

THE EFFECT OF VITAMIN D  
ON PLACENTAL  
DEVELOPMENT AND  
PREGNANCY SUCCESS

Jessica Ann Laurence

A thesis submitted for the degree of Doctor of Philosophy

Discipline of Obstetrics and Gynaecology,  
School of Paediatrics and Reproductive Health,  
The University of Adelaide

October 2014

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### III. Abstract

Vitamin D<sub>3</sub> deficiency is prevalent around the world, with 30-60% of Australians having 25(OH)D<sub>3</sub> levels below 50 nmol/L. There has been a resurgence of interest in vitamin D<sub>3</sub> in the last decade as its deficiency has been shown to be associated with an increasing number of diseases, including pregnancy complications. The role of vitamin D<sub>3</sub> in pregnancy is unclear, although vitamin D<sub>3</sub> metabolism genes are expressed in the placenta and circulating active vitamin D<sub>3</sub> increases 3-fold during pregnancy. As many pregnancy complications are associated with poor placental development, we hypothesise that vitamin D<sub>3</sub> deficiency may impair placental development and thereby contribute to the pathogenesis of pregnancy complications.

To determine the effect of dietary vitamin D<sub>3</sub> and calcium deficiency on the placenta we used a mouse model. Mice were fed diets deficient in vitamin D<sub>3</sub> and/or calcium, mated with normal males and killed at d18.5 *post-coitus*. Pregnant and non-pregnant mice had altered vitamin D<sub>3</sub> metabolism, namely alterations in biochemistry and kidney gene expression. During pregnancy serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels are elevated. The level of expression of the gene for the enzyme responsible for activation of vitamin D, vitamin D<sub>3</sub> 25-hydroxylase (*CYP27B1*), was highest in kidneys of pregnant mice compared to levels in non-pregnant mice as well as to levels in the placenta of pregnant mice, indicating that increased renal 1,25(OH)<sub>2</sub>D<sub>3</sub> production is a feature of pregnancy. Pregnant mice consuming diets deficient in both vitamin D<sub>3</sub> and calcium resulted in higher incidence of preterm birth (PTB) as defined by a delivery before d18.5 of gestation. While pregnant mice had comparable placental weights regardless of vitamin D and calcium deficient diets, the placental morphometry was altered such that there was increased

capacity for feto-maternal exchange. Consistent with this, the average fetal weight was significantly greater in those dams consuming a low calcium diet regardless of the dietary vitamin D level, suggesting that placental adaptations allowed greater fetal growth.

We next examined the human placental expression profile of vitamin D<sub>3</sub> metabolism and the interacting insulin-like growth factor (IGF) pathway genes across gestation. We found that vitamin D<sub>3</sub> pathway genes (*VDR*, *CYP2R1*) increase while IGF genes (*IGF1R*, *IGF2*) decrease from early to late gestation, reflecting the most important timeframes in gestation for the action of these pathways. Correlations in gene expression were found between *IGF2* and *VDR*, as well as *IGF1R* and *VDR*, suggesting new interactions between the pathways. Immunohistochemistry revealed reducing *CYP27B1*, *VDR* and *IGF2* protein across gestation, while *CYP24A1* was not altered. As *VDR* protein is reduced, with high mRNA levels, this indicates a high turnover of *VDR* protein in term placentas.

Human placental gene expression from pregnancies with medical complications was also compared to placentas from normal term-delivered controls. The mRNA level for *VDR* was reduced in placentas of pregnancies with preeclampsia and spontaneous preterm deliveries. In addition, *CYP24A1* mRNA levels were reduced in placentas from pregnancy cases with gestational diabetes and fetuses that were considered small for gestational age. This suggests that placental vitamin D<sub>3</sub> metabolism is altered both across gestation and in pregnancy complications. Despite these changes in gene expression for vitamin D related genes, there was only one altered IGF-family gene, with *IGF1* increased in placentas from pregnancies with preterm deliveries. As *IGF1* interacts with the vitamin D pathway, this could be an interacting pathway in pathology of preterm birth.



In conclusion, vitamin D<sub>3</sub> and calcium metabolism are altered by pregnancy, with deficiency of both vitamin D<sub>3</sub> and calcium resulting in PTB in mice. Dietary vitamin D and calcium deficiency increased fetal weight which was associated with changes in both placental morphometry and gene expression. In human placenta, the expression of vitamin D<sub>3</sub> metabolic pathway components is altered both across gestation and between complicated and uncomplicated pregnancies. These studies together strongly indicate a role for vitamin D<sub>3</sub> action in normal and complicated pregnancy.

## IV. Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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## V. Abstracts arising from this thesis

1. Laurence JA, Bianco-Miotto T, Anderson P, Roberts CT, Placental expression of the vitamin D<sub>3</sub> and insulin-like growth factor families across gestation, Fetal and Neonatal Workshop, 2014, Yanchep, W.A, Australia (Oral)
2. Laurence JA, Anderson P, Roberts CT, Dietary vitamin D<sub>3</sub> and calcium restriction and its effects on murine placental morphometry, Health Sciences Postgraduate Research Conference, The University of Adelaide, 2013, Adelaide, S.A., Australia (Poster)
3. Laurence JA, Anderson P, Bianco-Miotto T, Roberts CT, Murine placental and kidney gene expression is altered by dietary vitamin D<sub>3</sub> and calcium deficiency, Australian Society for Medical Research SA Annual Scientific Meeting, 2013, Adelaide, S.A., Australia (Oral)
4. Laurence JA, Anderson P, Bianco-Miotto T, Roberts CT, Dietary vitamin D<sub>3</sub> and calcium restriction and its effects on murine placental morphometry, Fetal and Neonatal Workshop, 2013, Barossa Valley, S.A., Australia (Oral)
5. Laurence JA, Anderson P, Bianco-Miotto T, Roberts CT, Effect of dietary calcium on vitamin D<sub>3</sub> related genes in mouse kidney and placenta, Australian Health and Medical Research Council Annual Meeting, 2012, Adelaide, S.A., Australia (Poster)
6. Laurence JA, Anderson P, Roberts CT, Pregnancy alters body composition, vitamin D<sub>3</sub> and calcium levels in mice, The Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology, 2012, Gold Coast, QLD, Australia (Oral)
7. Laurence JA, Bianco-Miotto T, Andraweera P, Anderson P, Dekker GA, Roberts CT, Placental gene expression of vitamin D<sub>3</sub> and insulin-like growth

- factor (IGF) genes in pregnancy complications, Australian Society for Medical Research SA Annual Scientific Meeting, 2012, Adelaide, S.A., Australia (Oral)
8. Laurence JA, Anderson P, Furness D, Roberts CT, Effect of dietary vitamin D<sub>3</sub> and calcium deficiency on pregnancy success in C57Bl6 mice, Annual Meeting of the Society for Reproductive Biology, 2011, Cairns, QLD, Australia (Oral)
  9. Laurence JA, Anderson P, Furness D, Roberts CT, Effect of dietary vitamin D<sub>3</sub> and calcium deficiency on pregnancy success in C57Bl6 mice, Faculty of Health Sciences Post Graduate Research Conference, 2011, Adelaide, S.A., Australia (Poster)
  10. Laurence JA, Anderson P, Furness D, Roberts CT, Effect of dietary vitamin D<sub>3</sub> and calcium deficiency on pregnancy success in C57Bl6 mice, Robinson Institute Research Centre for Reproductive Health Research Day, 2011, Adelaide, S.A., Australia (Poster)
  11. Laurence JA, Anderson P, Furness D, Roberts CT, Effect of dietary vitamin D<sub>3</sub> and calcium deficiency on pregnancy success in C57Bl6 mice, ANZSCDB Adelaide Cell and Developmental Biology Meeting, 2011, Adelaide, S.A., Australia (Poster)

## VI. Acknowledgements

I would firstly like to thank my primary supervisor Claire for her endless support during and before my PhD. She has generously given her time, expertise and money to ensure my project was a success. I also want to thank her for all her help with my animal training including increasing my confidence and ability to handle and care for my animals. Secondly I would like to thank the rest of my supervisory team: Tina, Paul, Denise and Gus for all of their support and the amount of time they have given me over the last few years.

To Tina and Gary, thanks for all of your help in the lab through teaching me basic and advanced laboratory skills and encouraging my troubleshooting of the numerous techniques I learnt in the course of my PhD.

I couldn't have made it sanely through this time without the support of my lab and work-mates. Thanks to Dylan, Gary, Amanda, Prabha, Sam, Shalem, Sean, Lucky, Bec, Ben, Zimin, Fleur, Alison, Ang, Steve, Jamie and Anita for all the support. Also thanks to my lab-neighbours Amy, Hong, Kathy and Damien for making life interesting. Thanks also to the rest of the Obstetrics & Gynaecology Department who were supportive and provided smiles and coffee when they were needed most.

I would like to thank my family for their endless support and patience over the last few years, in particular to my loving fiancé, Darryle. You have helped me through the tough times and now we can enjoy ourselves with weekends off, a wedding to plan and a bright future ahead. Thanks too to Mum, Dad, Kathryn and Stephanie, as well as Wendy, Michael, Jay and Beryl for all your support.

Finally I would like to thank my funding sources, without which I could not have carried out this research: Australian Post-graduate Award, Robinson Institute

Top-up Scholarship, NHMRC project grant as well as travel awards from the Department of Obstetrics & Gynaecology and the Robinson Research Institute to allow me to present my work.

## VII. Abbreviations

1,25(OH)<sub>2</sub>D<sub>3</sub> - 1α,25-dihydroxy vitamin D<sub>3</sub>

25(OH)D<sub>3</sub> - 25-hydroxy vitamin D<sub>3</sub>

AGA - appropriate for gestational age

ANOVA - analysis of variance

ANCOVA - analysis of covariance

CYP - cytochrome P450 enzyme

CYP2R1 - vitamin D<sub>3</sub> 25-hydroxylase

CYP27B1 - 25 hydroxy vitamin D<sub>3</sub> 1α-hydroxylase

CYP24A1 - 1,25 dihydroxy vitamin D<sub>3</sub> 24-hydroxylase

DNA - deoxyribonucleic acid

GDM - gestational diabetes mellitus

GH - gestational hypertension

IGF1 - insulin-like growth factor 1

IGF1R - insulin-like growth factor receptor 1

IGF2 - insulin-like growth factor 2

IGF2R - insulin-like growth factor receptor 2

IHC - immunohistochemistry

IQR - interquartile range

IUGR - intrauterine growth restriction

LBW - low birth weight

OR - odds ratio

PBS - phosphate buffered saline

PE - preeclampsia

PTB - preterm birth

PTH - parathyroid hormone

PTHrP - parathyroid hormone related protein

qPCR - quantitative real time PCR

RNA - ribonucleic acid

RR - relative risk

RQI - RNA Quality Index

SD - standard deviation

SEM - standard error of the mean

SGA - small for gestational age

sPTB - spontaneous preterm birth

VDR - vitamin D receptor

*Vdr* null - VDR knockout mice

VDRE - vitamin D responsive element

WT - wild type



# Chapter 1: Literature Review

## 1.1 General introduction

Over the past decade research has highlighted the importance of vitamin D<sub>3</sub> in human health, beyond that of bone. In particular, it has become apparent that there is a role for vitamin D<sub>3</sub> in fertility and pregnancy success. Initial studies demonstrated the role of vitamin D<sub>3</sub> in fertility after incidental findings of severely reduced fertility in vitamin D receptor knockout mice (*Vdr* null) were reported (Yoshizawa, Handa et al. 1997). Increasing numbers of studies have focused on associations between poor maternal vitamin D<sub>3</sub> status and a spectrum of pregnancy complications including preeclampsia (PE) (Bodnar, Catov et al. 2007), preterm birth (PTB) (Perez-Ferre, Torrejon et al. 2012), small for gestational age (SGA) babies (Leffelaar, Vrijkotte et al. 2010) and gestational diabetes mellitus (GDM) (Zhang, Qiu et al. 2008). Preterm birth affected an estimated 15 million pregnancies worldwide in 2010 (March of Dimes, 2012), with prematurity being the largest cause of newborn deaths worldwide (Save the Children, 2013). An estimated 800 women a day die in childbirth, with 3 million babies dying in their first month of life in 2011, most due to pregnancy complications (Save the Children, 2013). Combined, PE, PTB, SGA and GDM affect approximately 25% of first pregnancies with the aetiology of these pregnancy complications, as well as methods of prediction, still under investigation.

Vitamin D<sub>3</sub> deficiency (defined as serum 25-hydroxyvitamin D levels below 50 nmol/L, see 1.5.3 Vitamin D<sub>3</sub> deficiency) affects up to 60% of Australians (van der Mei, Ponsonby et al. 2007) dependent on such factors as the latitude of residence of the individual and the season in which testing is done. Vitamin D<sub>3</sub>

deficiency also affects approximately 50% of women of reproductive age (20-39 year olds) in winter (Pasco, Henry et al. 2001). This equates to a large number of women vulnerable to vitamin D<sub>3</sub> deficiency in pregnancy. As many pregnancy pathologies are also associated with poor placental development, there is an urgent need to understand how vitamin D<sub>3</sub> acts in pregnancy and its effects in the placenta.

## **1.2 Pregnancy**

During pregnancy, the placenta is the lifeline between the mother and fetus. Hence, a key factor in pregnancy success is development of a functional placenta. The placenta is a semi-permeable barrier responsible for transfer of nutrients and wastes between maternal and fetal circulations, as well as producing hormones and modifying the maternal immune response to the fetus (Gude, Roberts et al. 2004). Poor placental development results in a spectrum of complications from miscarriage to high blood pressure and preterm labour (Roberts 2010).

### **1.2.1 Determinants of pregnancy success**

A successful pregnancy is one resulting in a healthy, appropriately grown fetus delivered at term (37-40 weeks after the last menstrual period), without maternal or neonatal morbidity or mortality. This is determined by a range of factors, some of which will be subsequently discussed, for example embryonic implantation, maternal nutrition, physiological maternal adaptation to pregnancy, pregnancy hormone levels and development of a mature placenta.

#### **1.2.1.1 Embryonic implantation**

The embryo implants into the receptive uterus or occasionally other sites such as the fallopian tubes which results in ectopic pregnancies that require

termination to prevent catastrophic haemorrhage. The placenta forms from the trophoblast of the blastocyst plus extra-embryonic mesoderm and endoderm. The placental extravillous trophoblasts (EVTs) invade deeply into the endometrium and myometrium to sequester a blood supply for the mature placenta (Gude, Roberts et al. 2004). Deep invasion of placental EVT into the endometrium and myometrium ensures constant and adequate maternal blood flow to the placenta, allowing the fetus to successfully grow and extract nutrients from the mother (Pijnenborg, Vercruyssen et al. 2008).

### **1.2.1.2 Maternal adaptation to pregnancy**

Maternal physiology is altered from the onset of pregnancy under the influence of hormones from the ovaries and, subsequently, the placenta. In early pregnancy, haemodilution affects levels of serum constituents. Circulating albumin concentrations decrease from early gestation, leaving the free, ionized calcium fraction at a stable concentration, but decreasing total calcium levels, to maintain calcium homeostasis (Kovacs and Kronenberg 1997). There is also an associated increase in cardiac output of 50% and basal metabolic rate of 80 kcal/day. Insulin secretion is increased in early gestation by 60% and is followed by a rise in insulin resistance in mid pregnancy, with a reduction in insulin sensitivity of 45-70% (Newbern and Freemark 2011). These changes are attributed to alterations in pregnancy hormone levels, such as estrogen ( $E_2$ ), progesterone ( $P_4$ ), placental lactogen (hPL) and growth hormone variant (Sibai and Frangieh 1995).

### **1.2.1.3 Placental hormones**

One of the main roles of the placenta is to produce hormones to modify the fetal and maternal environments, such as human chorionic gonadotropin (hCG),

hPL, E<sub>2</sub> and P<sub>4</sub>. Human chorionic gonadotropin (hCG) is secreted by the syncytiotrophoblast. The main target of hCG in early pregnancy is the ovaries, initially maintaining the corpus luteum and stimulating maternal production of E<sub>2</sub> and P<sub>4</sub>. In the placenta, hCG induces cytotrophoblasts to fuse into the syncytiotrophoblast layer. The syncytiotrophoblast secretes hPL which increases insulin resistance in pregnancy, it may also play a role in nutrient homeostasis (Newbern and Freemark 2011). Progesterone production is stimulated by hCG and maintains uterine quiescence during pregnancy. It is initially produced by the ovaries, with placental production taking over from eight weeks' gestation (Malassine, Frenedo et al. 2003).

#### **1.2.1.4 Fetal nutrient supply**

Nutrient supply to the fetus is determined by maternal nutrition during pregnancy, maternal physiological adaptations to pregnancy and placental metabolism and transport of nutrients (Belkacemi, Nelson et al. 2010). Adequate maternal nutrition in pregnancy is essential for a healthy, appropriately grown baby, for example undernourished mothers deliver smaller babies (Barker 2001). Micronutrients such as vitamin D<sub>3</sub> and folate are important for fetal bone and neurological development, respectively (Botto, Moore et al. 1999). Maternal adaptations to pregnancy also influence fetal nutrient supply, for example the development of an insulin resistant state increasing maternal blood sugar levels (Newbern and Freemark 2011). Placental transfer of nutrients is dependent on the concentration of nutrients and the method of transfer; for example glucose is transported by facilitated diffusion along a concentration gradient, aided by the transport proteins GLUT1, 2 and 3, from the maternal circulation across the trophoblast layer to the fetal capillaries (Gude, Roberts et al. 2004).

Vitamin D metabolites have differing methods of placental transport as vitamin D<sub>3</sub> is undetectable in fetal plasma (Hollis and Pittard 1984), 25-hydroxyvitamin D<sub>3</sub> (25(OH)<sub>2</sub>D<sub>3</sub>) is found at similar or up to 20% higher concentrations in maternal compared to fetal plasma and probably crosses the placenta readily by diffusion (Kovacs 2008). Active vitamin D<sub>3</sub> 1 $\alpha$ ,25-dihydroxy vitamin D<sub>3</sub> 1,25(OH)<sub>2</sub>D<sub>3</sub> does not readily cross the placenta as maternal plasma concentrations are higher than fetal concentrations (Fleischman, Rosen et al. 1980, Wieland, Fischer et al. 1980, Hollis and Pittard 1984, Seki, Furuya et al. 1994), the fetus does not readily produce its own 1,25(OH)<sub>2</sub>D<sub>3</sub> as low PTH and high phosphorus inhibit this (Kovacs 2008). Ions such as calcium, potassium and phosphate are higher in the fetal circulation than maternal circulation. Calcium ions diffuse from maternal blood into trophoblasts, then are transported through the cytoplasm bound to calbindin-D<sub>9k</sub> then are actively transported into the fetal circulation by Ca-ATPase (PMCA) on the basement membrane. Calbindin levels increase across gestation and may be responsible for the increase in materno-fetal calcium transfer. Potassium is transferred by the Na<sup>+</sup>/K<sup>+</sup>/ATPase pump across into trophoblasts and then into the materno-fetal circulation. Whereas phosphate is transferred by a NaPi-2a transporter channels (Desforges and Sibley 2010).

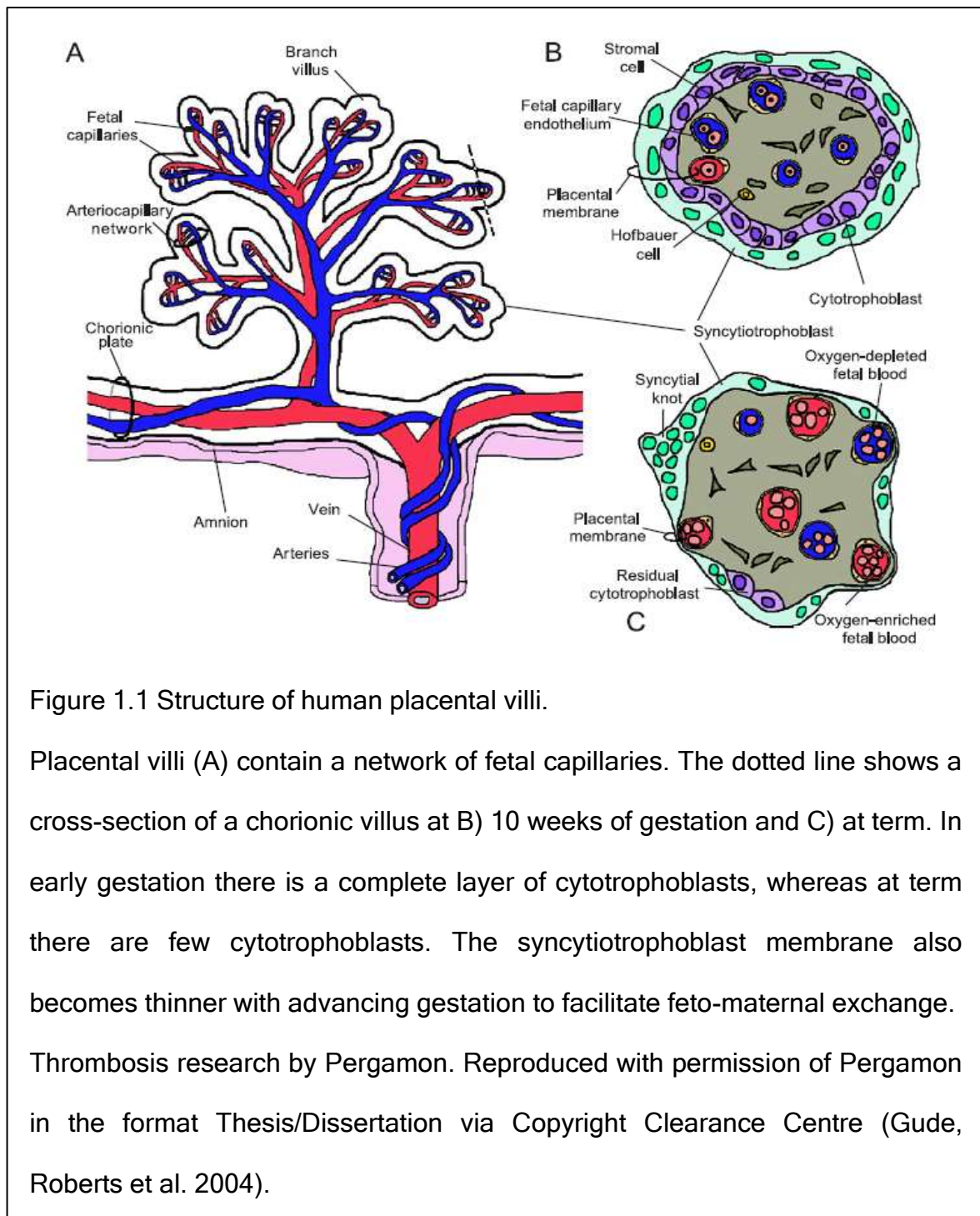
### **1.3 The human placenta**

The placenta is a complex, transient organ that forms the fetomaternal interface. It has many roles including providing nutrition and removing waste from the fetal circulation, provision of an active state of immune tolerance, as well as forming a physical barrier between maternal and fetal circulations and is an endocrine organ. The placenta has a complex structure composed of both maternal and fetal tissue. The placenta is derived from the conceptus shortly after

implantation and the mature organ develops rapidly over the first weeks of pregnancy. Poor placental development, including shallow trophoblast invasion early in pregnancy, results in placental insufficiency. This has been associated with a spectrum of pregnancy pathologies from miscarriage (Khong, Liddell et al. 1987) to PTB (Morgan, Tolosa et al. 2013), small for gestational age (SGA) babies and PE (Khong, De Wolf et al. 1986). All aspects of placental development, from blastocyst implantation to deep endometrial and myometrial invasion, are essential for development of a mature, functional placenta.

