate gene expression via vitamin D receptor, blocking the activity of the cyclic AMP response element in the promoter[27]. This may explain the observed negative association between 25(OH)D and *SNAT1* mRNA expression, a gene regulated by cyclic AMP at the cyclic AMP response element[28]. Vitamin D can also directly affect gene transcription by an interaction between vitamin D receptor and

Table 2. The associations between placental amino acid transporter mRNA expression and maternal serum 25-hydroxyvitamin D and vitamin D binding protein levels

	34-week vitamin D (nmol/l)		Vitamin D binding protein (mg/l)		34-week vitamin D (nmol/l)		Vitamin D binding protein (mg/l)	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
TAT1	0·07	0·50	0·23*	0·03*	0·14	0·21	0·12*	0·10*
LAT3	0·31*	0·003*	0·22*	0·04*	0·37*	0·003*	0·22*	0·05*
LAT4	−0·12	0·25	0·28*	0·01*	−0·13	0·26	0·28*	0·01*
SNAT1	−0·23*	0·03*	0·25*	0·02*	−0·20*	0·07*	0·23*	0·05*
SNAT2	0·01	0·96	0·23*	0·03*	0·04	0·70	0·23*	0·04*
SNAT4	0·14	0·19	0·08	0·45	0·12	0·30	0·12	0·29
ASCT1	0·23*	0·03*	0·06	0·62	0·20*	0·07*	0·11	0·33
ASCT2	0·04	0·74	0·18	0·10	0·05	0·63	0·17	0·14
y⁺LAT1	0·31*	0·003*	0·03	0·81	0·36*	0·001*	0·02	0·99
y⁺LAT2	0·04	0·73	0·26*	0·02*	−0·08	0·94	0·33*	0·003*
EAAT2	0·12	0·26	−0·07	0·53	0·06	0·56	−0·01	0·91
EAAT3	0·09	0·39	0·30*	0·01*	0·12	0·24	0·29*	0·009*
LAT1	−0·14	0·19	0·21*	0·06*	−0·17	0·12	0·23*	0·04*
LAT2	−0·08	0·44	0·18	0·10	−0·07	0·54	0·17	0·14
4F2HC	−0·12	0·26	0·25*	0·02*	−0·08	0·48	0·23*	0·04*
	Adjusted for sex and dGA				Adjusted for sex, dGA and maternal confounding factors			

4F2HC, type-II membrane glycoprotein heavy chain; dGa, days gestational age.
*$P < 0.05$.

Table 3. The associations between placental amino acid transporter mRNA expression and 4-year-old dual-energy X-ray absorptiometry (DXA) measurements of body composition

	Total lean (kg) (*n* 46)		Total Prentice BMD (g), without heads (*n* 42)	
4 year DXA	*r*	*P*	*r*	*P*
TAT1	0·33*	0·03*	−0·17	0·28
LAT3	0·38*	0·01*	−0·15	0·33
LAT4	−0·09	0·57	0·41*	0·01*
SNAT1	−0·12	0·45	0·06	0·72
SNAT2	0·06	0·68	−0·11	0·49
SNAT4	0·08	0·62	−0·18	0·26
ASCT1	0·23	0·13	−0·27	0·09
ASCT2	0·24	0·11	−0·42*	0·01*
y$^+$*LAT1*	0·31*	0·04*	−0·25	0·11
y$^+$*LAT2*	0·20	0·18	−0·27	0·09
EAAT2	0·28	0·07	0·04	0·82
EAAT3	−0·04	0·80	−0·59*	0·00005*
LAT1	−0·21	0·16	0·09	0·59
LAT2	−0·04	0·82	−0·02	0·90
4F2HC	0·05	0·73	0·14	0·38

BMD, bone mineral density; *4F2HC*, type-II membrane glycoprotein heavy chain.
*$P < 0·05$.

histone acetyltransferases, leading to an open/active chromatin state[29]. The amino acid transporter genes could therefore be in a region of DNA, affected by vitamin D-mediated epigenetic changes, or could be regulated indirectly via an effect on another gene in the placenta.

The relationship between vitamin D and placental function may be more complex than vitamin D receptor-mediated changes in placental gene expression, and could be very indirect via an effect on maternal physiology or metabolism. It could be that vitamin D levels are influencing aspects of the maternal environment, which in turn regulate placental gene expression. Alternatively, maternal factors could simply be regulating both vitamin D levels and placental amino acid transporter expression in a similar manner. Plasma vitamin D status is known to be related to factors such as maternal smoking, parity and BMI[30]. It could be that maternal body composition is influencing the placenta, as a signal reflecting the mother's nutrient reserves and capacity to support the pregnancy. We have previously demonstrated an association between maternal muscle mass and placental amino acid transfer, indicating that maternal body composition can affect placental amino acid handling[31].