### **1.3.1 Placental cell types**

The major placental cells are the trophoblasts (cytotrophoblasts and syncytiotrophoblasts), the fetal endothelium and immune cells (Figure 1.1). Cytotrophoblasts are the major trophoblastic cell type in early pregnancy, arising from the trophoblast and giving rise to the other trophoblastic cell populations. The syncytiotrophoblast is a single multi-nuclear cell which envelops the implanting embryo and, in the mature placenta, provides the interface between the maternal intervillous space and placental villi (Gude, Roberts et al. 2004). The syncytium is maintained and increases in size through fusion of cytotrophoblasts, with removal of nuclear and cellular debris by formation of syncytial buds (Huppertz 2010). The syncytiotrophoblast becomes thinner with advancing gestation decreasing the diffusion distance between the maternal and fetal circulations to as little as 2-4  $\mu\text{m}$  in some parts after 20wks' gestation. Extravillous cytotrophoblasts (EVTs) are those which invade both endovascularly and interstitially into the maternal endometrium and myometrium, recruiting and transforming the maternal spiral arterioles to provide the maternal blood supply to the placenta (Pijnenborg, Vercruyssen et al. 2006) (see Figure 1.2).



### 1.3.2 The intervillous space and spiral arteries

The intervillous space is initially formed when EVT's invade into uterine capillaries, leaving small maternal blood spaces in the syncytiotrophoblast, called lacunae. These form between villi and later merge with uterine spiral arteries, forming the intervillous space of the placenta (Jauniaux, Watson et al. 2000).

During early placental development, invasive EVT's invade through the maternal endometrium (decidua) and up to a third of the way through the myometrium (Burton, Woods et al. 2009), encountering the maternal spiral arteries which are recruited to supply the placenta. EVT's invade the arteries both

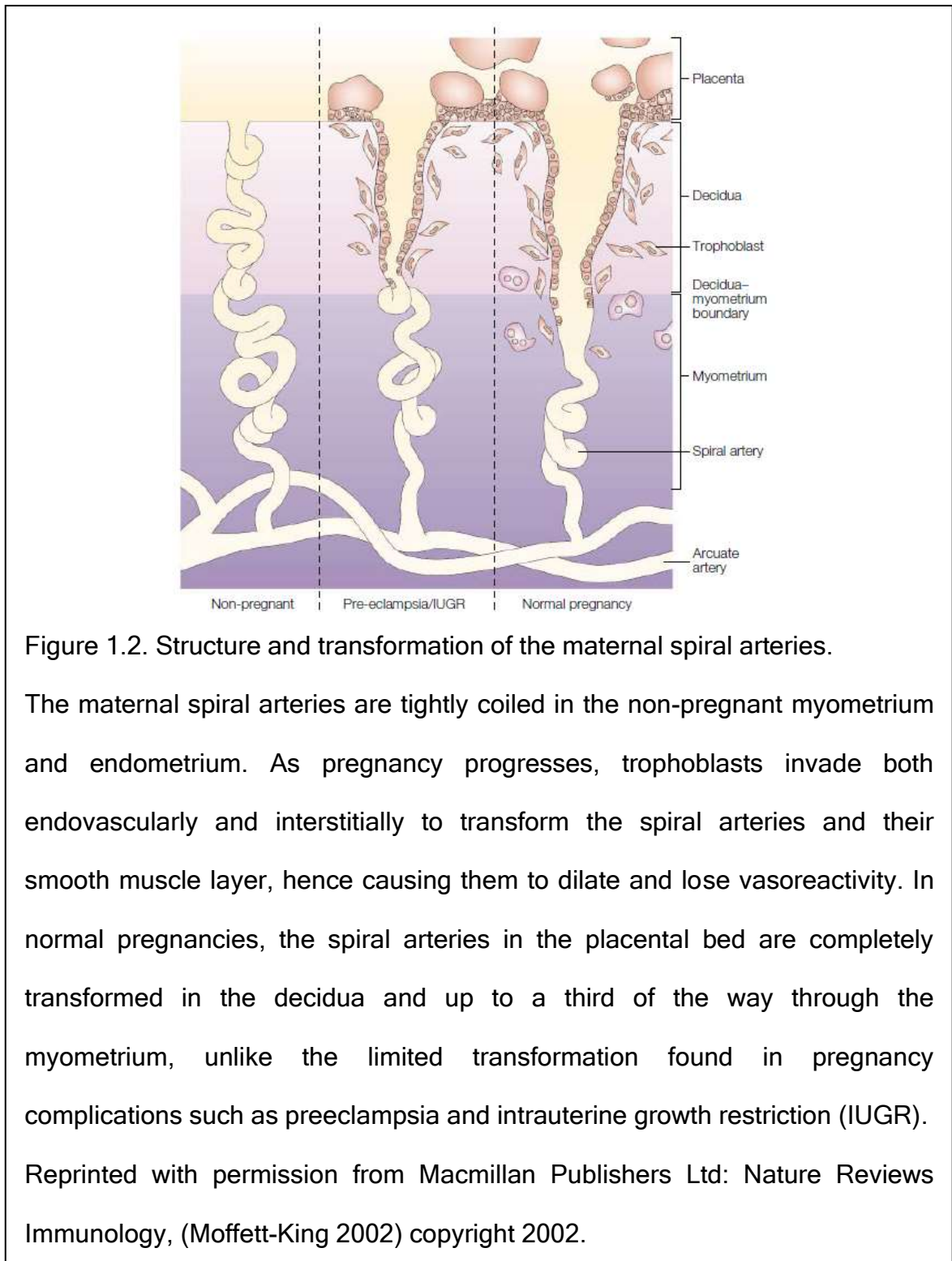


Figure 1.2. Structure and transformation of the maternal spiral arteries.

The maternal spiral arteries are tightly coiled in the non-pregnant myometrium and endometrium. As pregnancy progresses, trophoblasts invade both endovascularly and interstitially to transform the spiral arteries and their smooth muscle layer, hence causing them to dilate and lose vasoreactivity. In normal pregnancies, the spiral arteries in the placental bed are completely transformed in the decidua and up to a third of the way through the myometrium, unlike the limited transformation found in pregnancy complications such as preeclampsia and intrauterine growth restriction (IUGR).

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interstitially and endovascularly and transform them by replacing smooth muscle and endothelial cells which causes maternal arteries to lose vasoreactivity. This results in dilated vessels which slow the flow rate of blood entering the placenta (Burton, Woods et al. 2009).

Placental development occurs initially in a physiologically low oxygen environment due to the early occlusion of the maternal spiral arteries by EVT's. During this time the placental villi are not exposed to maternal blood but may be bathed in plasma filtrate (Hustin and Schaaps 1987) or uterine gland secretions (Burton, Jauniaux et al. 1999). Placental oxygen tension in the intervillous space rises from <20 mmHg at 8 weeks' gestation to 50 mmHg at 12 weeks' gestation with a corresponding increase in placental expression of antioxidant enzymes (Jauniaux, Watson et al. 2000). Ultrasound observations and histological investigations agree that maternal blood flow is established by 11-12 weeks' gestation (Hustin and Schaaps 1987, Burton, Jauniaux et al. 1999, Jauniaux, Greenwold et al. 2003).

Impaired placental development leads to pregnancy complications; these include shallow invasion of spiral arteries, premature initiation of placental blood flow and poorly transformed spiral arteries (Figure 1.2). These modifications are important to ensure an uninterrupted, low velocity blood flow to the placenta as perturbed blood flow can cause ischemia-reperfusion injury to the placenta, damaging the placental villi and hence, reducing the opportunity for fetomaternal exchange (Burton and Jauniaux 2004).

## **1.4 Complications of pregnancy**

In Australia approximately 25% of pregnancies are affected by a major complication such as PTB (8.2%), fetal growth restriction (for example 6.1% low

birth weight, LBW), pregnancy-induced hypertension (including PE, 5%) and GDM (5%) (Li, Hilder et al. 2013). Some women will have more than one complication in the same pregnancy. Currently we have no way to predict which women will develop these complications in their first pregnancy, other than family history. Insights into how these complications manifest and how they can be treated will improve obstetric care.

### **1.4.1 Preeclampsia**

Preeclampsia is directly associated with 2-8% of annual worldwide maternal deaths (Duley 2009). In the mother, PE can have various degrees of severity and is associated with hypertension, organ failure and higher risk of cardiovascular disease later in life. Fetuses are at risk of iatrogenic premature delivery, fetal growth restriction, stillbirth and oligohydramnios (Hawfield and Freedman 2009). Currently there are no effective screening tools to predict PE, treatments are based on alleviation of symptoms and the only cure is to deliver the placenta.

In Australia, PE is defined as high blood pressure (above 140/90 mmHg) after 20 weeks' gestation and resolving by 3 months postpartum, with at least one multisystem complication of proteinuria, renal insufficiency, neurological symptoms, liver disease, haematological disturbances or fetal growth restriction (Tranquilli, Dekker et al. 2014). Severe PE can occur late in pregnancy or early in pregnancy (early onset severe PE (EOSPE); <34 weeks' gestation). It is characterized by very high blood pressure (above 160/110) with proteinuria and/or other multi-system complications, including seizures (classified as eclampsia), visual disturbances, renal dysfunction and haematological complications (Tranquilli, Brown et al. 2013).

The pathogenesis of PE involves reduced placental blood flow, due to poorly transformed spiral arteries, which in turn causes placental ischemia and infarctions. The necrotic tissue then releases factors into the maternal blood that, cause placental endoplasmic reticulum stress, oxidative stress and inflammation (Chaiworapongsa, Chaemsaitong et al. 2014). Hence, PE has been associated with poor trophoblast invasion (Khong, De Wolf et al. 1986), immune maladaptation (Dekker and Robillard 2007), genetic and other factors and is considered a multifactorial disease. Women with PE are at increased risk of chronic disease later in life, for example cardiovascular disease (Williams 2011).

#### **1.4.2 Preterm birth**

PTB is defined as birth before 37 completed weeks of gestation, affecting 9.6% of pregnancies worldwide (Beck, Wojdyla et al. 2010) with over 1 million children dying annually due to PTB and its complications (WHO, 2012). Preterm birth can be further divided into iatrogenic (30% of PTBs) and spontaneous PTB (sPTB, 70% of PTBs) (Romero, Dey et al. 2014). Iatrogenic (indicated) PTBs are usually due to complications such as placenta previa, gestational hypertension, growth restriction (twins and singletons) and PE, where the baby is delivered preterm through induction of labour or caesarean section (ACOG, 2013). Conversely, sPTB occurs following the onset of preterm labour before 37 weeks' gestation, it can be precipitated by a range of factors such as uterine over distension (in multi-fetal pregnancies), poor placentation (Morgan, Tolosa et al. 2013) or infection and inflammation (Keelan, Blumenstein et al. 2003). The latter causes are associated with the majority of early sPTB (<34 weeks gestation), with the associated inflammation being responsible for triggering the onset of labour (Romero, Espinoza et al. 2007). Prematurity confers many risks to the baby, such

as respiratory distress, disability, poorer health outcomes during childhood and adulthood including obesity, diabetes mellitus, and heart disease. Mothers of preterm infants also have an increased risk of stroke and cardiovascular disease later in life (Irgens, Reisaeter et al. 2001) as well as an increased risk of subsequent preterm birth (Edlow, Srinivas et al. 2007).

### **1.4.3 Small for gestational age**

Fetal growth restriction (FGR) during pregnancy affects approximately 10% of births depending on the definitions used, which makes comparisons between studies difficult. Although fetal sex, gestation and ethnicity are known to affect birth weight, many clinical settings use low birth weight (LBW, <2500g) as their definition of growth restriction. Serial ultrasound measurements are used to identify FGR which is a more accurate, but time consuming measure. By examining the baby's weight as compared to others for its gestational age while accounting for sex the lightest 10% of babies are considered SGA (Lausman, Gagnon et al. 2013).

This can be further refined by using customized centiles, which account for maternal ethnicity, height and weight, as well as the baby's gestation, birth weight and gender. Customised centiles are more accurate in identifying babies that are truly SGA, and not just small due to ethnicity or other constitutional factors (Figueras and Gardosi 2009). SGA is associated with poor trophoblast invasion (Khong, De Wolf et al. 1986) and results in increased risk of intrauterine death and other complications such metabolic disorders in adulthood (Godfrey and Barker 2001).

#### **1.4.4 Gestational diabetes mellitus**

GDM is diabetes diagnosed in pregnancy in women who did not previously have diabetes (Schneiderman 2010). Diagnosis of GDM in Australia is a fasting plasma glucose of  $\geq 5.5$  mmol/L or a 2 hour plasma glucose  $\geq 8.0$  mmol/L (after a glucose load of 75 g) (Hoffman, Nolan et al. 1998). Recently new guidelines have been released defining GDM (after a 75 g oral glucose tolerance test) as fasting plasma glucose levels of 5.1-6.9 mmol/L,  $\geq 10$  mmol/L at 1 hour and 8.5-11 mmol/L at 2 hours. Diabetes mellitus in pregnancy is diagnosed as of  $\geq 7$  mmol/L when fasting and  $\geq 11.1$  at 2 hours (WHO 2013, RANZCOG 2014). Maternal adaptations in normal pregnancy cause insulin resistance but in GDM normal beta cell function is impaired and glucose homeostasis cannot be maintained (Schneiderman 2010).

Maternal and fetal morbidities are associated with GDM. Fetal complications include macrosomia, which can result in shoulder dystocia, and hypoglycaemia and intrauterine demise (Schneiderman 2010). Maternal risks include injury during delivery or increased risk of Caesarean section due to macrosomia (large babies) resulting from increased blood sugar levels. Mothers also have an increased risk of developing type 2 diabetes later in life compared to those without GDM (Damm, Kuhl et al. 1992). Later in life there is an increased risk of metabolic and cardiovascular diseases for the child, such as obesity and type 2 diabetes (Catalano, Kirwan et al. 2003), as has also been found in various animal models (Nathanielsz, Poston et al. 2007).

### **1.5 Vitamin D<sub>3</sub>**

Vitamin D<sub>3</sub> is a secosteroid hormone that is predominantly produced through sun exposure, with cholesterol in the skin converted to vitamin D<sub>3</sub> through the action of UV-B radiation. Vitamin D was first investigated in 1919 when a trace

compound in cod liver oil was known to prevent the bone disease known as rickets (Mellanby 1919), it was only in the 1930's that the active forms of plant and human derived vitamin D<sub>3</sub> were characterised (Windaus, Linsert et al. 1932, Brockman 1936). Since this time, vitamin D<sub>3</sub> structure, synthesis and action have been well characterised; vitamin D<sub>3</sub> regulates calcium and phosphate homeostasis and prevents the bone diseases rickets and osteoporosis. More recently, vitamin D<sub>3</sub> has been suggested to function in a wide range of tissues and its deficiency has been associated with many diseases including pregnancy pathologies.

### 1.5.1 Vitamin D<sub>3</sub> metabolism

Although there are some dietary sources, vitamin D<sub>3</sub> is predominantly produced in the skin (Figure 1.3). Pre-vitamin D<sub>3</sub> is produced from 7-dehydrocholesterol (a cholesterol precursor produced by keratinocytes (Glossmann 2010)) through the action of UV-B on the skin, which then undergoes a structural change, catalysed by body heat, to form vitamin D<sub>3</sub>. Vitamin D<sub>3</sub>, bound to the vitamin D binding protein (VDBP) enters the circulation and is hydroxylated in the liver to form 25(OH)D<sub>3</sub>, catalysed by CYP2R1 (Cheng, Levine et al. 2004). The 25(OH)D<sub>3</sub> metabolite is the major circulating form of vitamin D<sub>3</sub>, is used as a marker of vitamin D status and has a half-life of fifteen days (Jones 2008). When required, 25(OH)D<sub>3</sub> is then further hydroxylated in the kidney, catalysed by 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase (CYP27B1), producing the active circulating form of vitamin D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) with a half-life of a few hours.

CYP27B1 activity was thought to be restricted to the kidney, however expression and activity of CYP27B1 has been demonstrated in many cell types (Anderson, Hendrix et al. 2008), including placental trophoblasts (Diaz, Sanchez et

al. 2000), suggesting actions in a wide variety of tissues for  $1,25(\text{OH})_2\text{D}_3$ . The regulation of  $1,25(\text{OH})_2\text{D}_3$  activity is, in part, through catabolism by 24-hydroxylase (CYP24A1, encoded by *CYP24A1*) to form inactive 1,24,25-dihydroxyvitamin  $\text{D}_3$  which is the first step in formation of calcitroic acid which is excreted in the urine. *CYP24A1* is expressed in all vitamin D responsive tissues, including the placenta (Avila, Diaz et al. 2004). Further metabolism and excretion of vitamin  $\text{D}_3$  products involves side-chain hydroxylation of  $1,25(\text{OH})_2\text{D}_3$  by various liver cytochrome P450 enzymes (CYPs), metabolites are primarily excreted through the bile into faeces

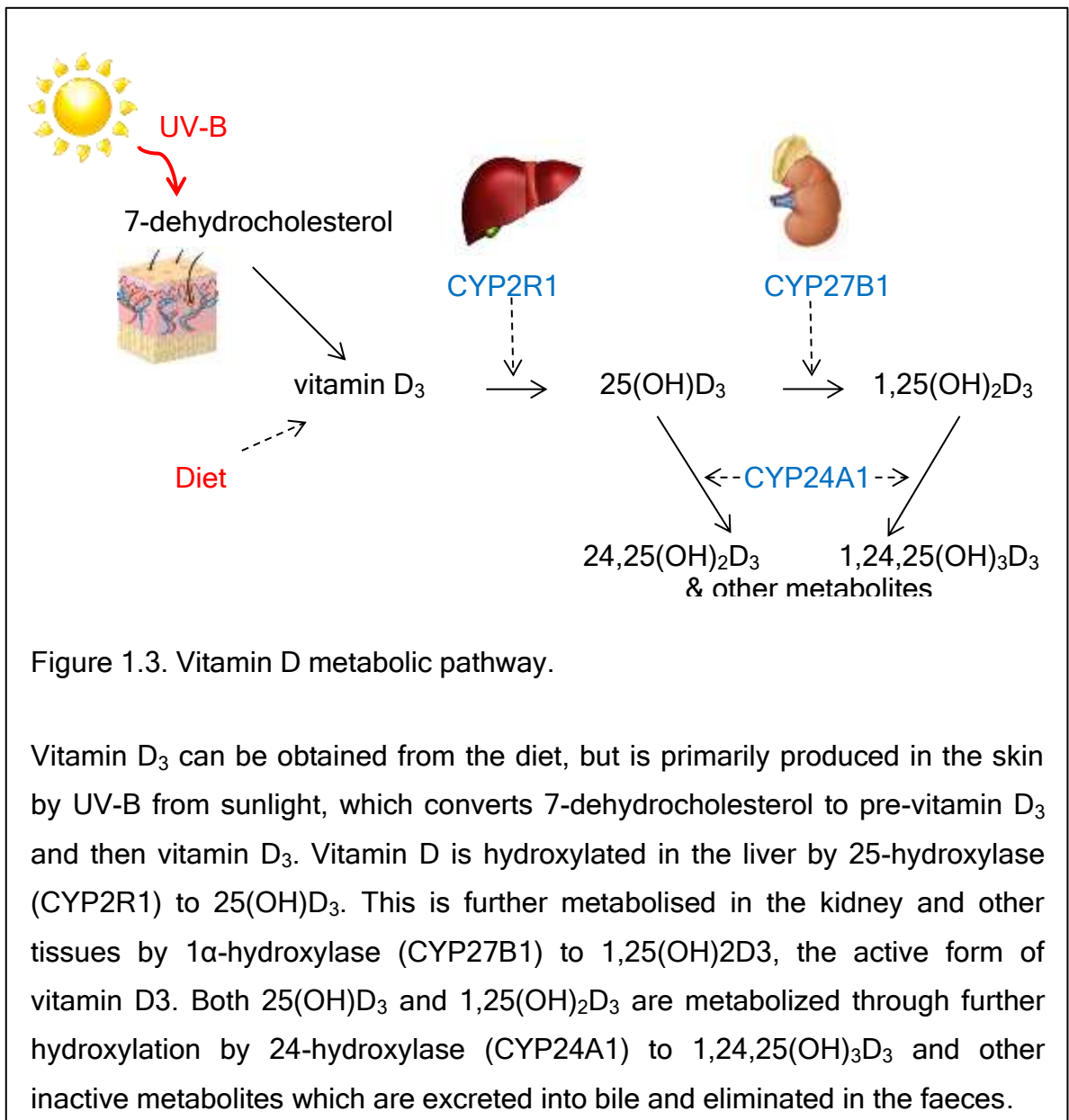


Figure 1.3. Vitamin D metabolic pathway.

Vitamin  $\text{D}_3$  can be obtained from the diet, but is primarily produced in the skin by UV-B from sunlight, which converts 7-dehydrocholesterol to pre-vitamin  $\text{D}_3$  and then vitamin  $\text{D}_3$ . Vitamin D is hydroxylated in the liver by 25-hydroxylase (CYP2R1) to  $25(\text{OH})\text{D}_3$ . This is further metabolised in the kidney and other tissues by  $1\alpha$ -hydroxylase (CYP27B1) to  $1,25(\text{OH})_2\text{D}_3$ , the active form of vitamin  $\text{D}_3$ . Both  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  are metabolized through further hydroxylation by 24-hydroxylase (CYP24A1) to  $1,24,25(\text{OH})_3\text{D}_3$  and other inactive metabolites which are excreted into bile and eliminated in the faeces.

rather than in the urine as DBP-bound metabolites are recovered by the kidney (IOM, 2011).

### 1.5.2 Vitamin D<sub>3</sub> action

Active vitamin D<sub>3</sub> has wide-ranging effects. 1,25(OH)<sub>2</sub>D<sub>3</sub> acts mainly through the vitamin D receptor (VDR, encoded by *VDR*) to alter expression of genes such as vitamin D pathway genes (*CYP24A1*, *VDR*). VDR is a nuclear transcription factor which forms a heterodimer with retinoid X receptor (RXR) and other transcription factors. This complex binds to vitamin D responsive elements (VDREs), with or without 1,25(OH)<sub>2</sub>D<sub>3</sub> binding, in responsive genes to influence their transcription. A study using CHIP analysis showed 913 genes had VDREs in human SCC25 cells (squamous carcinoma) (Wang, Tavera-Mendoza et al. 2005). VDREs have been shown to be located within the promoter regions of genes as well kilobases upstream or downstream of coding genes, suggesting a complex role for VDR-mediated gene transcription (Pike and Meyer 2014).

### 1.5.3 Vitamin D<sub>3</sub> deficiency

Measurement of 25(OH)D<sub>3</sub> is used as a surrogate marker of vitamin D<sub>3</sub> status as its levels are relatively stable due to its long half-life. In comparison, 1,25(OH)<sub>2</sub>D<sub>3</sub> has a short half-life, and with stimulation of production by various hormones such as parathyroid hormone (PTH) to correct calcium homeostasis. Risk of 25(OH)D<sub>3</sub> deficiency is associated with reduced exposure to sunlight (Morris, Morrison et al. 1984) and other factors such as dark pigmented skin (Grover and Morley 2001, Bodnar, Simhan et al. 2007), obesity (Need, Morris et al. 1993), living at a high latitude (Webb, Kline et al. 1988), thin skin in the elderly (Need, Morris et al. 1993), extensive use of sunscreen (Faurischou, Beyer et al.



2012) and low vitamin D<sub>3</sub> consumption (Nowson and Margerison 2002). Supplementation of foods with vitamin D<sub>3</sub> is practiced in many countries, with Australia mandated to include 55 µg/kg in edible oil spreads such as margarine since the year 2000 (Food Standards Australia New Zealand, 2011). However, other countries (for example the United States of America) have more prevalent food fortification, with cereals and milk mandated to include vitamin D<sub>3</sub> (Calvo, Whiting et al. 2004).

#### 1.5.4 Definition of vitamin D<sub>3</sub> deficiency

There has been much discussion in the past decade concerning both the required intake levels of vitamin D<sub>3</sub> to achieve sufficient Table 1.1 circulating concentrations and also to define vitamin D<sub>3</sub> sufficiency itself. The recommended daily intake has increased in the last decade as more is discovered about vitamin D<sub>3</sub> and rates of deficiency. In 2011, the recommended daily intake (RDI) was increased by the Institute of Medicine (IOM) from 400 IU/day to 600 IU/day in the USA (Table 1.1) (IOM, 2011).

Vitamin D<sub>3</sub> sufficiency is currently considered as a serum concentration of

Table 1.1. Current recommended vitamin D and calcium intakes for various age groups.

<b>Age Group</b>	<b>Vitamin D RDI <sup>1</sup></b>	<b>Calcium RDI <sup>1</sup></b>
0-12 months	400 IU <sup>2</sup>	200-260 mg
1-18 years	600 IU	700 mg (1-3 years) 1000 mg (4-8 years) 1300 mg (9-18 years)
19-50 years	600 IU	1000 mg
51-71 years	600 IU	1000 mg
≥ 71 years	800 IU	1200 mg
Pregnant & lactating women	600 IU	1300 mg (14-18 years) 1000 mg (19-50 years)

Note: (modified from (Ross, Manson et al. 2011)); <sup>1</sup> RDI - recommended daily intake (per day); <sup>2</sup> Based on EAR (estimated adequate requirements) as limited data

$\geq 50$  nmol/L of 25(OH)D<sub>3</sub> by the Australian National Health and Medical Research Council (NHMRC), which is based on bone health to prevent rickets and osteomalacia as other diseases lacked evidence on the level of vitamin D required to prevent them (Ross, Manson et al. 2011). Although the NHMRC has not updated their 2005 guidelines (2006), there was a position statement released by a working group representing the Australian and New Zealand Bone and Mineral Society, the Endocrine Society of Australia and Osteoporosis Australia in 2012 consistent with the IOM recommendations (Paxton, Teale et al. 2013). During this period, researchers have suggested various cut-offs for vitamin D sufficiency based on their own studies, while some suggest that vitamin D<sub>3</sub> sufficiency should be based on prevention of bone disease alone (Morris 2005) (Table 1.2), as optimum 25(OH)D<sub>3</sub> concentrations to prevent other diseases are still undefined. Additionally, the South Australian Child Health Clinical Network reported that they consider deficiency as  $< 60$  nmol/L (SA CHCN, 2013). 25(OH)D<sub>3</sub> definitions for deficiency and insufficiency vary greatly between studies (Table 1.4, Table 1.5, Table 1.6, Table 1.7), with deficiency ranging from a serum concentration of  $< 12.5$  nmol/L (NHMRC, 2006) to  $< 80$  nmol/L of 25(OH)D<sub>3</sub> (Bodnar, Simhan et al. 2007) depending on the study, making interpretation of the prevalence of vitamin D<sub>3</sub> deficiency difficult.

### **1.5.5 Epidemiology of vitamin D<sub>3</sub> deficiency**

An estimated 1 billion people worldwide are vitamin D<sub>3</sub> deficient (Holick 2011). Van der Mei *et al.* reported vitamin D<sub>3</sub> status in three Australian regions in winter and spring and found vitamin D<sub>3</sub> deficiency (25(OH)D<sub>3</sub>  $< 50$  nmol/L) affected a significant proportion of the population: 40.5% in south east Queensland, 37.4% in Geelong and 67.3% in Tasmania (van der Mei, Ponsonby et al. 2007). The

Table 1.2. Recommended definitions of vitamin D deficiency based on bone health.

Vitamin D status	Serum 25(OH)D <sub>3</sub> (nmol/L)
Severely deficient	< 12.5
Moderately deficient	12.5-29
Mildly deficient	30-49
Sufficient	≥ 50
Elevated	> 250
Toxic	> 500

Note: modified from (Morris 2005) and (Paxton, Teale et al. 2013)

1996-7 National Adults Nutrition Study in New Zealand found that 30.4% of New Zealanders were vitamin D<sub>3</sub> deficient (<37.5 nmol/L) (unpublished, reviewed in 2006). In comparison, the American NHANES 2000-2004 survey found 20-49 year olds had an adjusted mean serum 25(OH)D<sub>3</sub> of 62 nmol/L with only 5% below 25 nmol/L of 25(OH)D<sub>3</sub> (Looker, Pfeiffer et al. 2008). At a population level, differences in proportion of deficiency may be due to latitude, sun exposure, ethnic, dietary and supplementation differences between populations. Interestingly, the Geelong Osteoporosis Study found that 19.8% of women of reproductive age (20-39 years old) had frank deficiency (<20 nmol/L) and 43% had marginal deficiency (25(OH)D<sub>3</sub> 25-50 nmol/L) at the end of winter (Pasco, Henry et al. 2001). This clearly demonstrates that many people, including women of reproductive age are vitamin D<sub>3</sub> deficient according to current definitions.

## 1.6 Calcium

Classically, the role of vitamin D<sub>3</sub> has been seen as the maintenance of calcium homeostasis through regulation of various calciotropic pathways in order to maintain bone health. As vitamin D<sub>3</sub> deficiency is associated with an increasing number of diseases, both during and independent of pregnancy, further roles for calcium and calciotropic pathways are emerging.

### 1.6.1 Calcium homeostasis

Serum calcium concentration is strictly maintained at 2.25-2.5 nmol/L (Guyton and Hall 1996) through interplay of calciotropic hormones and trafficking of calcium between compartments, such as the uptake from intestines and bone (Figure 1.3). The calcium sensing receptor (CaSR) in the parathyroid glands monitors calcium levels and when levels drop parathyroid hormone (PTH) is released from the parathyroid glands (Favus, Bushinsky et al. 2006). PTH then acts on the kidney to increase renal calcium reabsorption and increase  $1,25(\text{OH})_2\text{D}_3$  production through stimulation of *CYP27B1* gene expression.  $1,25(\text{OH})_2\text{D}_3$  then increases calcium by increasing intestinal expression of calcium binding proteins (CaBP; calbindin-D9k and calbindin-28), possibly through VDREs which increase intestinal calcium absorption (Favus, Bushinsky et al. 2006, David and Quarles 2011). Both  $1,25(\text{OH})_2\text{D}_3$  and PTH also act on bone directly to release calcium into the circulation. Alternatively, low serum phosphate levels induce release of fibroblast growth factor 23 (FGF23) from bone. This is triggered by the release of calcium phosphate from bone, where phosphate is then passively excreted by the kidney. FGF23 then acts to reduce PTH secretion, hence reducing circulating  $1,25(\text{OH})_2\text{D}_3$  and calcium concentrations. Other calciotropic hormones are also involved such as calcitonin, which oppose the actions of PTH, and PTH related protein (PTHrP) which regulates placental calcium transfer and is under control of CaSR (Wysolmerski 2012).

Vitamin D<sub>3</sub> also regulates many of the genes involved in calcium homeostasis. Through VDREs,  $1,25(\text{OH})_2\text{D}_3$  regulates the expression of CaBP in enterocytes (*S100G*), the FGF23/Klotho receptor gene (*FGFBP*) and PTHrP (*PTHrP*) (Wang, Tavera-Mendoza et al. 2005) as well as inhibiting calcitonin expression (*CALCA*) through a negative VDRE, which reduces expression through

similar mechanisms (Peleg, Abruzzese et al. 1993). In bone, osteoclastic bone resorption is stimulated through upregulation of RANKL expression and down regulation of OPG expression and direct suppression of osteoblast activity (Ross 2011).

### **1.6.2 Calcium deficiency and excess**

The recommended dietary calcium levels range between 200 mg and 1300 mg per day dependant on age and pregnancy status (Table 1.2). Calcium deficiency cannot be detected by measuring serum calcium concentration as this can only reflect extreme deficiencies as levels are tightly regulated. Prolonged low calcium intake can result in osteomalacia (bone softening due to poor mineralization) and lead to osteoporosis and fractures. Symptoms of calcium deficiency include numbness and tingling of fingers, convulsions and abnormal heart rhythms. Excessive calcium intake can result in constipation, impaired zinc and iron absorption, kidney stones and cardiovascular disease (Emkey and Emkey 2012).

### **1.6.3 Calcium homeostasis in pregnancy**

Calcium regulation is dramatically altered in pregnancy (Figure 1.3). By the end of first trimester, intestinal calcium absorption has doubled compared to the non-pregnant state (Kovacs 2009). Although intestinal calcium absorption increases there is also increased bone turn-over in late compared to early pregnancy as indicated by markers of bone resorption such as urinary deoxypyridinoline (DPD) excretion (Kovacs and Kronenberg 1997). Serum total calcium levels decrease in pregnancy, although the active fraction of calcium, ionised calcium, remains comparable to that in the non-pregnant state, due to haemodilution and decreased serum albumin (35% of total calcium is bound to albumin) (Guyton and Hall 1996).

It is interesting that calcium metabolism is so drastically altered at the onset of pregnancy, as it is not until the third trimester that fetal calcium demand is high. It may be that the maternal skeleton is being fortified in preparation for the high calcium demands of the fetus later in pregnancy (Kovacs 2008). This is supported by the low levels of PTH and high levels of the calciotropic hormones, calcitonin and PTHrP, which increase dramatically during early pregnancy (Ardawi, Nasrat et al. 1997), protecting the skeleton from calcium resorption and increasing calcium reabsorption in kidney and intestines, respectively (Figure 1.4).

## **1.7 Vitamin D<sub>3</sub> in pregnancy**

Vitamin D<sub>3</sub> in pregnancy has regained the interest of health professionals due to a resurgence of rickets around the globe (Elder and Bishop 2014). Vitamin D<sub>3</sub> deficiency affects many women worldwide and has been associated with pregnancy complications such as PE and PTB.

### **1.7.1 Vitamin D<sub>3</sub> metabolism in pregnancy**

Vitamin D<sub>3</sub> metabolism is altered in pregnancy (Figure 1.5). Although there is only a minor decrease in serum 25(OH)D<sub>3</sub> concentration across gestation, there is a marked increase in circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels with concentration doubling in early pregnancy rising to 300-700 pmol/L in healthy pregnant women near term (Ardawi, Nasrat et al. 1997, Hollis and Wagner 2013). Although renal production of circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> is usually under control of PTH this may not be the case in pregnancy as PTH levels decrease to half the pre-pregnancy values and remain at low-normal levels throughout pregnancy, which is dependent on race and diet (Kovacs 2009). Some non-calciotropic hormones also mirror the rise of 1,25(OH)<sub>2</sub>D<sub>3</sub> across gestation such as prolactin and hPL (Ardawi, Nasrat et al.

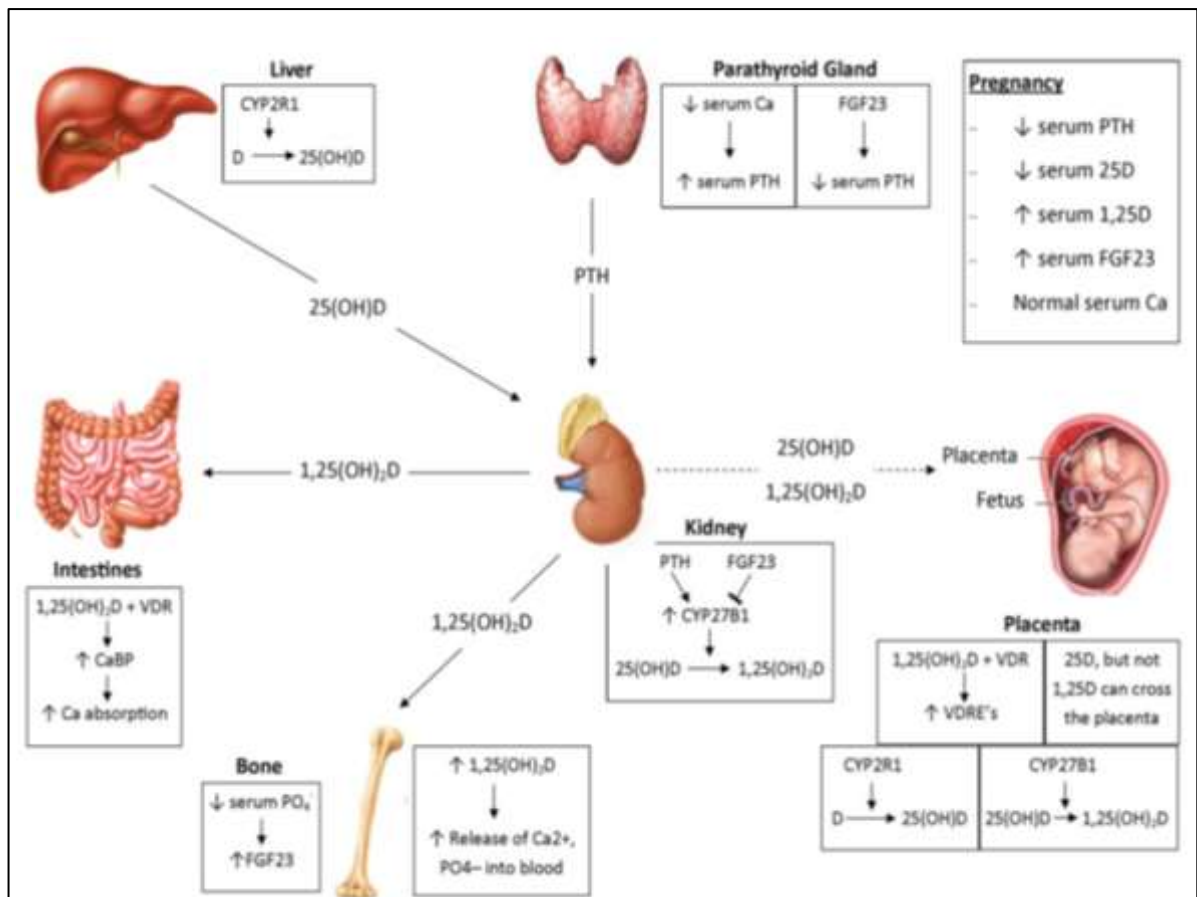


Figure 1.4. Calcium homeostasis and vitamin D metabolism in pregnancy.

Circulating vitamin D is activated by CYP2R1 in the liver, then metabolised by renal CYP27B1 to the active 1,25(OH)<sub>2</sub>D<sub>3</sub>. The parathyroid gland releases PTH into the circulation in response to low serum calcium concentrations (Ca) and stimulates renal CYP27B1 expression. 1,25(OH)<sub>2</sub>D<sub>3</sub> acts in the intestines, increasing levels of calcium binding protein (CaBP) to increase calcium absorption, and in bones where it releases Ca and phosphate (PO<sub>4</sub><sup>-</sup>) from osteoclasts. Bones release fibroblast growth factor 23 (FGF23) in response to low serum PO<sub>4</sub><sup>-</sup>, negatively regulating PTH and CYP27B1 protein expression. In pregnancy, vitamin D metabolism is altered. The placenta converts vitamin D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub> and alters expression through vitamin D responsive elements (VDREs) in genes. Only 25(OH)D<sub>3</sub>, not 1,25(OH)<sub>2</sub>D<sub>3</sub> is able to cross the placenta to the fetal circulation.

1997). As 1,25(OH)<sub>2</sub>D<sub>3</sub> regulates placental hPL production (Stephanou, Ross et al.

1994) and both prolactin and hPL genes, PRL and CSH1, have VDREs

(Stephanou and Handwerger 1995, Castillo, Jimenez-Lara et al. 1999). Serum levels of these hormones may be regulated by  $1,25(\text{OH})_2\text{D}_3$ .

The insulin-like growth factors play a major role in placental development and maternal adaptation to pregnancy (Roberts, Owens et al. 2008). IGF levels are altered across gestation (Figure 1.5), with circulating IGF1 initially decreasing within the normal range then increasing in third trimester (Moller, Streym et al. 2013), while IGF2 falls to half its non-pregnant levels at 7-8 weeks of gestation, then rises to 150% of non-pregnant levels and is maintained across gestation, falling to non-pregnant levels during lactation (Gargosky, Moyse et al. 1990). IGF1 has been found to stimulate  $1,25(\text{OH})_2\text{D}_3$  production, although it appears unlikely that IGF1 is responsible for the increased  $1,25(\text{OH})_2\text{D}_3$  in pregnancy as IGF1 levels increase later in pregnancy (Moller, Streym et al. 2013).

Vitamin  $\text{D}_3$  supply to the fetus is also important and occurs by passive diffusion of  $25(\text{OH})\text{D}_3$  across the placenta (Kovacs 2008).  $1,25(\text{OH})_2\text{D}_3$  cannot cross the placenta into the fetal circulation (Salle, Delvin et al. 2000). As this is the only source of fetal vitamin  $\text{D}_3$ , maternal vitamin  $\text{D}_3$  deficiency causes rickets (Elder and Bishop 2014) and is associated with increased rates of allergy in children (Litonjua 2012).

### **1.7.2 Vitamin $\text{D}_3$ deficiency in pregnancy**

In Adelaide, South Australia, 56.8% of women screened in early pregnancy were vitamin  $\text{D}_3$  deficient (<60 nmol/L). Of the women in this study who were deemed to be at low risk of deficiency (not veiled, dark skinned or housebound), nearly half (46.2%) were deficient (De Laine, Matthews et al. 2013). A high prevalence of deficiency was also found in pregnant women in the UK, with 96% deficient (<50 nmol/L) at 12 and 20 weeks of gestation (Holmes, Barnes et al.



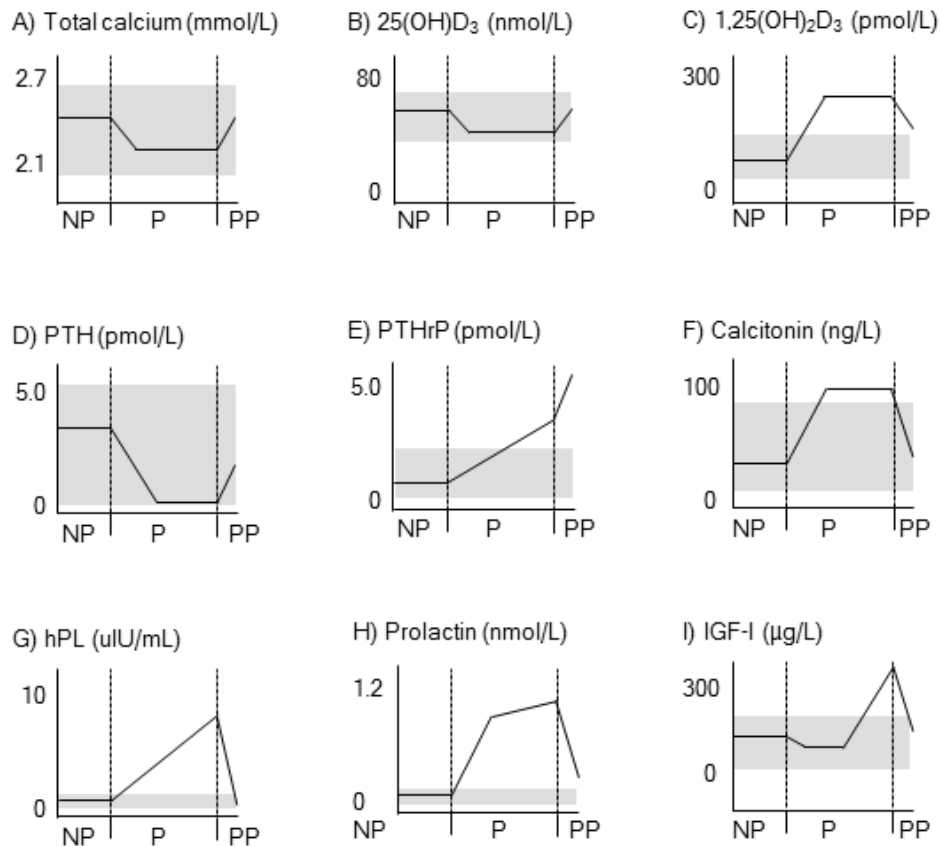


Figure 1.5. Schematic of vitamin D, calcium and hormone levels across pregnancy.