Vitamin D levels could therefore be a proxy for another aspect of the maternal environment, and not a direct mediator of amino acid transporter expression levels. When we corrected our correlation analysis to adjust for maternal factors, we did indeed see that the amino acid transporters *ASCT1* and *SNAT1* were no longer related to the maternal 25(OH)D levels. These transporters may therefore be regulated by aspects of maternal body composition rather than vitamin D status, or vitamin D levels may be mediating the effects of body composition on the placenta. *LAT3* and *y*$^+$*LAT1* did still show strong associations with maternal 25(OH)D levels, suggesting that it is the vitamin D rather than body composition that affects their regulation. Further studies are needed to establish the mechanisms underlying this association.

Interestingly, there were a number of positive associations between VDBP and amino acid transporter expression levels. This suggests that the delivery of the vitamin D to the placenta by its binding protein may be an important determinant of vitamin D action, possibly mediated by receptor-mediated endocytosis[32]. Further investigation into the uptake of vitamin D and levels of the active 1,25 dihydroxyvitamin D within the placenta is needed. This will help us understand and improve the effects of 25(OH)D supplementation during pregnancy, which may also require the VDBP to be upregulated.

Postnatal outcome

We previously reported that placental *TAT1* and *LAT3* mRNA expression levels in this cohort are positively related to measures of foetal growth, with *TAT1* mRNA being associated with foetal growth in terms of lean mass[18]. Consistent with these observations we found that *y*$^+$*LAT1*, *TAT1* and *LAT3* mRNA expression in placentas are positively related to 4-year-old lean mass. As lean mass contains a high proportion of muscle, a protein-rich tissue, its growth will require a substantial amino acid supply, and so it may rely on appropriate amino acid supply in early development.

Limitations

The present study has the advantage of using a well characterised population representative of the general population, with detailed phenotyping of mother–offspring pairs. The placentas and offspring included in this study were of the mothers who allowed DXA measurements to be undertaken. The women whose offspring had DXA measures, compared to those that did not, were slightly older and tended to be better educated. They do represent a wide range of maternal age and family backgrounds, and all comparisons were internal to the selected subset. When comparing the vitamin D levels in the women with placental samples *v.* the whole cohort they look very similar with a slightly higher mean, but a similar standard deviation; 71·7 (SD 32·1) nmol/l, *n* 91 *v.* 64·2 (SD 30·9) nmol/l, *n* 2178. In the present study we were only able to measure the inactive 25(OH)D, which is thought to be the best measure of vitamin D status. Further studies would be enhanced by measuring the level of active 1,25 dihydroxyvitamin D within the placental tissue, and relating this directly to gene expression. The exploratory nature of the present study, small sample size and the possibility of chance findings need to be acknowledged. In particular, we had reduced numbers at 4 years of age (42–46 mother–offspring pairs) due to participants' not returning for measurement. The measures made in this sub-set at 4 years of age were representative of the whole cohort, for example the mean lean mass was 11·8 (SD 1·6) kg, *n* 46 compared to 12·0 (SD 1·5) kg, *n* 743. These numbers did, however, give us greater than 90 % power to detect a correlation coefficient of 0·5. Compared to adults, DXA assessment of body composition in children is more problematic due to their smaller size and tendency to move. These DXA measures were,

however, validated previously in piglets using biochemical assessment of carcass N content and lipid extraction to determine lean and fat mass, respectively[33]. In the present study specific paediatric software was used, and movement artefacts were minimal. While the present study focused on the actions of vitamin (as a transcription factor) on the expression of key placental genes, it would also have been interesting to study the effect of a wider range of factors including maternal and foetal amino acid levels. It is important to remember that the regulation of gene function and physiology are complex and will rarely be dependent on a single factor. It is not possible in this observational study to determine whether the observed associations are causal. Nevertheless, the patterns of observations are indicative of a role for vitamin D in the regulation of placental amino acid transporter expression, and it forms, we think, the basis for future studies.

Conclusion

In conclusion the present study demonstrates relationships between maternal vitamin D levels, and in particular VDBP and placental gene expression. As there are associations between vitamin D and body composition, these observations provide a possible mechanism by which maternal factors influence placental function. Further work needs to be undertaken to investigate the association between maternal VDBP and placental gene expression, and whether these are direct or indirect effects.

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The authors' contributions are as follows: J. K. C., R. M. L., C. L. S. and N. C. H. formulated the specific research question and the design of the study. H. M. I., K. M. G., M. A. H., C. C., N. C. H. and the SWS Study Group designed the cohort (SWS) study. The experiments were carried out by P. E. D., J. K. C., R. M. L. and P. A. M. J. K. C. and S. J. B. analysed the data. The article was written by J. K. C., P. E. D., R. M. L. and N. C. H. with input from all other authors. The final manuscript was read and approved by all the authors.

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