Serum levels in non-pregnant (NP), pregnant (P) and postpartum (PP) women of: A) total calcium, B) 25(OH)D<sub>3</sub>, C) 1,25(OH)<sub>2</sub>D<sub>3</sub>, D) PTH, E) PTHrP, F) calcitonin, G) hPL, H) prolactin and I) IGF1. Grey bars represent the normal range in the non-pregnant general population; adapted from (Ardawi, Nasrat et al. 1997, Kovacs 2009, Moller, Streym et al. 2013).

2009). Similarly, pregnant American women have high rates of vitamin D<sub>3</sub> deficiency, with 29.2% of non-Hispanic black women being deficient (<37.5 nmol/L) and 54.1% insufficient (37.5-80 nmol/L) (Bodnar, Simhan et al. 2007). As vitamin D<sub>3</sub> deficiency is so common in pregnant women and is associated with pregnancy complications (see below), it is clear that there is a need for better

definitions of vitamin D deficiency in pregnancy and a greater understanding of the role of vitamin D<sub>3</sub> in pregnancy.

### 1.7.3 Vitamin D<sub>3</sub> supplementation in pregnancy

A number of trials have examined supplementation of vitamin D (either with standard or high oral doses) to increase serum 25(OH)D<sub>3</sub> levels in pregnancy (Table 1.3). These trials have consistently showed that high dose vitamin D<sub>3</sub> supplementation is effective at raising vitamin D<sub>3</sub> levels in pregnancy compared to low dose supplementation. 1,25(OH)<sub>2</sub>D<sub>3</sub> levels were found to plateau in women taking 4000 IU/day vitamin D<sub>3</sub> in late pregnancy, although this plateau effect has not been observed outside of pregnancy (Wagner, McNeil et al. 2013). This study also confirmed that there are no identifiable safety issues with the levels of vitamin D<sub>3</sub> supplemented, as side effects such as hypercalciuria (>250 mg/24 hours of urinary calcium) or 25(OH)<sub>2</sub>D<sub>3</sub> over 100 nmol/L, were monitored as well as serum calcium, PTH and creatinine and phosphorus levels (Dawodu, Saadi et al. 2013, Roth, Al Mahmud et al. 2013, Wagner, McNeil et al. 2013).

Table 1.3. Vitamin D oral supplementation trials during pregnancy.

Location	Total (n)	Gestation of supplementation	Vitamin D supplement (IU)	25(OH)D <sub>3</sub> achieved (nmol/L)	Reference
United Arab Emirates	192	12-16 wks to term	4000/day	88	(Dawodu, Saadi et al. 2013)
			400/day	65	
			200/day	47	
United States of America	257	12-16 wks to term	4000/day	95	(Wagner, McNeil et al. 2013)
			2000/day	90	
Iran	130	24-26 wks to	400/day +	119	(Hashemipour, Lalooha et al. 2013)
		36-38 wks	50,000/wk <sup>†</sup> 400/day <sup>†</sup>	39	
Bangladesh	160	26-30 wks to term	35,000/wk	134	(Roth, Al Mahmud et al. 2013)
			0	48	

<sup>†</sup> also received 200 mg calcium

### 1.7.4 Vitamin D<sub>3</sub> deficiency and pregnancy complications

In the last decade, many studies have described associations between vitamin D<sub>3</sub> deficiency and pregnancy complications. Studies are difficult to compare due to differences in sampling gestation and definitions of sufficiency, although two recent systematic reviews have examined associations between 25(OH)D<sub>3</sub> and pregnancy complications (Aghajafari, Nagulesapillai et al. 2013, Wei, Qi et al. 2013).

#### 1.7.4.1 Preeclampsia

Maternal serum vitamin D<sub>3</sub> levels have been investigated in many preeclamptic populations to date (Table 1.4). Recent meta-analyses have determined that vitamin D<sub>3</sub> deficiency (<50 nmol/L) increases risk for PE (OR 1.79 (95% CI 1.25-2.58) (Aghajafari, Nagulesapillai et al. 2013); OR 2.78 (95% CI 1.45-5.33) (Tabesh, Salehi-Abargouei et al. 2013), OR 2.09 (95% CI 1.50-2.90) (Wei, Qi et al. 2013). The relationship between severe PE [as described previously (Tranquilli, Brown et al. 2013)] and 25(OH)D<sub>3</sub> levels has also been investigated, with strong evidence for a negative association (Table 1.4).

Many studies have investigated calcium supplementation to reduce the incidence of PE. A recent Cochrane review examining calcium supplementation in pregnancy found supplementation reduced PE incidence by 52% in women on low calcium diets (RR 0.45, 95% CI 0.31-0.65) (Hofmeyr, Lawrie et al. 2014). This is consistent with the fact that women who develop PE have lower calcium excretion from 28 weeks' gestation than women who do not develop PE (Pal, Roy et al. 2012). On the other hand, a study that used supplementation of 375 mg/day calcium and 1200 IU/day vitamin D<sub>3</sub> compared to placebo found no difference in PE incidence, although blood pressure was reduced (Marya, Rathee et al. 1987).

Table 1.4. Comparison of serum 25(OH)D<sub>3</sub> levels in preeclamptic and uncomplicated pregnancies

Complication	Serum 25(OH)D <sub>3</sub> (nmol/L)		Odds Ratio (OR (95% CI)) and cut-off	Gestational age (weeks)	References
	Control (mean ± SD)	PE (mean ± SD)			
PE	62 (± 3)	60 (± 3) <sup>^</sup>	3.9 (1.2-3.2) (<75 nmol/L)	Delivery	(Ullah, Koch et al. 2013)
	57 (± 28)	<b>44 (± 34)</b>	<b>24 (2.1-274.8)</b> (<25 nmol/L)	37-40	(Abedi, Mohaghegh et al. 2014)
	56 (± 19)	51 (± 15)	1.2 (0.6-2.7) (<50 nmol/L)	12-18	(Wei, Audibert et al. 2012)
	57 (± 19)	<b>49 (± 17)</b>	<b>3.2 (1.4-7.7)</b> (<50 nmol/L)	24-26	
	50 (36-68) <sup>IQR</sup>	<b>43 (33-72)<sup>IQR</sup></b>	<b>1.4 (0.5-3.5)</b> (<50 nmol/L)	10-20	(Shand, Nassar et al. 2010)
	72 (± 23)	68 (± 31)	2.5 (0.9-6.9) (<37.5 nmol/L)	11	(Powe, Seely et al. 2010)
	53 (47-60) <sup>CI</sup>	<b>45 (39-53)<sup>CI</sup></b>	<b>5.0w (1.7-14.1)</b> (<37.5 nmol/L)	4-21	(Bodnar, Catov et al. 2007)
Severe PE	107 (90-121) <sup>IQR</sup>	<b>75 IQR (53-107)</b>	n/a	16-18	(Woodham, Brittain et al. 2011)
	98 (68-113) <sup>IQR</sup>	<b>75 IQR (47-107)</b>	<b>5.4 (2.0-14.5)</b> (<50 nmol/L)	16-19	(Baker, Haeri et al. 2010)
	80 (52-110) <sup>IQR</sup>	<b>45 (32-77)<sup>IQR</sup></b>	n/a	28-31	(Robinson, Alanis et al. 2010)

These negative findings may be due to the low dose of vitamin D<sub>3</sub> and calcium employed resulting in women not achieving sufficiency over the ten weeks of supplementation. A significant reduction in a composite of pregnancy complications (P=0.044), defined as PTB, GH, PE, GDM and/or infection, was found when supplementing with 4000 IU/day compared to 2000 IU/day vitamin D<sub>3</sub> from 12 weeks' gestation until delivery (Wagner, McNeil et al. 2013). As calcium supplementation is effective at reducing the risk of PE in women with low calcium diets, and vitamin D<sub>3</sub> supplementation may protect against PE by increasing

calcium uptake, further trials are needed to determine whether calcium and 25(OH)D<sub>3</sub> supplementation may be effective at preventing PE.

#### 1.7.4.2 Spontaneous preterm birth

The association between vitamin D<sub>3</sub> deficiency and spontaneous PTB has been investigated in a limited number of studies (Table 1.5). Due to the mixed results, a recent meta-analysis concluded that there is an association between vitamin D<sub>3</sub> deficiency and PTB (OR 1.58 (95% CI 1.08-2.31) (Wei, Qi et al. 2013). No supplementation studies have been conducted to determine the effectiveness of vitamin D<sub>3</sub> at preventing PTB.

Complication	Serum 25(OH)D <sub>3</sub> (nmol/L)		Odds Ratio (OR (95% CI)) (cut-off)	Gestational age (weeks)	References
	Control (±SD)	PTB (±SD)			
PTB	n/a	n/a	<b>3.3 (1.52-7.19)</b> (<50 nmol/L)	24-28	(Perez-Ferre, Torrejon et al. 2012)
	n/a	n/a	1.0 (0.5-2.2) (<50 nmol/L)	10-20	(Shand, Nassar et al. 2010)
Recurrent PTB	73 (± 32.6)	71 (± 30.7)	n/a	16-22	(Thorp, Camargo et al. 2012)
Threatened PTB	39 (± 13)	<b>28 (± 8)</b>	n/a	>30	(Shibata, Suzuki et al. 2011)

Note: OR = adjusted odds ratio, odds of delivering a growth restricted baby

#### 1.7.4.3 Small-for-gestational age babies

Maternal 25(OH)D<sub>3</sub> status has been associated with fetal growth and SGA incidence in a few studies (Table 1.6), with the association recently confirmed by meta-analyses (OR 1.52 (95% CI 1.08-2.15) (Wei, Qi et al. 2013); OR 1.85 (95%

CI 1.52-2.26) (Aghajafari, Nagulesapillai et al. 2013). Bodnar *et al.* found a U-shaped risk curve for vitamin D insufficiency or excess increasing the risk of delivering a SGA infant in white American women (<58 nmol/L OR 2.7 (95% CI 1.1-6.8), >90.7 nmol/L OR 3.9 (95% CI 1.6-9.7)) (Bodnar, Catov et al. 2010). Although Morley *et al.* found no association between birth weight and vitamin D<sub>3</sub> deficiency further investigation revealed that infant VDR genotypes were associated with birth weight as the Fok1 polymorphism (rs2228570 C > T) FF and Ff genotypes were associated with lower birth weight when mothers were vitamin D<sub>3</sub> deficient (Morley, Carlin et al. 2006, Morley, Carlin et al. 2009). Another study found a reduction in birth weight of 198 g in vitamin D<sub>3</sub> deficient mothers (<28

Table 1.6. Comparison of serum 25(OH)D<sub>3</sub> levels in pregnancies complicated by fetal growth restriction with uncomplicated pregnancies

Complication	Serum 25(OH)D <sub>3</sub> (nmol/L)		Odds Ratio (OR (95% CI)) (cut-off)	Gestational age (weeks)	References
	Control (range)	Growth Restriction (range)			
SGA	n/a	n/a	<b>1.8 (1.3-2.5)</b> (<30 nmol/L)	12-14	(Leffelaar, Vrijkotte et al. 2010)
	72 (64-80) <sup>CI</sup>	73 (70-78) <sup>CI</sup>	7.5 (1.8-31.9) (<37.5 nmol/L)	<22	(Bodnar, Catov et al. 2010)
Severe IUGR	50 (36-68) <sup>IQR</sup>	<b>41 (25-65)</b> <sup>IQR</sup>	2.3 (0.65-8.49) (<50 nmol/L)	10-20	(Shand, Nassar et al. 2010)
Severe SGA	47 (28-70) <sup>IQR</sup>	<b>30 (20-51)</b> <sup>IQR</sup>	n/a	11-13	(Ertl, Yu et al. 2012)

Note: OR = adjusted odds ratio, odds of delivering a growth restricted baby when serum 25(OH)D<sub>3</sub> levels are below the defined level; SGA was defined as birth weight less than the 10th centile; severe IUGR was defined as less than the 3rd centile; severe SGA was defined as <5th centile; bold denotes significant difference (P<0.05); CI - geometric mean (95% confidence interval); IQR - median (interquartile range).

nmol/L) compared to those with sufficient vitamin D<sub>3</sub> status, although there was no association with fetal growth restriction (Bowyer, Catling-Paull et al. 2009).

Vitamin D<sub>3</sub> supplementation studies have also examined the incidence of SGA, with some studies finding increased birth weights. Asian women in London were supplemented with 1000 IU/day of vitamin D<sub>3</sub> or placebo in the last trimester of pregnancy, with no difference in birth weight found; although there were nearly twice as many low birth weight (LBW) babies in the unsupplemented group (Maxwell, Ang et al. 1981). During follow up, infants from supplemented mothers were heavier and longer from 3-12 months of age (Brooke, Butters et al. 1981). Wagner *et al.* recently compared 4000 IU/day to 2000 IU/day of vitamin D<sub>3</sub> from 12-16 weeks throughout pregnancy and found that mothers consuming 4000 IU had 2.4-fold greater chance of having a baby of average weight (at the 50<sup>th</sup> centile) (Wagner, McNeil et al. 2013). Another study examined vitamin D<sub>3</sub> intake during pregnancy, with <200 IU/day (compared to >200 IU/day) associated with 60 g lighter babies (Scholl and Chen 2009). Most studies suggest an association between vitamin D<sub>3</sub> sufficiency and improved fetal growth, although few have examined SGA.

#### **1.7.4.4 Gestational diabetes mellitus**

There has been much interest in the last few years on the potential role of vitamin D<sub>3</sub> in diabetes mellitus and more recently in GDM (Table 1.7). A meta-analysis was conducted by Poel *et al.* in 2012 comparing vitamin D<sub>3</sub> deficiency in GDM in seven studies and concluded that the prevalence of GDM was greater amongst vitamin D<sub>3</sub> deficient women (25(OH)D<sub>3</sub> <50 nmol/L O.R. 1.61 (95% CI 1.19-2.17) (Poel, Hummel et al. 2012). Obesity is a risk factor for GDM and as obese pregnant women are more likely to have low vitamin D levels as vitamin D is

fat soluble (Bodnar, Catov et al. 2007); hence it is not surprising that obese, vitamin D deficient women have a higher risk of GDM .

Table 1.7. Comparison of serum 25(OH)D<sub>3</sub> levels in pregnancies complicated with GDM and uncomplicated pregnancies.

Serum levels (nmol/L) (control vs. complication)		Odds ratio (OR)	Gestation (weeks)	References
Control (±SD)	GDM (±SD)			
26 (19-35)^	<b>22.4</b> <b>(18-29)^ *</b>	1.6 ( <25 nmol/L)	26-28	(Wang, Nie et al. 2012)
n/a	n/a	3.7 (<50 nmol/L)	24-28	(Perez-Ferre, Torrejon et al. 2012)
n/a	n/a	2.2 (<25 nmol/L)	26-28	(Burris, Rifas- Shiman et al. 2012)
48 (± 27)	47.2 (± 27)	n/a	<14	(Makgoba, Nelson et al. 2011)
86 (22)^	97.0 (29)^	1.25 (<50 nmol/L)	11-14	(Baker, Haeri et al. 2012)
32 (23)^	23.7 (21)^ *	2.0 (<50 nmol/L)	24-28	(Soheilykhah, Mojibian et al. 2010)
38 (n/a)	38.8 (n/a)	n/a	30	(Farrant, Krishnaveni et al. 2009)
55 (± 23)	48.6 (± 25) *	1.9 (<50 nmol/L)	26-30	(Clifton-Bligh, McElduff et al. 2008)
75 (± 24)	60.4 (± 21) *	2.66 (<20 nmol/L)	16	(Zhang, Qiu et al. 2008)
23 (± 18)	17 (± 10) *	n/a	20-30	(Maghbooli, Hosseini-Nezhad et al. 2008)

Note: OR - odds of GDM when 25(OH)D<sub>3</sub> is below the defined level; \* indicates significant difference in 25(OH)D<sub>3</sub> between control and GDM pregnancies; ^ - median (interquartile range)



## 1.8 Vitamin D<sub>3</sub>'s role in the human placenta

Recently, there have been a number of investigations into the role of vitamin D<sub>3</sub> in the placenta, following the confirmation of placental *CYP27B1* expression (Diaz, Sanchez et al. 2000). Studies of vitamin D<sub>3</sub>'s function in humans have employed human tissue, cell cultures and placental explants to explore cellular and molecular changes induced by vitamin D<sub>3</sub> in the placenta.

Components of the vitamin D<sub>3</sub> metabolic pathway have been analysed at the protein and mRNA levels in early and late gestation placentas, with some conflicting results. Expression of *VDR* mRNA has been investigated across gestation, with 3-fold and 10.9-fold increases in first and second trimester, respectively, compared to third trimester (Zehnder, Evans et al. 2002). As *VDR* mRNA levels are not necessarily a predictor of VDR protein levels, the protein has also been investigated by immunohistochemistry and western blot analyses. VDR protein was located to the syncytiotrophoblast in term placentas, with another study also reporting localization to the vascular smooth muscle cells (VSMC) (Zehnder, Evans et al. 2002, Ma, Gu et al. 2012). Another histochemical study compared the staining pattern obtained using two antibodies. The N-terminus VDR antibody labelled VSMC, while C-terminus labelled syncytiotrophoblast (Barrera, Avila et al. 2008), which may account for variations in staining patterns between studies. It may also suggest different functions for VDR or different isoforms within tissues. When comparing pregnancy complications, placentas from PE pregnancies had less syncytiotrophoblast staining than normal term placentas; also early gestation placentas had stronger staining than at term (confirmed by western blot) (Ma, Gu et al. 2012). These findings suggest reduced VDR localisation in placentas from PE pregnancies, possibly indicating altered vitamin D

metabolism. Also the higher level of VDR expression in early gestation, suggesting that vitamin D signalling may be important in early pregnancy.

The role for *CYP27B1* in placental function has also been investigated. *CYP27B1* mRNA was found to be over 75-fold higher in first and second trimester placentas compared to term (Zehnder, Evans et al. 2002). *CYP27B1* mRNA expression levels at term were found to be correlated with mid-gestation serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels, while CYP27B1 protein abundance was correlated with serum 25(OH)D<sub>3</sub> levels at delivery (O'Brien, Li et al. 2014). Interestingly, CYP27B1 and CYP24A1 protein levels were found to be positively correlated (O'Brien, Li et al. 2014). CYP27B1 protein was localized to fetal endothelium in one study (Ma, Gu et al. 2012), while in two other studies it was localized to the syncytiotrophoblast (Zehnder, Evans et al. 2002, Barrera, Avila et al. 2008). There was stronger CYP27B1 protein localisation in early gestation placentas and in the syncytiotrophoblast of PE placentas (confirmed by western blot) (Zehnder, Evans et al. 2002, Ma, Gu et al. 2012). These studies agree that CYP27B1 protein and mRNA are highest in early pregnancy, although they have conflicting protein localisation. This may indicate that the placenta can synthesise its own 1,25(OH)<sub>2</sub>D<sub>3</sub> in its early development.

One study also examined protein levels of CYP24A1, VDBP and CYP2R1 by immunohistochemistry and western blot analyses. CYP24A1 was localized to the syncytium, with stronger staining in PE and early gestation placentas compared to term. VDBP was also localized to the syncytium, with stronger staining in early gestation and weaker staining in PE placentas compared to term. CYP2R1 was localized to syncytium, stromal cells and fetal endothelial cells and, similar to that of VDBP, labelling was stronger in early gestation and weaker in PE placentas, compared to term (Ma, Gu et al. 2012).

## 1.9 Animal models of vitamin D<sub>3</sub> deficiency

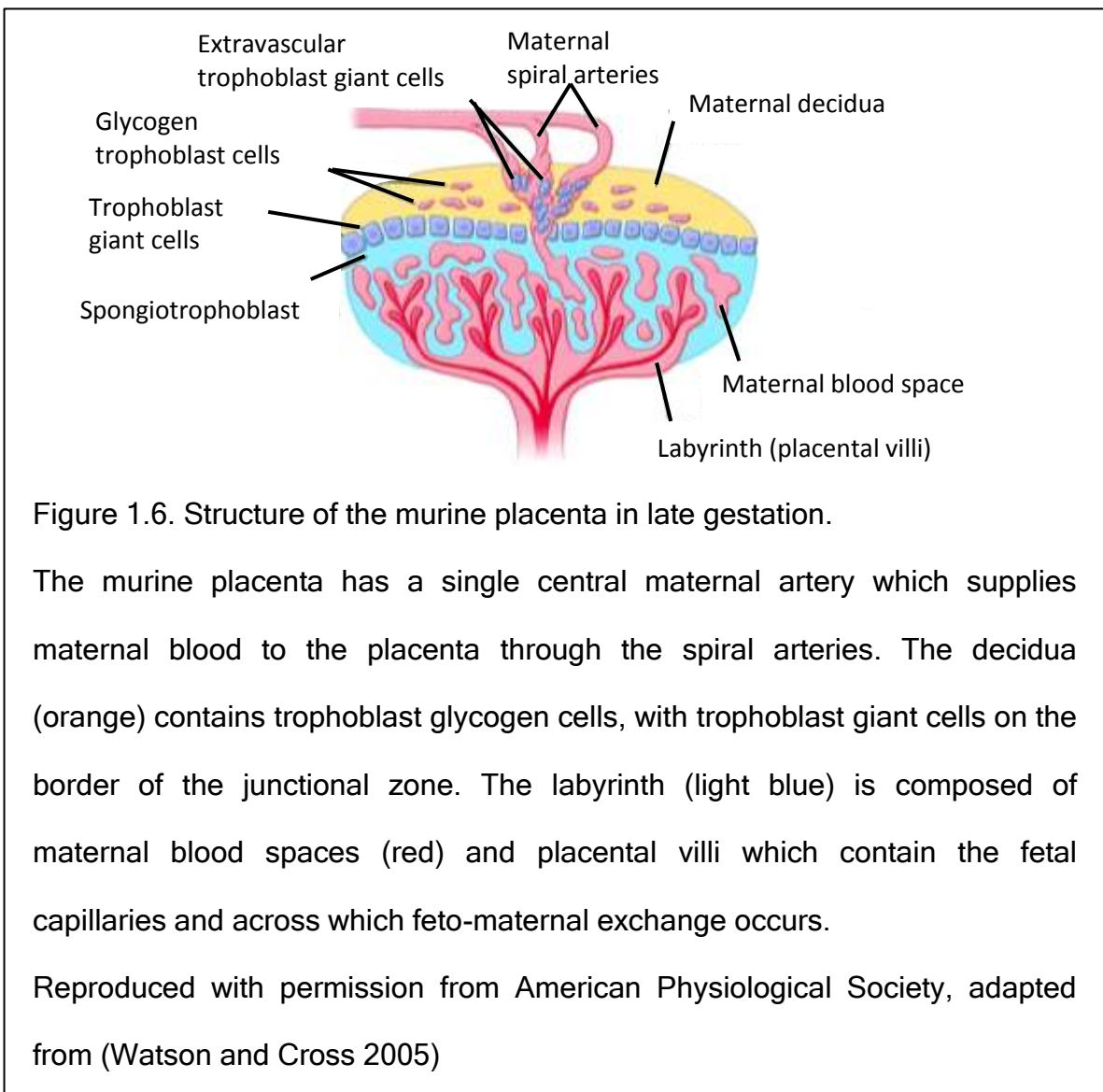
While investigations in humans can answer many questions about placental vitamin D<sub>3</sub> metabolism and function, animal studies are vital in providing insight into the effects of vitamin D<sub>3</sub> and calcium deficiency.

### 1.9.1 Comparative anatomy of the human and mouse placenta

Both the human and murine placentas are discoid in shape and haemochorial with maternal blood in direct contact with trophoblasts. When mature, they are both haemotrophic; that is, they rely on circulating maternal blood to provide nutrition, with human placenta being haemotrophic from the second trimester and that of mice from gestational day 10.5 (d10.5) (Georgiades, Ferguson-Smith et al. 2002). Both species have three defined placental regions: decidua, maternal and fetal; although there are differences in both terminology and physiology between human and murine placenta.

There are structural differences between placental architectures in human and mouse placenta, although they perform the same functions. The human chorionic villi are highly organized, with branching, terminal and anchoring villi resembling a tree-like structure (Burton, Jauniaux et al. 1999). Human terminal villi “float” in the intervillous space, whereas in mice the placenta has a maze-like labyrinth of villi with an interconnected structure, resulting in structural differences in maternal blood spaces between species (Figure 1.6) (Georgiades, Ferguson-Smith et al. 2002). Due to spiral artery transformation both have slow-moving maternal blood to allow ample opportunity for exchange. The structure of the trophoblasts covering the villi also varies between human and mouse placentas. Humans have a haemomonochorial placenta with only one layer of trophoblasts between the intervillous space and fetal capillaries. The mouse placenta is

haemotrichorial, with mononuclear trophoblasts in contact with maternal blood, with two layers of syncytiotrophoblasts (Malassine, Frendo et al. 2003). Although there are differences in villous structure, the villi of both species function to exchange nutrients and wastes between the maternal and fetal circulations. Furthermore, there are also differences in the decidua between human and mouse, as the human placenta invades deeply into the decidua through the endometrium and up to a third of the way through the myometrium (Burton, Woods et al. 2009), whereas murine placental invasion only reaches the distal region of the decidua basalis (Adamson, Lu et al. 2002).



The murine placenta, unlike that of the human, can be clearly divided into functionally different compartments including the labyrinth (LZ) and junctional (JZ) zones. The LZ is the region of feto-maternal exchange following the delivery of maternal blood from the maternal central artery (Adamson, Lu et al. 2002). The JZ is made up of trophoblast giant cells (TGC), spongiotrophoblasts and glycogen cells; it contains placental stem cells and is the primary hormone producing zone. Unlike in humans, the murine syncytiotrophoblasts in the labyrinth do not produce hormones; this is instead achieved by TGCs which produce the mouse placental lactogen 1 and 2. Unlike the human placenta, the mouse placenta does not produce estradiol or progesterone, as the ovaries maintain this function throughout gestation (Malassine, Frendo et al. 2003). TGCs mark the border between the decidua and junctional zone from about d10, of gestation when the chorio-allantoic placenta develops. These cells appear to be responsible for spiral artery transformation (Adamson, Lu et al. 2002). Glycogen cells, which have large glycogen-containing vacuoles, appear at d 10.5 of gestation and are maximal at d16, with a gradual decrease in the presence of glycogen cells until almost none are seen at d18.5 (Coan, Conroy et al. 2006). Their function within the placenta is unknown.

While the duration of normal human pregnancy is 37-41 weeks, mouse pregnancy lasts approximately 20 days, dependant on mouse strain (Murray, Morgan et al. 2010). The mouse has two placentas, the first functional placenta is the yolk sac placenta which forms from the mural trophoctoderm of the blastocyst. This forms a membranous cone-like placenta which invades shallowly. The second placenta becomes functional around mid-gestation (d10.5-d12.5) and forms on the opposite side of the blastocyst from the polar trophoctoderm. This is the chorio-

allantoic placenta which is similar to the human placenta, with delayed maternal blood flow to the placenta observed.

The yolk-sac placenta initially forms from the mural trophoblast of the blastocyst on the opposite side from where the chorionic placenta will later form. It forms a membranous cone-like placenta in a uterine crypt, which later inverts to cover the fetal membranes (Jollie 1990). By mid-gestation (d10.5) the chorio-allantoic placenta forms, becoming functional between d10.5 and d12.5 of gestation (Muntener and Hsu 1977). The chorio-allantoic placenta can be compared to the human placenta and, as in human pregnancy there is evidence of a delay in maternal blood flow. From d14.5 to d16.5 in the murine placenta there is a rapid expansion of labyrinth zone volume and surface area, with maximal placental volume achieved by d16.5. From d16.5 to d18.5 there is a reduction in the junctional zone as the labyrinth expands, possibly due to the decreased proportion of glycogen cells which invade the decidua (Coan, Ferguson-Smith et al. 2004).

Although there are many differences between the human and mouse placentas, they are similar enough to make it a useful model. There are similarities in both overall structure and function, such as in the production of hormones and the transfer of nutrients (Georgiades, Ferguson-Smith et al. 2002, Malassine, Frenzo et al. 2003). Due to these similarities the mouse placenta is a well characterized alternative to the study of human placentas, although findings in this model need to be verified in humans. Other advantages of using mice are that there are many knockout models available, as well as their short time to maturity and short gestation. This also allows us to examine organs and test treatments which would not be ethical in humans, especially in pregnancy.

### 1.9.2 Animal studies of vitamin D<sub>3</sub> in pregnancy

Animal studies of vitamin D<sub>3</sub>'s action in bone, specifically in *Vdr* knockout (*Vdr* null) mice, have revealed vitamin D<sub>3</sub>'s involvement in pregnancy and supported other early human work on vitamin D<sub>3</sub> in pregnancy such as the finding of vitamin D<sub>3</sub> deficiency in PE (August, Marcaccio et al. 1992). For example, *Vdr* null mice were found to have uterine hypoplasia, poor folliculogenesis and infertility (Yoshizawa, Handa et al. 1997). This led to studies on the role of vitamin D<sub>3</sub> in pregnancy through vitamin D<sub>3</sub> depletion and *Vdr* null studies that have provided us with an insight into the effects of vitamin D<sub>3</sub> and calcium in pregnancy and placentation.

Although fertility was reduced in vitamin D<sub>3</sub> deficient rats and *Vdr* null mice (Halloran and DeLuca 1980, Johnson and DeLuca 2001, Kovacs, Woodland et al. 2005), dietary interventions appeared to restore fertility. Diets were modified by addition of lactose (10-20%) to increase intestinal calcium absorption, or increased calcium from 0.8% standard diets up to 2% (Johnson and DeLuca 2001, Kovacs, Woodland et al. 2005), with a rescue diet later recommended to normalise plasma calcium and maintain fertility in *Vdr* null mice (2% calcium, 1.25% phosphate, 20% lactose) (Rummens, van Cromphaut et al. 2003). Litter size was also found to be affected, with a 30% decrease in vitamin D<sub>3</sub> deficient rats, but not in *Vdr* null mice (Halloran and DeLuca 1980, Johnson and DeLuca 2001, Rummens, van Cromphaut et al. 2003, Kovacs, Woodland et al. 2005), although one study reported fewer viable fetuses from *Vdr* null mice on a standard diet (Kovacs, Woodland et al. 2005).

There was no difference in birth weights of vitamin D<sub>3</sub> deficient rats or mice, although vitamin D<sub>3</sub> deficient mice had increased fetal weight during pregnancy (d14 and d18), with a reduction in neonatal weight (Halloran and DeLuca 1980,

Liu, Ouyang et al. 2013). This was also seen in *Vdr* null mice, as fetal weights were lower in *Vdr* null than wild type (WT) at d18.5 of pregnancy (Rummens, van Cromphaut et al. 2003); weaning weights were also lower in *Vdr* null mice than WT mice (Masuyama, Nakaya et al. 2001). As reductions in growth may be, in part, due to alterations in placental development, some studies have also investigated placental effects of vitamin D<sub>3</sub> deficiency.

These studies have found that low dietary calcium in the absence of VDR affects fertility and while increasing calcium corrects fertility problems, fetal growth is reduced. These findings may help us to better identify vitamin D's role in fetal growth restriction in human pregnancies.

### **1.9.3 Animal studies of vitamin D<sub>3</sub> in placenta**

Although a few studies have investigated vitamin D deficiency in mouse pregnancy, few report any placental findings. Placental weight was reported to be unchanged in vitamin D<sub>3</sub> deficient or *Vdr* null mice (Dardenne, Prud'homme et al. 2001, Panda, Miao et al. 2001, Liu, Ouyang et al. 2013). None of the papers examined fetal:placental weight ratio which is a surrogate marker of placental efficiency, although the scant data available suggest that as there was no change in placental weight, and that fetal weight was higher in mice on vitamin D<sub>3</sub> deficient diets, that placental efficiency was increased (Liu, Ouyang et al. 2013). On the other hand, *Vdr* null mice had reduced fetal weight and therefore may have reduced placental efficiency (Panda, Miao et al. 2001). Placental morphology has received minimal attention in vitamin D<sub>3</sub> deficient mice. Lower diameter vessels have been observed in the placental labyrinth of deficient mice (Liu, Ouyang et al. 2013); while *Vdr* null placentas had no gross abnormalities and exhibited similar placental lactogen localisation to WT placentas (Dardenne, Prud'homme et al.



2001). Examination of placental gene expression found no difference in expression of *Cyp27b1* between *Vdr* null and WT placentas (Kovacs, Woodland et al. 2005). Interestingly, placental calcium transfer was increased in *Vdr* null mice compared to other genotypes (Dardenne, Prud'homme et al. 2001), which may indicate a mechanism to preserve fetal mineralisation during maternal hypocalcaemia.

These studies have demonstrated poor fertility and reproduction in vitamin D<sub>3</sub> deficient and *Vdr* null rats and mice. However, comparison of these studies is complicated by the various diets and genotypes involved, as some studies have investigated heterozygotes as well as null mice. While placental weight and gross morphology of *Vdr* null placentas appears normal, placental gene expression and calcium transport are perturbed. Further studies must be conducted to separate the effects of dietary vitamin D<sub>3</sub> and calcium, and to determine how these changes affect pregnancy, placental function and fetal growth.

## 1.10 Conclusion

There is strong evidence for a role for vitamin D<sub>3</sub> in pregnancy, such as associations between pregnancy complications and 25(OH)D<sub>3</sub> deficiency. There is also evidence to suggest that the placenta may be the source of the increased circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations in pregnancy, rather than altered kidney production. Infertility of vitamin D<sub>3</sub> deficient rats and *Vdr* null mice and epidemiological studies linking maternal vitamin D<sub>3</sub> deficiency with PE, SGA and GDM, provide strong evidence for a role for vitamin D<sub>3</sub> in maintaining placental function and ensuring pregnancy success. However we do not know the precise biochemical pathways by which vitamin D<sub>3</sub> acts in the placenta. To investigate this both mouse models and human tissue studies of placental vitamin D<sub>3</sub> metabolism will be employed. The aim is to determine in combination the roles of vitamin D<sub>3</sub>

and calcium in murine pregnancy and its effects on the placenta (see Chapter 2, Chapter 3). We also aim to investigate vitamin D<sub>3</sub> metabolism in the human placenta across gestation and assess changes and differences between complicated and uncomplicated term placentas (see Chapter 4).

# Statement of Authorship

Title of Paper:	Chapter 2 - Effect of pregnancy on vitamin D and calcium restriction
Publication Status	<input type="radio"/> Published, <input type="radio"/> Accepted for Publication, <input type="radio"/> Submitted for Publication, <input checked="" type="radio"/> Publication style
Publication Details	Written in publication style, currently being prepared for publication.

## Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Jessica Laurence	
Contribution to the Paper:	Experimental design Experimental work - mouse, PCR Data analysis Manuscript preparation	
Signature	Date	23/10/14

Name of Co-Author	Tina Bianco-Miotto	
Contribution to the Paper	Experimental troubleshooting - PCR Analysis of data Critical comments on manuscript	
Signature	Date	17.10.14

Name of Co-Author	Paul Anderson	
Contribution to the Paper	Experimental design Experimental work - blood biochemistry Interpretation of data Critical comments on manuscript	
Signature	Date	22/10/14

Name of Co-Author	Claire Roberts	
Contribution to the Paper	Experimental design Interpretation of data Critical comments on manuscript	
Signature	Date	23.10.14

## Chapter 2: Effects on pregnancy of maternal dietary vitamin D<sub>3</sub> and calcium restriction

### 2.1 Abstract

Impaired placental development and function are associated with pregnancy complications. Vitamin D and calcium deficiency are also associated with pregnancy complications, with vitamin D deficiency increasing the risk of preeclampsia and calcium supplementation reducing the risk of preeclampsia in women with low calcium diets.

As vitamin D is a major regulator of calcium homeostasis, we aimed to assess placental development in C57Bl6 mice fed diets sufficient or deficient in vitamin D and/or calcium for 10 weeks before mating. At d18.5 of pregnancy mice were sacrificed, with placentas collected for histology and gene expression analyses.

Placental weight was unaffected by diet but fetal weight and fetal:placental weight ratios were increased in calcium deficient mice compared to controls. Placental labyrinthine (exchange region) morphometric analyses revealed mice consuming diets deficient in either nutrient had increased maternal blood space and trophoblast (feto-maternal) barrier surface area, with reduced barrier thickness compared to controls. These suggest increased placental efficiency and increased opportunity for feto-maternal exchange in these mice. These modifications are indicative of positive placental compensatory responses to maternal vitamin D and calcium dietary restriction. Placental gene expression analyses showed increased *Cyp24a1* and *Vdr* expression in calcium deficient mice, suggesting altered placental vitamin D metabolism.

Our data demonstrate the complexity of vitamin D and calcium metabolism in the placenta and indicate that these micronutrients should be studied together in the placenta and in pregnancy and not in isolation. Interactions between these micronutrients may underlie the discrepancies reported in the literature regarding the effect of vitamin D deficiency on pregnancy complications, particularly those between different populations. Calcium status should clearly be included in the analyses.

## 2.2 Introduction

Vitamin D<sub>3</sub> deficiency is associated with increased risk of pregnancy complications such as preeclampsia (PE) (Woodham, Brittain et al. 2011) and preterm birth (PTB) (Shibata, Suzuki et al. 2011). Supplementation studies have had mixed results, dependent on the level of vitamin D<sub>3</sub> supplementation and timing of administration during pregnancy. Supplementation of pregnant women with 4000 IU/day vitamin D<sub>3</sub> (as opposed to 400 IU/day) throughout pregnancy, resulted in an overall decrease in composite complications (PTB, infection, gestational diabetes, PE/gestational hypertension) (Hollis and Wagner 2013), whereas supplementation with only 400 IU/day (vs. 0 IU/day) from 12 weeks gestation resulted in no change in incidence of pregnancy complications (Cockburn, Belton et al. 1980). The most recent Cochrane review on calcium supplementation in pregnancy found that  $\geq 1$  g/day during pregnancy reduced the rates of PE in women who had calcium deficient diets, as well as reducing blood pressure during pregnancy (Hofmeyr, Atallah et al. 2006). As dietary vitamin D<sub>3</sub> and calcium deficiency are both associated with pregnancy complications, both need to be examined in combination.

Vitamin D<sub>3</sub> is a calcitropic hormone and is a major regulator of calcium metabolism (see Figure 1.3). UVB converts 7-dehydrocholesterol to vitamin D<sub>3</sub> in the skin, where it enters the circulation bound to vitamin D binding protein (VDBP). It then undergoes hydroxylation in the liver to the storage form of vitamin D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>). Vitamin D<sub>3</sub> activation is under the influence of parathyroid hormone (PTH) and occurs in the kidney, where 25(OH)D<sub>3</sub> is converted to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) by the enzyme 25-hydroxy vitamin D<sub>3</sub>-1 $\alpha$  hydroxylase, CYP27B1, and metabolized by 1,25-dihydroxy vitamin D<sub>3</sub>-24-hydroxylase, CYP24A1. 1,25(OH)<sub>2</sub>D<sub>3</sub> then acts on the intestines to increase calcium absorption and on the bones to augment calcium reabsorption. There are also other calcitropic mechanisms to be considered, such as the FGF23/Klotho axis. FGF23 is released from bones in response to increased 1,25(OH)<sub>2</sub>D<sub>3</sub> and acts on the kidneys to both reduce phosphate reabsorption and vitamin D<sub>3</sub> activation (David and Quarles 2011).  $\alpha$ -Klotho regulates PTH expression in the parathyroid glands, with most of its actions mediated through binding to both FGF23 and the FGF receptor to affect gene transcription (Ohata, Arahori et al. 2011). While vitamin D<sub>3</sub> and calcium metabolism is well understood in general, little work has been done on adaptations in pregnancy.

Vitamin D<sub>3</sub> and calcium metabolism are altered in human pregnancy, with a rise in 1,25(OH)<sub>2</sub>D<sub>3</sub> throughout gestation that is not stimulated by a rise in PTH (Kirby, Ma et al. 2013), while total serum calcium and phosphorous levels are stable. Calcitropic and pregnancy hormones follow the up-regulation of 1,25(OH)<sub>2</sub>D<sub>3</sub> including PTHrP, calcitonin, human placental lactogen and prolactin (Ardawi, Nasrat et al. 1997). Currently there is no known mechanism behind the PTH-independent increase in 1,25(OH)<sub>2</sub>D<sub>3</sub> levels. It would appear that either kidney negative feedback mechanisms are altered in pregnancy, allowing high

levels of  $1,25(\text{OH})_2\text{D}_3$  to be produced, or that production is stimulated by an, as yet unknown, factor. As  $\alpha$ -Klotho is expressed in the placenta, as well as the kidney, the FGF23/Klotho axis is a candidate regulator of  $1,25(\text{OH})_2\text{D}_3$  in pregnancy (Kuro-o, Matsumura et al. 1997).

As vitamin  $\text{D}_3$  deficiency is associated with pregnancy complications, such as PTB, it is plausible that alterations in calcium metabolism are further exacerbated by deficiency and contribute to complications. Although there are various causes of PTB, the majority of cases of early spontaneous PTB (<34 weeks of gestation) are attributed to infection, with the associated inflammation being held responsible for inducing onset of preterm labour (Romero, Espinoza et al. 2007). As vitamin  $\text{D}_3$  has immunomodulatory properties, there may be a higher risk of infection in pregnancies with deficient maternal circulating  $25(\text{OH})\text{D}_3$  levels (Equils and Hewison 2010); alternately a poorly formed placenta at the onset of pregnancy may not be able to sustain a fetus to term, as placental insufficiency has been found to contribute to PTB (Morgan, Tolosa et al. 2013).

We hypothesised that vitamin  $\text{D}_3$  deficiency would exert flow-on effects on the vitamin  $\text{D}_3$  and calcium metabolic changes of pregnancy. We used a mouse model to investigate the effects of dietary vitamin  $\text{D}_3$  and calcium deficiency on calcium homeostasis in pregnancy. We examined diet-induced differences between pregnant and non-pregnant mice as well as the effects of deficiency on serum composition, kidney gene expression and measures of pregnancy success (fetal and placental weights, gestation).

## **2.3 Methods**

### **2.3.1 Animals**

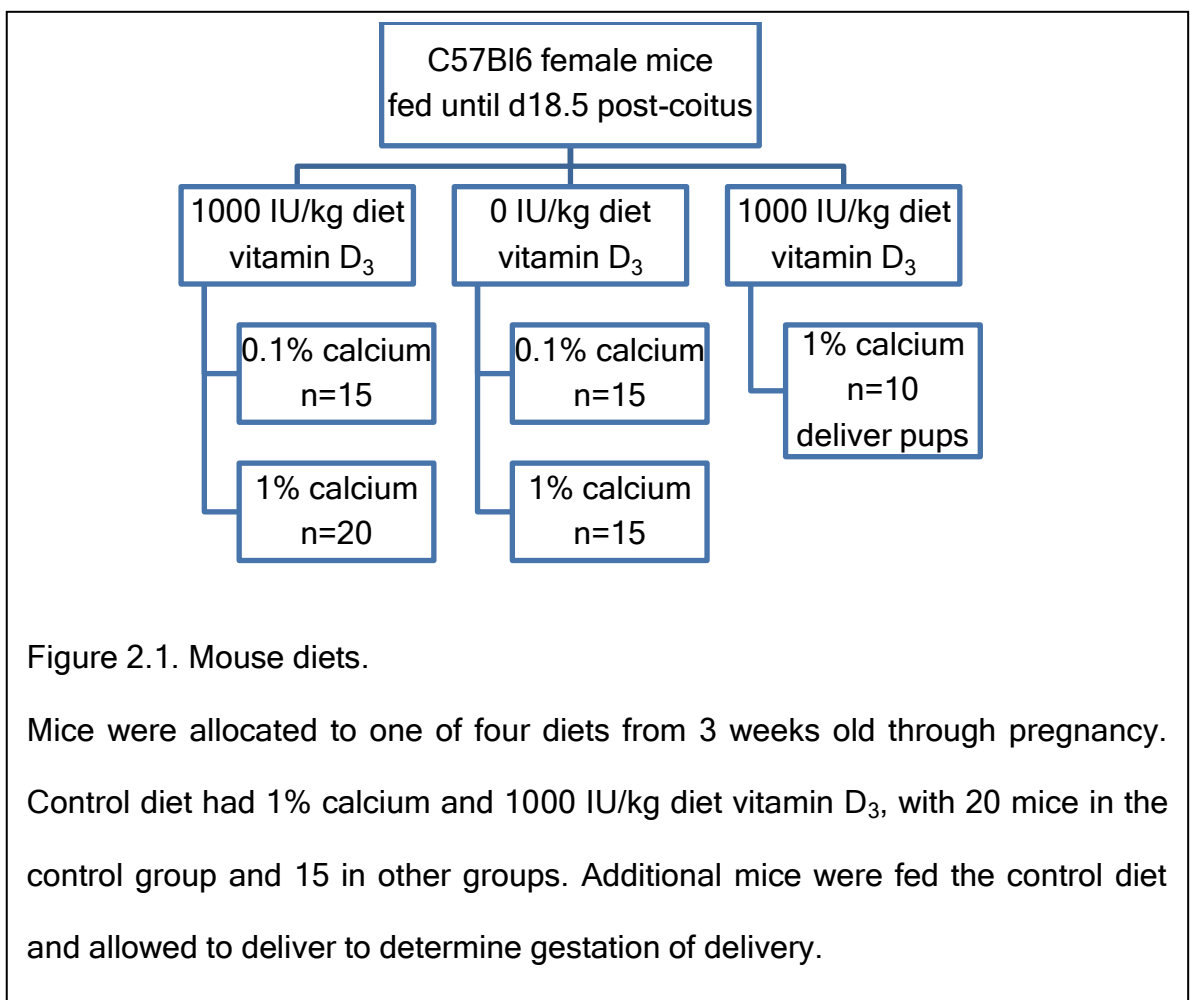
Ethics approval was obtained from the University of Adelaide Animal Ethics Committee and the IMVS/CNAHS Animal Ethics Committee with animal work complying with the Australian Code of practice for the care and use of animals. C57Bl6 female mice were maintained at a 12 h/12 h light/dark cycle and fed ad libitum either a standard diet of 1000 IU vitamin D<sub>3</sub>/kg diet with 1% calcium (control, AIN93G (Reeves, Nielsen et al. 1993)), or a deficient diet with reduced vitamin D<sub>3</sub> (0 IU/kg diet) and/or calcium (0.1%), with 15 mice per diet (20 mice on control diet) diets were from Specialty Feeds, Australia (4 groups, Figure 2.1). Mice consumed their allocated assigned diet from 3 weeks old (at weaning), through mating at 13 weeks of age, until post-mortem at day 18.5 post-coitus (d18.5). Mating was performed with C57Bl6 males maintained on chow, with day 0.5 post-coitus indicated by the presence of a vaginal plug. At post-mortem maternal serum was collected, maternal organs were weighed for body composition and snap frozen in liquid nitrogen, fetuses and placentas were weighed, then snap-frozen.

### **2.3.2 Serum Analyses**

Maternal blood was obtained by sub-orbital bleed at post-mortem into untreated tubes and was incubated on ice for an hour to induce clotting. Blood was centrifuged at 4000 rpm (1500 xg) for 5 min at 4°C, the blood clot was removed by ringing, with remaining serum re-centrifuged. Serum was transferred to a fresh tube and frozen at -80°C prior to analysis.



Vitamin D<sub>3</sub> was measured using a RIA kit for 25(OH)D<sub>3</sub> (Immuno Diagnostic Systems, UK). Total calcium, inorganic phosphorus and albumin were measured using detection kits (Fisher Diagnostics, ThermoFisher, USA). Calcium levels were corrected for serum albumin using the standard formula [corrected Ca (mg/dL) = measured total Ca (mg/dL) + 0.8 (4 - serum albumin (g/dL))] where 40 is the average human albumin level in g/L (Goldstein 1990), but as the average mouse albumin level in C57Bl6 is reported as 37 g/L in males and 46 g/L in females, we retained 40 g/L in the calculations (Quimby and Luong 2007).



### 2.3.3 RNA Extraction

RNA was extracted from kidneys of mice that had either undergone post-mortem on d18.5 while still pregnant (n=25), or had not become pregnant at d18.5

postcoitus (n=27), for gene expression analysis. Mice that had delivered pre-term (before d18.5) also had RNA extracted (n=5) and were analysed separately from other pregnant mice. Maternal left kidneys had RNA extracted by the TRIzol method. Gene expression was analysed for vitamin D<sub>3</sub> pathway genes (*Cyp2r1*, *Cyp27b1*, *Cyp24a1*, and *Vdr*) and for reporters of the FGF23/Klotho axis (*Napi2a*, *Napi2c*) by TaqMan® Assay.

Briefly, tissues were snap frozen in liquid nitrogen at post-mortem and stored at -80°C. Tissues were rapidly bisected transversely over ice without thawing and immersed in TRIzol. Tissue was homogenised in TRIzol by a Powerlyser (MoBio) with ceramic beads (CK14, Geneworks), with RNA extracted as per TRIzol manufacturer's instructions. Rigorous DNase treatment was performed using a TURBO DNase kit (Ambion, Life Technologies), with protocol modifications. Briefly, 2 µg of RNA was incubated for an hour at 37°C with 0.1 volume of TURBO DNase and of 10x TURBO DNase buffer, with another 0.1 volume of TURBO DNase added after 30 min, followed by quenching of the reaction by 0.2 volumes of DNase Inactivation Reagent. RNA integrity was assessed by Experion (BioRad) with a RQI of >4 being acceptable for real time PCR (Fleige and Pfaffl 2006). Absence of genomic DNA was assessed by PCR of DNase-treated RNA with genomic DNA primers (F: 3'-GGCACTGACTGAGGTCAAAC-5'; R: 3'-GTCACAATCACAGAGACTTTGA-5'; amplicon length 120 bp, annealing temperature 60°C; Geneworks). 500 ng of RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad) as per manufacturer's instructions. cDNA was diluted 1/10 and stored at -20°C.

### 2.3.4 Gene expression

Real time qPCR was set up using a QIAgility (QIAGEN) liquid handling robot and run on a CFX-384 Real Time PCR machine (BioRad). 10  $\mu$ L PCR reactions were run containing 5  $\mu$ L TaqMan® Gene Expression Master Mix (Applied Biosystems), 0.5  $\mu$ L TaqMan® Assay, with 2  $\mu$ L of diluted cDNA. Cycling conditions were 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR efficiency was determined using a five point standard curve of pooled maternal kidney cDNA and gel electrophoresis was used to confirm amplicon size. TaqMan® Gene Expression Assays used were for vitamin D<sub>3</sub> metabolism - *Vdr* (Mm00437297\_m1), *Cyp2r1* (Mm00616774\_g1), *Cyp27b1* (Mm01165918\_g1) and *Cyp24a1* (Mm00487244\_m1); for FGF23/Klotho action *Napi2a* (*Slc34a1*, Mm00441450\_m1) and *Napi2c* (*Slc34a3*, Mm00551746\_m1); and for reference genes *Gusb* (Mm06003537\_s1), *Hmbs* (Mm01143545\_m1), *Gapdh* (Mm99999915\_g1) and *Hprt1* (Mm00446968\_m1).

Reference gene suitability was analysed by GeNorm (qBasePlus v2, Biogazelle), with *Hmbs* and *Hprt1* being optimum ( $M < 0.5$ ). PCR data was analysed using BioRad CFX Manager (BioRad), which normalised expression of the genes of interest to the geometric mean of the reference genes.

### 2.3.5 Fetal skeletal mineralisation

Snap-frozen fetuses were imaged by x-ray using a Faxitron (model 804) x-ray imager with Faxitron SR software (Faxitron Bioptics, AZ, USA). Fetuses were irradiated for 11-12 s at 37 kV with contrast adjusted to make bones clearly visible. Fetuses were arranged as weight-matched pairs from 1000 IU/kg + 1% Ca on the right of the image and 0 IU/kg + 0.1% Ca on the left of each image. No quantitative

analysis was undertaken as there appeared to be little mineralization of fetal bones at this time point (d18.5) and little difference between control dietary groups.

### **2.3.6 Statistics**

Fetal and placental weights were analysed by a Linear Mixed Model, Repeated Measures, as repeated measures of the mother with Sidak post-hoc. Gene expression, serum measurements and organ weights were analysed by ANOVA or by Kruskal-Wallis and Mann-Whitney test, as appropriate. Linear Mixed Models, ANOVAs and non-parametric tests were performed with IBM SPSS Statistics (IBM, v19). Chi-square and Fisher's Exact test were performed with GraphPad Prism (GraphPad Software v 5.01). Results are expressed as mean  $\pm$  SEM with  $P < 0.05$  as significant findings.

## **2.4 Results**

### **2.4.1 Pre-pregnancy**

On arrival mice were weighed, then group housed (5 mice per cage) with food consumption and weekly body weight recorded. Food consumption was similar across diets, as were the growth curves of mice on different diets. Despite this, body weight at mating was altered by calcium, with mice consuming 0.1% calcium being 0.5 g heavier than those on normal calcium diets ( $P = 0.035$ , corrected for arrival weight).

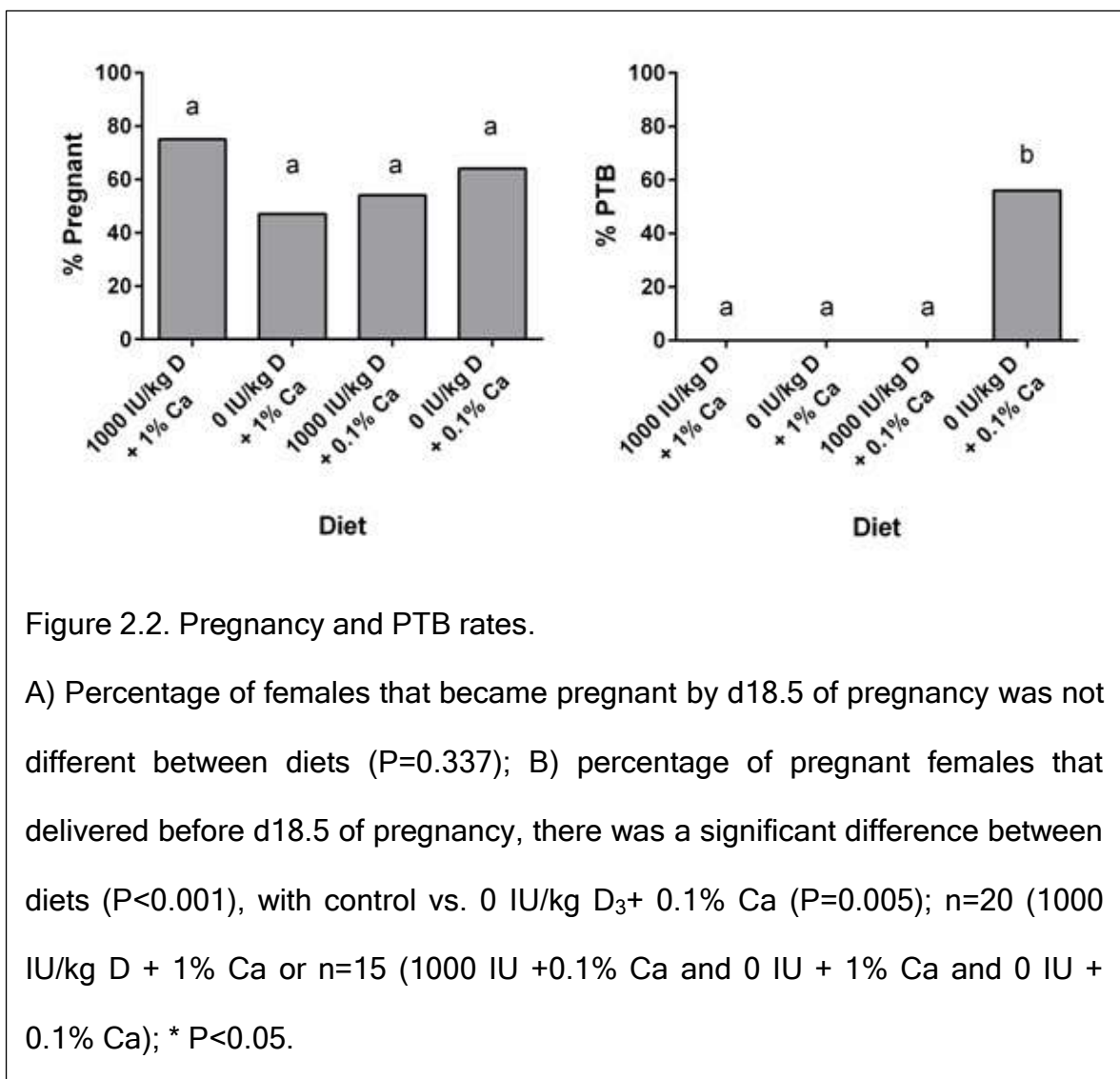
### **2.4.2 Pregnancy rate & PTB**

Mice were time-mated, with presence of a vaginal plug indicating day 0.5 of pregnancy. Only 60% of these mice became pregnant after plugs were identified

(Figure 2.2). There was no effect of diet on litter size or number of resorptions (Table 2.1Error! Reference source not found.).

Mice were sacrificed on d18.5 of pregnancy (midday of d18). Some mice delivered prior to post-mortem (d15-18) and were considered as pre-term. Rate of PTB was altered by diet ( $P < 0.001$ ), vitamin D<sub>3</sub> ( $P = 0.010$ ) and calcium ( $P = 0.014$ ), with PTB only occurring in mice consuming the most deficient diet (0 IU/kg + 0.1% Ca; (Figure 2.2)). Mice consuming the control diet had no incidence of PTB, which was significantly lower than those consuming 0 IU/kg + 0.1% which had a 56% PTB rate ( $P = 0.005$ ).

To confirm that these early deliveries were pre-term births a group of



control-fed mice (n=10), under the same conditions, was allowed to deliver their litters naturally. Of these 10 mice, 1 was non-pregnant at d18.5, leaving 9 pregnant mice; 2 of these had no plug detected so were of unknown gestation at delivery. Of the 7 mice with known delivery gestations, 2 delivered overnight between d17.5 and d18.5 (d18), while 5 delivered after d18.5 (Figure 2.3). This was unexpected, as none of our control mice had delivered before the scheduled d18.5 post-mortems prior to this. There was no difference in the incidence of PTB between deficient (0 IU/kg + 0.1% Ca) and control (1000 IU/kg + 1% Ca) delivered mice (P=0.358), although there was an overall difference in incidence between deficient mice and all control-fed mice (original and delivery; P=0.016; Figure 2.3).

### 2.4.3 Fetal & placental weights

At post-mortem fetal and placental wet weights were obtained, with mothers that delivered before d18.5 analysed separately (PTB) and mothers that had no plug identified excluded from analysis. Fetal weight was affected by diet (P<0.001), with low calcium diets resulting in a 3% increase in fetal weight compared to mice on normal calcium diets (P<0.001; Table 2.2). Although placental weight was

Table 2.1. Number of fetuses, resorptions and implantations at d18.5 of pregnancy.						
Diet	1000 IU/kg D <sub>3</sub> + 1% Ca	0 IU/kg D <sub>3</sub> + 1% Ca	1000 IU/kg D <sub>3</sub> + 0.1% Ca	0 IU/kg D <sub>3</sub> + 0.1% Ca	P-value	
n (litters)	15	7	8	10		
Viable fetuses	6.00 ± 0.79	6.57 ± 0.75	6.25 ± 0.96	6.70 ± 0.08	0.969	
Resorptions	0.47 ± 0.03	1.00 ± 0.31	1.13 ± 0.40	0.50 ± 0.27	0.352	
Total	6.57 ± 0.80	7.57 ± 0.69	7.38 ± 0.89	7.20 ± 0.87	0.779	
Note: Mean ± SEM.						

unaffected by diet, fetal:placental weight ratios were affected by diet ( $P < 0.001$ ), with the vitamin D-deficient diet decreasing the ratio by 2% ( $P = 0.020$ ) and low calcium increasing the ratio by 5% ( $P = 0.011$ ). There were also interactions for fetal weight analyses between litter size and diet / calcium and for fetal:placental weight ratio between litter size and diet / calcium / vitamin D<sub>3</sub> ( $P < 0.01$ ). The median serum 25(OH)D<sub>3</sub> level (20 nmol/L) was used to group pregnant mice, regardless of diet. Mice with  $>20$  nmol/L had 2% heavier placentas, as compared to mice with  $<20$  nmol/L ( $P = 0.028$ ), with no change in fetal weight.

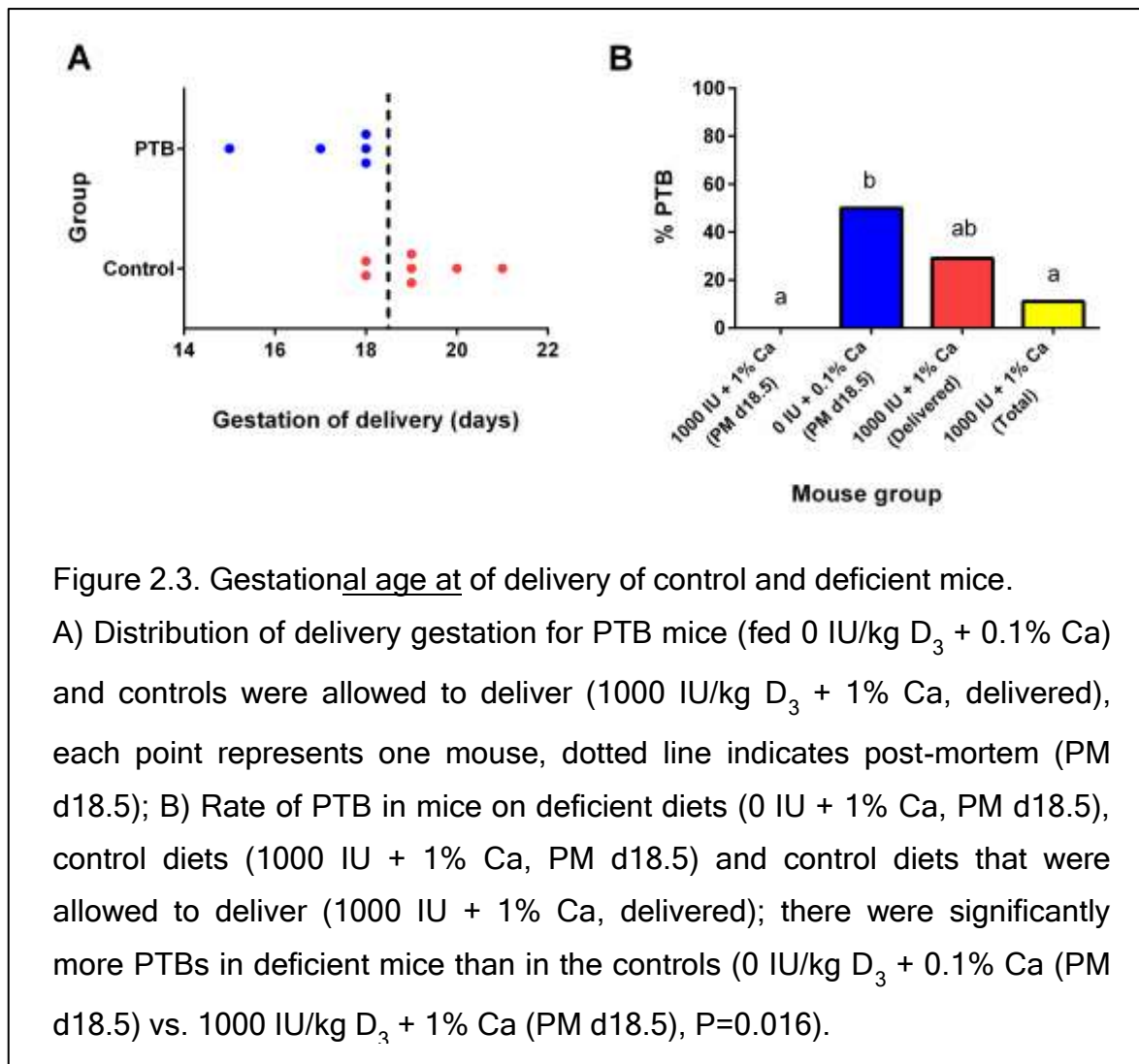
#### 2.4.4 Body composition

Maternal body composition was analysed separately for mice that were not pregnant at post-mortem (PM), pregnant at PM and those that delivered before PM (PTB). Organ weights were analysed as raw measures, percentage of PM body weight (%PM) or percentage of carcass weight (%carcass = PM weight - [fetal and placental weights]).

Differences were found, as expected, between pregnant and non-pregnant body composition by raw organ weights. Liver weight was increased 60% in pregnant mice ( $P < 0.001$ ), with a 68% increase in parametrial fat weight ( $P < 0.001$ , Table 2.3).

Non-pregnant mice body composition was altered by diet. Both the raw and %PM spleen weights were heavier in mice consuming low calcium, with raw spleen weight increased by 20% ( $P = 0.012$ ) in mice on calcium deficient compared to normal calcium diets.

Pregnant mice from each dietary treatment group demonstrated no differences in organ weights. However, there was a non-significant difference in %carcass weight for the spleen across diets ( $P = 0.056$ , corrected for litter size) and



liver weight was significantly increased with increasing litter size (P<0.01). When examining the body composition of PTB mice, it was found that they had significantly enlarged spleens compared to both pregnant and non-pregnant mice, with spleens weighing 42% more than in pregnant mice (P<0.001, Table 2.3).

#### 2.4.5 Serum measurements

Post-mortem serum measurements were performed for the first 40 mice in the study (n=10/diet). Measurements were made for serum 25(OH)D<sub>3</sub>, total calcium and inorganic phosphorus, with albumin measured to correct calcium



Diet	1000 IU/kg D <sub>3</sub> + 1% Ca	0 IU/kg D <sub>3</sub> + 1% Ca	1000 IU/kg D <sub>3</sub> + 0.1% Ca	0 IU/kg D <sub>3</sub> + 0.1% Ca	P-value
N Litters (fetuses)	8 (48)	7 (46)	7 (43)	4 (22)	
Fetal Weight (g)	1.08 ± 0.02 <sup>a</sup>	1.05 ± 0.02 <sup>a</sup>	1.12 ± 0.02 <sup>a</sup>	1.14 ± 0.03 <sup>a</sup>	<0.001
Placental Weight (mg)	92.3 ± 1.6 <sup>a</sup>	95.1 ± 1.60 <sup>a</sup>	95.8 ± 1.67 <sup>a</sup>	87.7 ± 2.40 <sup>b</sup>	0.800
Fetal to Placental Weight Ratio	11.47 ± 0.28 <sup>a</sup>	11.03 ± 0.28 <sup>a</sup>	11.77 ± 0.27 <sup>ab</sup>	13.01 ± 0.42 <sup>b</sup>	<0.001

Note: Mean ± SEM, fetal and placental weights corrected for litter size, different superscripts represent significantly different values P<0.05

measures, with mice analysed as non-pregnant, pregnant or PTB. Overall 25(OH)D<sub>3</sub> levels were found to be highest in mice consuming the control diet, with a median level of 20 nmol/L.

There was little effect of pregnancy status on serum measurements, regardless of diet. When comparing pregnant vs. non-pregnant mice, uncorrected total serum calcium concentrations were 18% higher in pregnant mice as compared to non-pregnant mice (P=0.013), while serum 25(OH)D<sub>3</sub>, phosphorus, corrected calcium and corrected calcium to phosphorous ratio (Ca:P) did not differ with pregnancy (Table 2.4).

Non-pregnant mice exhibited significantly altered serum 25(OH)D<sub>3</sub> levels due to changes in diet, with control mice having 2.4-fold higher 25(OH)D<sub>3</sub> levels and having significantly higher levels than mice fed the vitamin D- or calcium-deficient-diets (P=0.042). Serum phosphorus levels were reduced by 27-80% (per diet) in control mice compared to those on vitamin D- or calcium-deficient-diets (P=0.022), with a 50% increase in phosphate levels in mice consuming vitamin D-deficient diets (P=0.007). Albumin levels were non-significantly altered by diet (P=0.069) and were increased by 76% in mice on low calcium diets. Serum

Table 2.3. Organ weights of non-pregnant, pregnant and PTB mice at post-mortem.

Organ	Non-pregnant	Pregnant	Pregnant vs. Non-pregnant P-value	PTB	Pregnant vs. PTB P-value
N	28	25		5	
Liver (g)	0.99 ± 0.03	1.58 ± 0.03	<0.001	1.54 ± 0.07	0.969
Kidneys (g)	0.28 ± 0.01	0.28 ± 0.01	0.794	0.29 ± 0.01	0.978
Spleen (g)	0.06 ± 0.01	0.06 ± 0.01	0.983	0.08 ± 0.01	<0.001
Parametrial Fat (g)	0.26 ± 0.02	0.44 ± 0.02	<0.001	0.39 ± 0.04	0.568
Heart (g)	0.13 ± 0.01	0.14 ± 0.01	0.193	0.13 ± 0.01	0.695
Lungs (g)	0.17 ± 0.01	0.17 ± 0.01	0.940	0.17 ± 0.01	0.999

Note: Mean ± SEM; groups regardless of diet; pregnant mice were at d18.5 of gestation

calcium levels were increased on calcium-deficient diets by 19% (P=0.047), with no effect on corrected calcium levels or Ca:P ratio. This may be due to increased 1,25(OH)<sub>2</sub>D<sub>3</sub> which would increase bone resorption and intestinal calcium uptake release of calcium from bones to increase available serum calcium

Pregnant mice on the control diet had the highest serum 25(OH)D<sub>3</sub> levels, though they were lower than those in control-fed non-pregnant mice. Serum calcium, corrected calcium and phosphorous were unchanged across diets. Serum albumin levels were non-significantly altered across diets (P=0.060), with the highest levels found in the mice consuming the most deficient diet (0 IU/kg + 0.1% Ca). Serum albumin was also altered by dietary calcium, with low calcium consuming mice having 50% higher albumin concentrations (P=0.050). Serum Ca:P was non-significantly affected by dietary calcium, with mice consuming low calcium diets having a 35% decrease in Ca:P (P=0.083).

Table 2.4. Serum measurements of vitamin D and calcium in pregnant and non-pregnant mice.

Pregnancy Status	Non-pregnant					Pregnant					Pregnant vs. Non-pregnant
	1000 IU/kg	0 IU/kg	1000 IU/kg	0 IU/kg	P-value	1000 IU/kg	0 IU/kg	1000 IU/kg	0 IU/kg	P-value	
Dietary vitamin D <sub>3</sub>	1%	1%	0.1%	0.1%		1%	1%	0.1%	0.1%		
Dietary calcium											
N	3	4	4	3		3	5	5	2		
25(OH)D <sub>3</sub> (nmol/L)	48.667 ± 24.168 <sup>a</sup>	22.000 ± 2.041 <sup>ab</sup>	18.750 ± 1.315 <sup>b</sup>	20.667 ± 0.882 <sup>ab</sup>	0.046	41.333 ± 21.365 <sup>a</sup>	22.600 ± 5.046 <sup>a</sup>	17.200 ± 0.800 <sup>a</sup>	17.000 ± 3.000 <sup>a</sup>	0.315	0.634
Total Ca (nmol/L)	1.697 ± 0.316 <sup>a</sup>	1.980 ± 0.075 <sup>a</sup>	2.200 ± 0.117 <sup>a</sup>	2.087 ± 0.081 <sup>a</sup>	0.219	2.200 ± 0.067 <sup>a</sup>	2.222 ± 0.126 <sup>a</sup>	2.324 ± 0.206 <sup>a</sup>	2.445 ± 0.205 <sup>a</sup>	0.851	0.013
Corrected Ca (nmol/L)	1.417 ± 0.593 <sup>a</sup>	2.070 ± 0.106 <sup>a</sup>	1.628 ± 0.267 <sup>a</sup>	1.150 ± 0.121 <sup>a</sup>	0.236	1.893 ± 0.246 <sup>a</sup>	2.002 ± 0.125 <sup>a</sup>	1.798 ± 0.201 <sup>a</sup>	1.275 ± 0.205 <sup>a</sup>	0.202	0.283
P (nmol/L)	1.537 ± 0.072 <sup>a</sup>	2.778 ± 0.188 <sup>b</sup>	1.955 ± 0.159 <sup>ab</sup>	2.220 ± 0.478 <sup>ab</sup>	0.022	2.177 ± 0.159 <sup>a</sup>	1.858 ± 0.370 <sup>a</sup>	2.306 ± 0.148 <sup>a</sup>	2.310 ± 0.260 <sup>a</sup>	0.611	0.907
Ca:P Ratio	0.916 ± 0.395 <sup>a</sup>	0.748 ± 0.022 <sup>a</sup>	0.854 ± 0.156 <sup>a</sup>	0.587 ± 0.162 <sup>a</sup>	0.714	0.870 ± 0.089 <sup>a</sup>	1.277 ± 0.270 <sup>a</sup>	0.800 ± 0.112 <sup>a</sup>	0.549 ± 0.027 <sup>a</sup>	0.176	0.293
Albumin (g/L)	53.967 ± 15.967 <sup>a</sup>	35.425 ± 8.231 <sup>a</sup>	68.775 ± 13.836 <sup>a</sup>	86.900 ± 9.301 <sup>a</sup>	0.069	56.267 ± 12.248 <sup>a</sup>	51.100 ± 8.393 <sup>a</sup>	66.480 ± 5.712 <sup>a</sup>	98.800 ± 20.500 <sup>a</sup>	0.060	0.701

Note: Ca - calcium, P- inorganic phosphorus, Ca:P ratio - corrected calcium to phosphorus ratio; Mean ± SEM; different superscripts

denote significant differences; pregnant mice were at d18.5 of pregnancy.

## 2.4.6 Kidney gene expression

Kidney gene expression was analysed as this is the main site of 25(OH)D<sub>3</sub> metabolism, with proposed changes in metabolism during pregnancy. We compared expression in non-pregnant, pregnant and PTB mice.

### 2.4.6.1 Vitamin D<sub>3</sub> metabolism genes

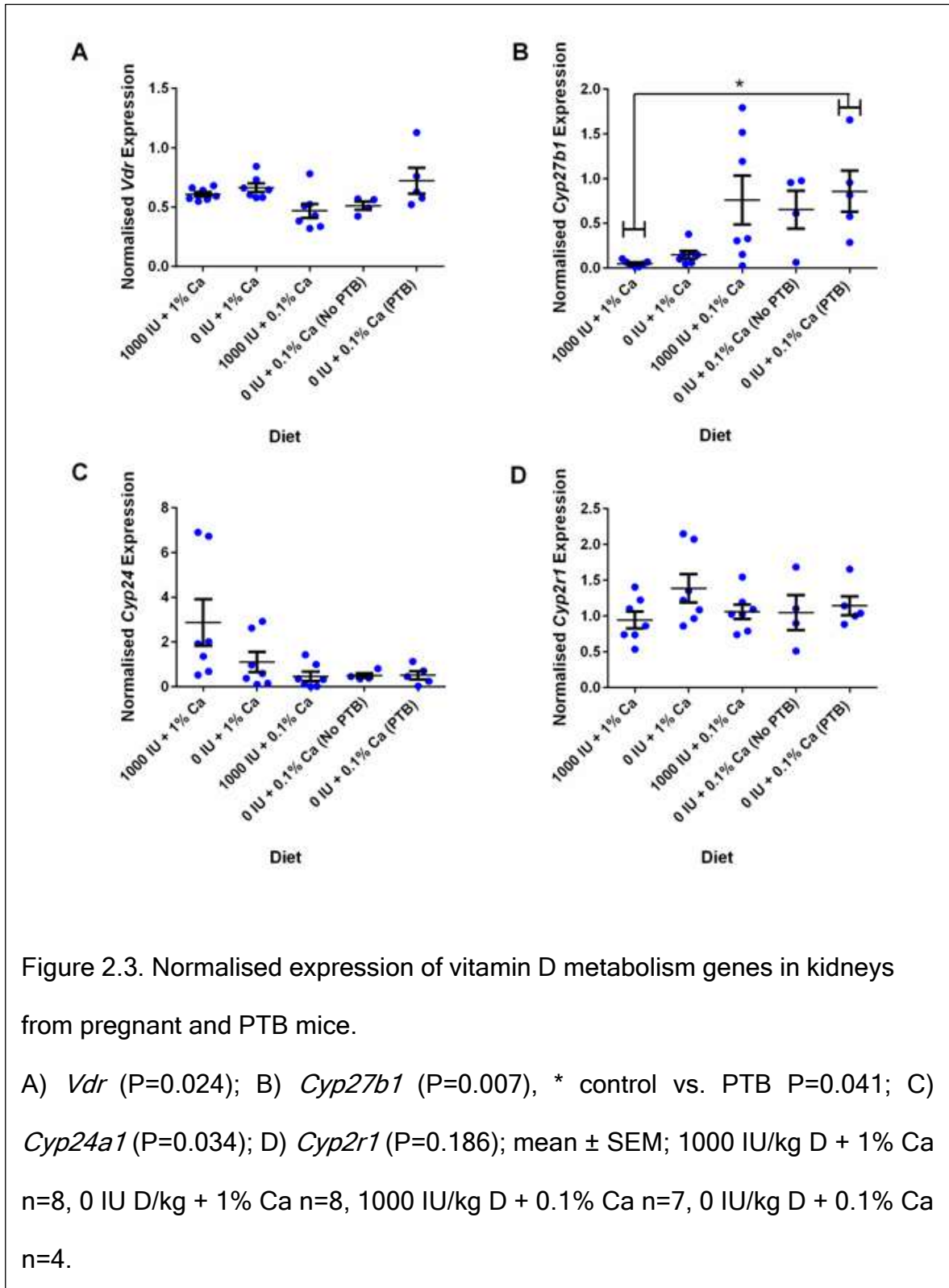
Non-pregnant mice consuming low in vitamin D-deficient diets had a 55% reduction in renal expression of *Cyp24a1* (P=0.034, Figure 2.4) compared to those consuming normal vitamin D<sub>3</sub> diets, while low calcium diets induced a non-significant 94% increase in *Cyp27b1* expression (P=0.071) compared to normal calcium diets. In comparison, pregnant mice had amplified gene expression responses to low calcium diets. *Cyp24a1* expression was reduced by 82% (P=0.016, Figure 2.5) and *Cyp27b1* expression was increased 7.4-fold (P<0.001) in pregnant mice consuming low calcium diets compared to normal calcium.

When comparing gene expression between pregnant and non-pregnant mice, similar expression levels were seen for *Vdr* and *Cyp24a1*. *Cyp27b1* expression was increased 3-fold in kidneys from pregnant compared to non-pregnant mice, irrespective of diets (P=0.015, Figure 2.6). *Cyp2r1* expression was not affected by diet in either pregnant or non-pregnant mice, but was affected by pregnancy with 40% higher expression in non-pregnant mice (P<0.001).

### 2.4.6.2 FGF23/Klotho axis genes

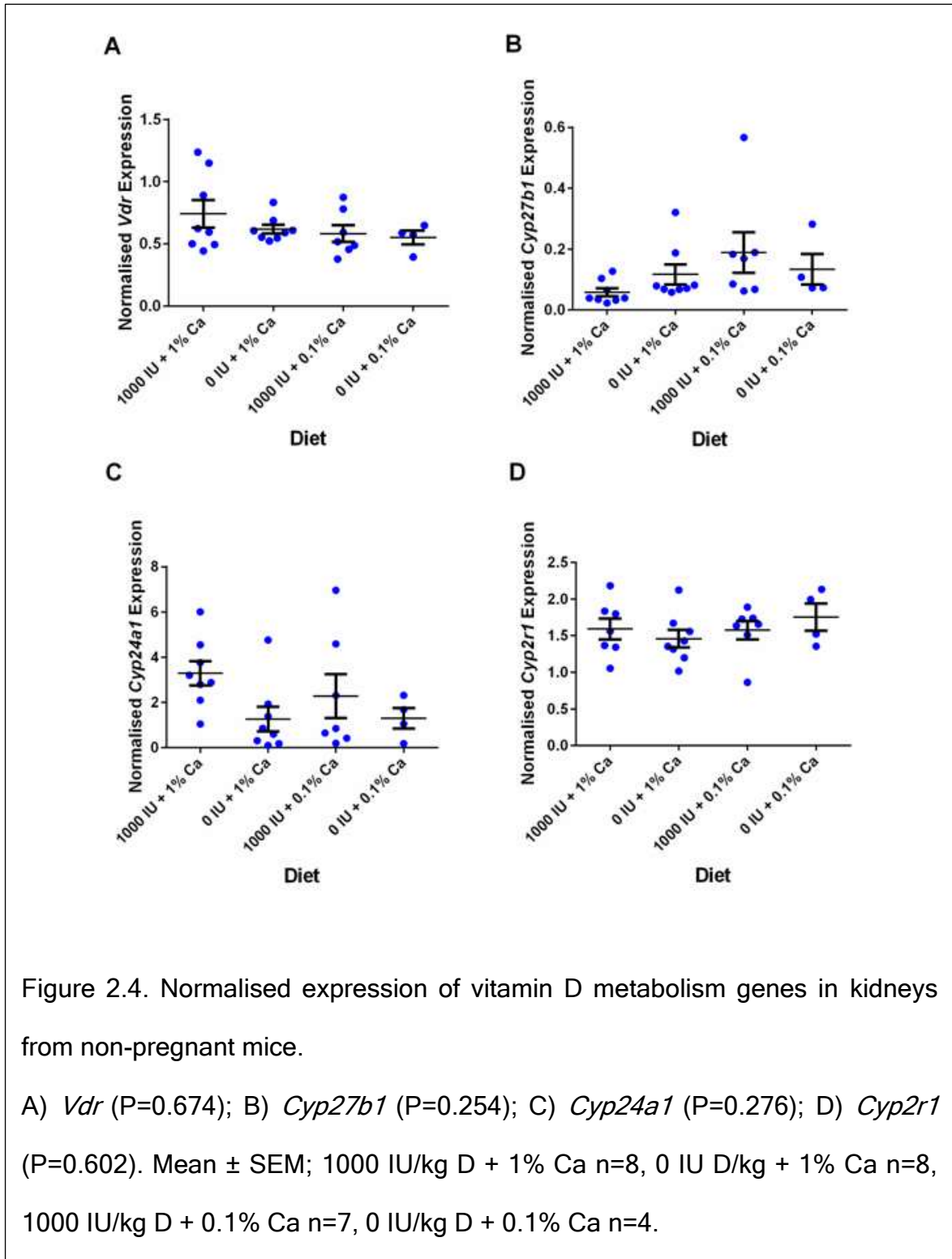
To determine whether calcium metabolism in pregnancy is influenced by the FGF23/Klotho axis which regulates circulating phosphate concentrations, gene expression of *Napi2a* and *Napi2c*, which are reporter genes of FGF23/Klotho

activity, were quantified. Expression was similar between diets (Figure 2.7, Figure 2.8) and between pregnant and non-pregnant mice (Figure 2.9).



## 2.4.7 PTB Kidney

Kidney gene expression from PTB mice was compared to expression in pregnant and non-pregnant mice. *Cyp27b1* expression was highest in kidneys from PTB mice, with pregnant mice having 66% lower expression ( $P=0.036$ ) and



non-pregnant mice 86% lower expression ( $P=0.001$ ; Figure 2.6). However, PTB kidney expression of *Cyp27b1* was similar to mice on the most deficient diet (0 IU/kg + 0.1% Ca). *Cyp24a1* was also reduced by 75% as compared to non-pregnant kidneys ( $P=0.001$ ), with similar expression levels to pregnant kidneys. Both *Napi2a* and *Napi2c* had significantly decreased expression in kidneys from mice that delivered pre-term, with *Napi2a* being 6.6-fold higher than in pregnant and 7-fold higher than in non-pregnant mice ( $P<0.001$ ), while *Napi2c* was 7.7-fold higher than in pregnant and 6.9-fold higher in non-pregnant mice ( $P<0.001$ ; Figure 2.9).

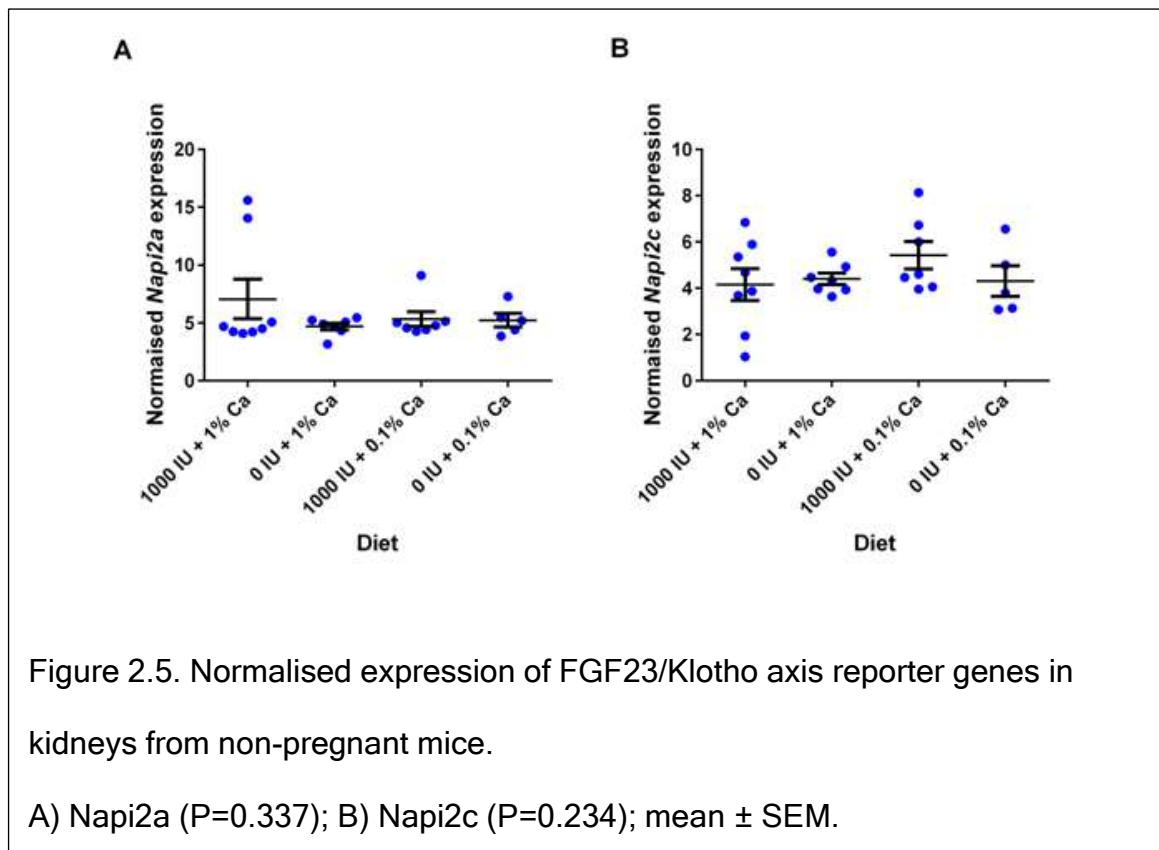
#### **2.4.8 Fetal skeletal mineralisation**

To determine whether diets that are vitamin D-deficient and low in calcium affected fetal skeletal mineralisation, we examined fetuses from control (1000 IU/kg + 1% Ca) and deficient diets (0 IU/kg + 0.1% Ca). We sought to confirm previous reports that there was no skeletal defect by x-ray imaging of fetuses. As there is variation between the largest and smallest fetuses in litters, we weight matched control and deficient fetuses and x-rayed each pair. There was no difference in fetal mineralization at this gestation (Figure 2.10) as determined by fifteen pairs of fetuses from nine litters.

## **2.5 Discussion**

Dietary restriction of vitamin D<sub>3</sub> and calcium, as well as pregnancy, affected vitamin D<sub>3</sub> metabolism, length of gestation, body composition, serum measurements of calcium homeostasis and kidney gene expression.

### **2.5.1 Preterm birth**



Although pregnancy rate was unaffected by vitamin D<sub>3</sub> or calcium deficiency, gestation of delivery was affected. Half of the mice fed the most deficient diet (0 IU/kg + 0.1% Ca) delivered pre-term (before d18.5 of gestation, PTB), with no early deliveries in other diet groups. The gestation of delivery of C57Bl6 mice has previously been determined to be d19.5 (Murray, Morgan et al. 2010); so to confirm that the early deliveries were not part of the normal gestational range for mice on our control diet, we allowed a group of control mice to deliver. Surprisingly, two of seven mice delivered before d18.5 and would have been deemed PTBs, while five mice delivered from d19-d21 of gestation. There was greater PTB incidence in deficient mice than in all control mice, so this suggests that delivery before d18.5 could be pathological, indicating PTB.

There are two overlapping mechanisms by which vitamin D and calcium deficiency could induce early labour: inflammation or altered immune function due to vitamin D deficiency. Inflammation is a major cause of spontaneous PTB



(Romero, Espinoza et al. 2007), with our PTB mice having evidence of inflammation due to their enlarged spleens. Studies of placental inflammation in the presence or absence of vitamin D signaling have shown that vitamin D in the placenta is anti-inflammatory and suppresses TLR-2, IL-6 and IFN- $\gamma$  release after LPS challenge (Liu, Kaplan et al. 2011). Trophoblasts express Toll-like receptors

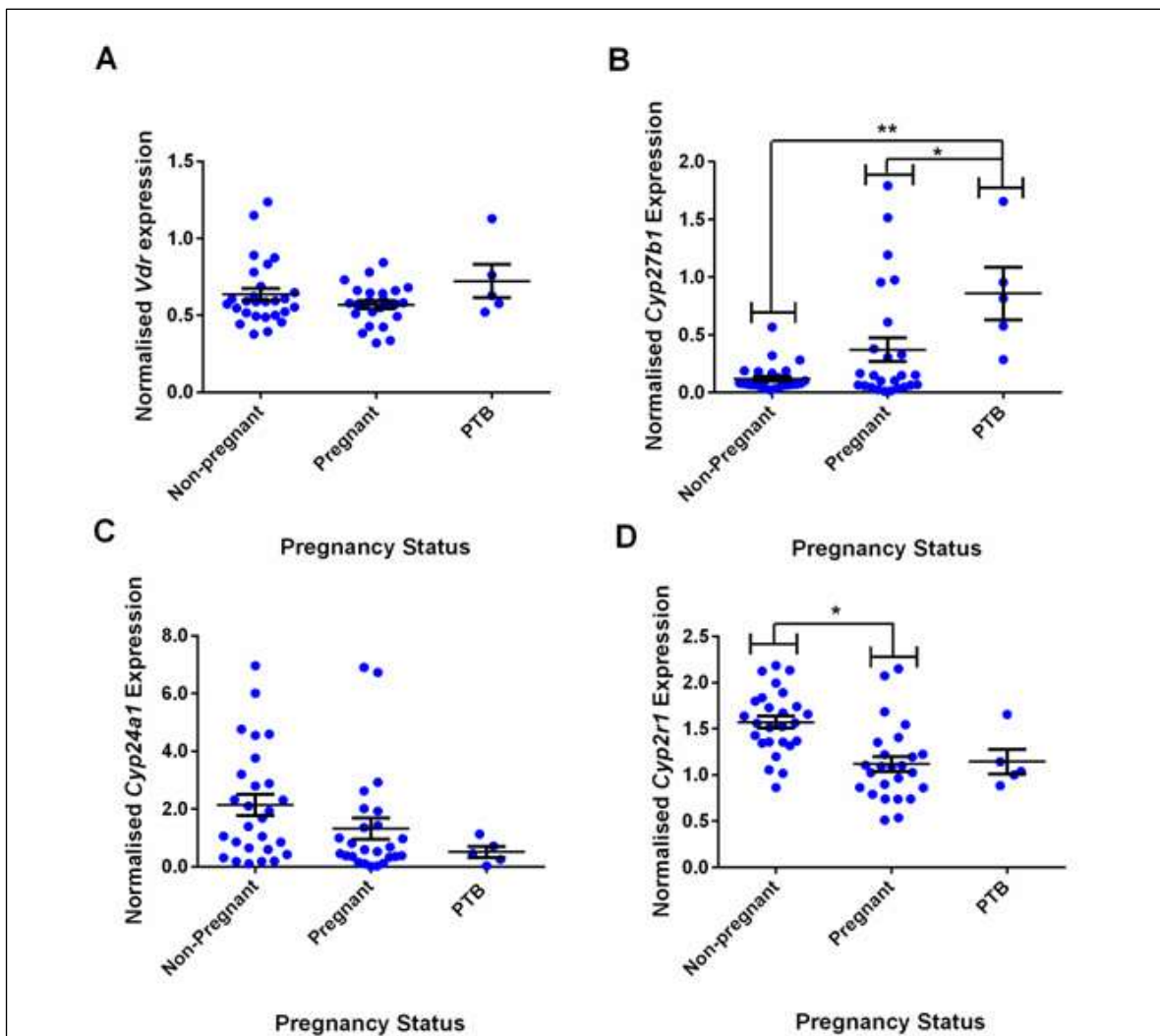


Figure 2.6. Normalised expression of vitamin D-related genes in kidneys from non-pregnant, pregnant and PTB mice.

A) *Vdr* (P=0.163); B) *Cyp27b1* (P=0.008), \* P=0.039, \*\* P=0.001 C) *Cyp24a1* (P=0.157); D) *Cyp2r1* (P<0.001), \* P<0.001; mean  $\pm$  SEM.

(TLRs) TLR-2&4 which allow the trophoblasts to respond to inflammation by cytokine production or apoptosis (Abrahams, Bole-Aldo et al. 2004), with activation causing production of inflammatory cytokines, antimicrobial peptides and chemokines, similar to the inflammation seen in term parturition (Keelan, Blumenstein et al. 2003).

Increased PTB in vitamin D<sub>3</sub> deficiency is in accordance with recent epidemiological evidence that vitamin D<sub>3</sub> deficiency increases the risk for PTB in humans (Perez-Ferre, Torrejon et al. 2012, Wagner, McNeil et al. 2013). Vitamin D<sub>3</sub> is suggested to reduce placental colonization with species common to bacterial vaginosis and hence could reduce PTB incidence (Grant 2011). Vitamin D<sub>3</sub> deficiency may play a role in the infectious aetiology of PTB as vitamin D<sub>3</sub> is immunomodulatory, increasing innate immune activity and reducing inflammation

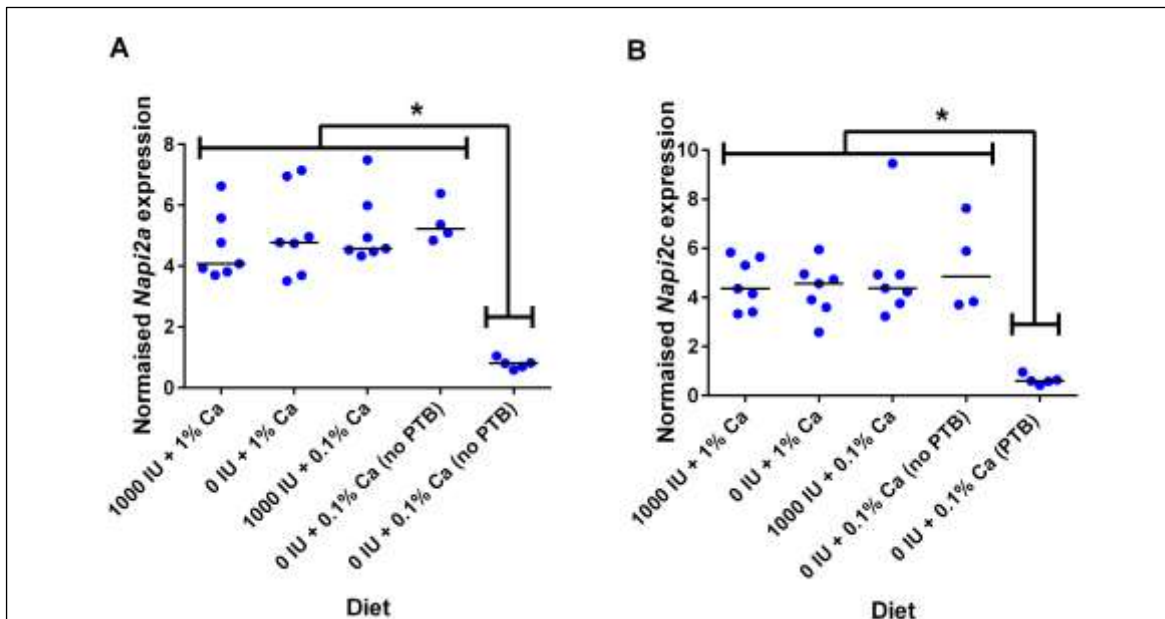


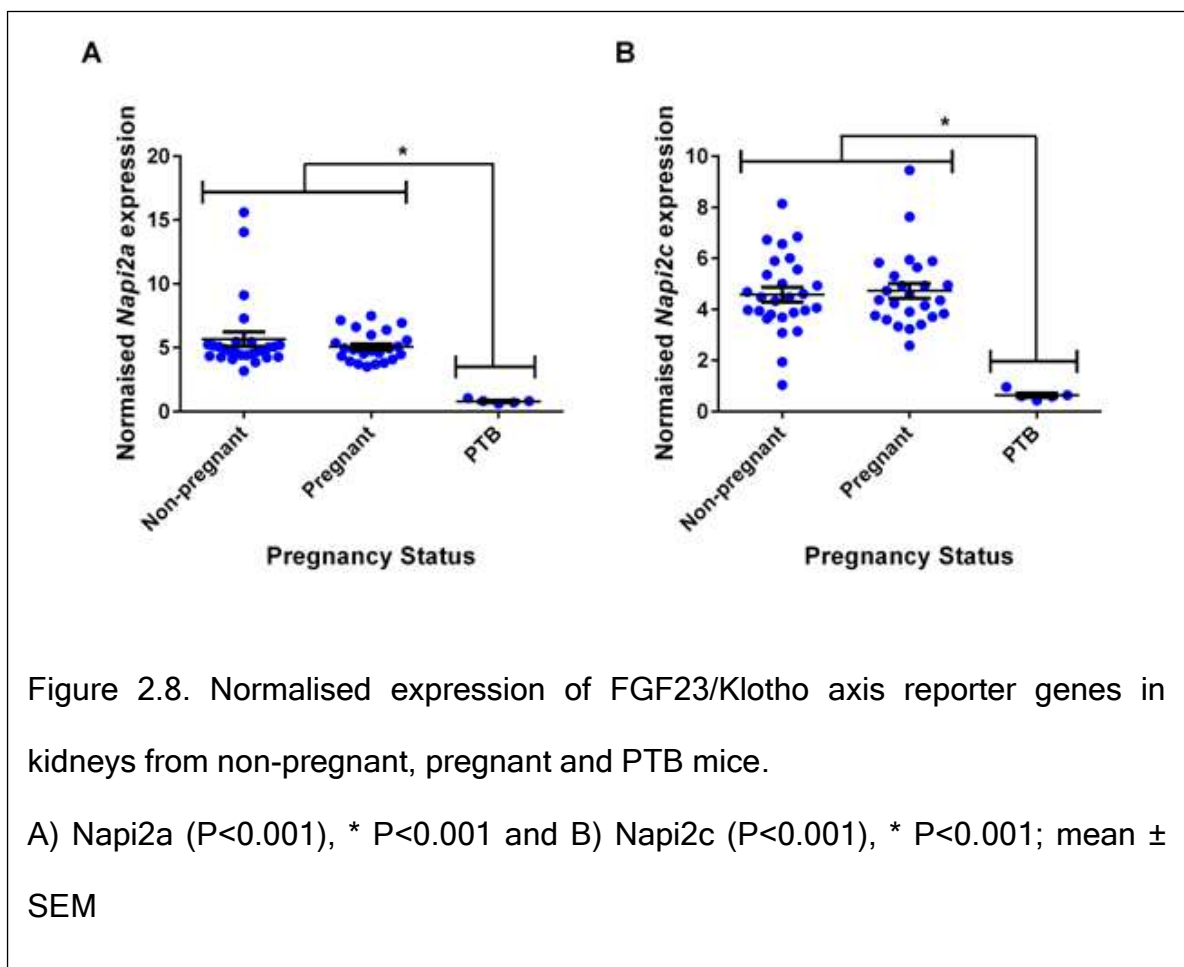
Figure 2.7. Normalised expression of FGF23/Klotho axis reporter genes in kidneys from pregnant mice.

A) Napi2a (P<0.001), \* P<0.001; B) Napi2c (P=0.001), \* P<0.001; mean ± SEM.

(Hewison 2012).  $1,25(\text{OH})_2\text{D}_3$  has also been found to increase production of cathelicidin, an antimicrobial protein, in human placental explants (Liu, Kaplan et al. 2009). Although the PTB mice and non-PTB mice on the most deficient diet (0 IU/kg + 0.1% Ca) had lower  $25(\text{OH})\text{D}_3$  levels than controls, there was no evidence of vitamin  $\text{D}_3$  metabolism being further altered as gene expression was similar between deficient mice with and without PTB. An infection/inflammation cause of PTB is suggested by the increased spleen weight in our PTB mice.

## 2.5.2 Fetal and placental weights

Fetal weight was increased in those mice that were consuming low calcium diets, as was fetal to placental weight ratio which is a measure of placental efficiency. Although there was increased fetal weight in mice on deficient diets,



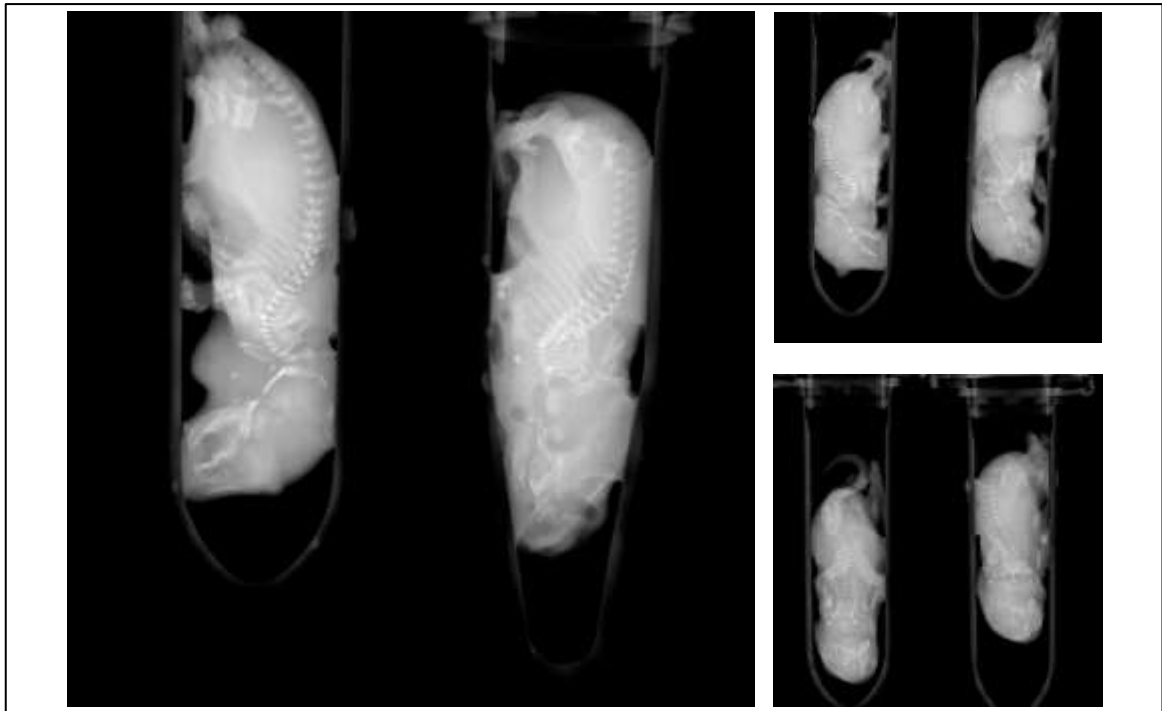


Figure 2.9. Representative X-ray images of fetal skeletons.

Fetuses (in tubes) are positioned as control fed mice (1000 IU + 1% Ca) on the left and deficient fed mice (0 IU + 0.1% Ca) on the right of each image. Each image is a pair of weight matched fetuses, 15 pairs of fetuses were analysed from 9 litters. There was no visible difference in skeletal mineralization at d18.5 of gestation.

there was no alteration in placental weight or dimensions, so there must be morphological changes in the placenta to support greater fetal growth (see Chapter 3). Placental weight was, however, marginally increased in mice with  $>20$  nmol/L of 25(OH) $D_3$ . Increased placental efficiency, as indicated by the increased fetal placental weight ratio, in calcium deficient mice indicates that this mild insult increases fetal growth without increasing placental mass. Placental efficiency has previously been reported to increase due to mild insults, such as moderate alteration of nutrients, with severe insults such as changes to vasculature or extreme alteration of nutrients causing decreased placental efficiency (Fowden, Sferruzzi-Perri et al. 2009). This was also seen in a recent study of vitamin  $D_3$

deficiency, with higher fetal weights and unaltered placental weights at d18 in mice consuming diets lacking vitamin D<sub>3</sub> (Liu, Ouyang et al. 2013). The study also found that maternal blood pressure was increased in mice on vitamin D<sub>3</sub> deficient diets, which may be a mechanism for alteration of placental morphology (see Chapter 3). Dietary vitamin D<sub>3</sub> and/or calcium deficiency appears to alter placental morphometry but not dimensions, possibly through increased maternal blood pressure and hence placental perfusion pressure, to increase placental efficiency and increase fetal weight at d18.5 of gestation.

### **2.5.3 Body composition**

There were differences in body composition during pregnancy, with pregnant mice having heavier livers and parametrial fat. This is expected as maternal physiology changes dramatically during pregnancy, mainly under the influence of estrogens and progesterone (Bacq 2000). A gradual increase in liver size is observed in rodent pregnancies due to the high demand of multiple fetuses, with a higher litter size resulting in a heavier liver, but may also facilitate a higher rate of conversion of vitamin D<sub>3</sub> to 25(OH)D<sub>3</sub> during pregnancy. Parametrial fat stores are also increased in pregnancy under the control of estrogens (Cooke and Naaz 2004).

### **2.5.4 Serum measurements**

Pregnancy induces increased blood volume, causing modification of many serum measures, although ionized (free) serum calcium concentration remains constant.

Post-mortem serum measurements were also compared, with higher uncorrected total calcium levels in pregnant mice compared to non-pregnant.

Other serum measures, such as phosphate, corrected calcium and albumin did not differ with pregnancy status. Increased total calcium in pregnancy is unusual, as human studies report mild decreases of total serum calcium across gestation (Ardawi, Nasrat et al. 1997, Kovacs 2009), although this was not significantly different from non-pregnant values when corrected for serum albumin as most circulating calcium is bound to albumin.

Non-pregnant mice had significantly altered 25(OH)D<sub>3</sub> by diet, with control mice having the highest levels. This was expected from our experimental design, but was not found in pregnant mice, as although the levels were highest in controls, there was no significant difference between diets. This may be due to a higher demand in pregnancy to convert 25(OH)D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub>, as in human and mouse pregnancy 1,25(OH)<sub>2</sub>D<sub>3</sub> increases across gestation, while 25(OH)D<sub>3</sub> has a mild decrease (Ardawi, Nasrat et al. 1997, Kirby, Ma et al. 2013). This is seen in other situations, where calcium has a sparing effect on the 25(OH)D<sub>3</sub> levels, protecting them from depletion, for example, in a study in rats animals were fed high calcium diets and didn't suffer 25(OH)D<sub>3</sub> depletion (Lips 2004, Anderson, Lee et al. 2010).

Albumin levels were altered by diet in both pregnant and non-pregnant mice, with low calcium-fed mice having higher albumin levels, regardless of pregnancy status. Albumin levels have previously been positively associated with blood pressure in human studies (Hostmark, Tomten et al. 2005), suggesting that the high albumin levels could be associated with, but not causative of, higher blood pressure in mice on low calcium diets. A previous study in mice has found increased blood pressure in vitamin D<sub>3</sub> deficient mice (Liu, Ouyang et al. 2013) and calcium supplementation has previously been successful in reducing the

incidence of gestational hypertension and preeclampsia in humans (Hofmeyr, Atallah et al. 2006).

### 2.5.5 Vitamin D<sub>3</sub> metabolism in pregnancy

Kidney metabolism of vitamin D<sub>3</sub> has been well studied previously, but had not been investigated in pregnant mice with vitamin D<sub>3</sub> and calcium deficiency. Non-pregnant control mice had baseline *Cyp27b1* expression as the diet contains adequate calcium, so PTH-induced expression of *Cyp27b1* wouldn't occur as additional 1,25(OH)<sub>2</sub>D<sub>3</sub> would not have been required to maintain intestinal calcium absorption. Kidney *Cyp24a1* expression is highly variable in these mice as it is directly up regulated in response to local 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations. In comparison to controls, the low calcium-fed mice had non-significantly higher *Cyp27b1* expression which indicates increased production of 1,25(OH)<sub>2</sub>D<sub>3</sub> to increase serum calcium levels. *Cyp24a1* expression is lower in low calcium consuming mice as the locally made 1,25(OH)<sub>2</sub>D<sub>3</sub> enters the circulation (Christakos, Ajibade et al. 2012).

Comparison of kidney gene expression between pregnant and non-pregnant mice suggests that 1,25(OH)<sub>2</sub>D<sub>3</sub> production is enhanced in pregnancy with a 3-fold increase in *Cyp27b1* expression, compared to non-pregnant gene expression. This corresponds with the increased circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels found in pregnancy. Interestingly, renal *Cyp2r1* expression was unaffected by diet, but was found to be decreased in pregnant compared to non-pregnant mouse kidneys.

In pregnant mice, low calcium diets induced high *Cyp27b1* and low *Cyp24a1* expression, with no change in *Vdr* or *Cyp2r1* expression. This is due to stimulated 1,25(OH)<sub>2</sub>D<sub>3</sub> production which enters the circulation to stimulate

intestinal calcium absorption and bone reabsorption to maintain calcium homeostasis and provide calcium to the fetus (Kovacs 2009). In non-pregnant animals, this increased  $1,25(\text{OH})_2\text{D}_3$  production would normally be attributed to PTH stimulation of *Cyp27b1* expression, but as PTH is greatly reduced in pregnancy, there must be other mechanisms responsible for the increased  $1,25(\text{OH})_2\text{D}_3$  in pregnancy. Until recently, the source of the increased circulating  $1,25(\text{OH})_2\text{D}_3$  was thought to be either of placental or renal origin, but recently our work and that of others (Kirby, Ma et al. 2013) have demonstrated that the increased  $1,25(\text{OH})_2\text{D}_3$  levels are predominantly due to increased renal production, rather than from the placenta.

Kidneys from PTB mice were shown to have similar levels of expression of *Vdr*, *Cyp2r1* and *Cyp24a1* as pregnant mice. Although they had a higher median *Cyp27b1* expression than in pregnant mice overall, there was high variation with a similar level of expression to the non-PTB mice from the same dietary group (0 IU/kg + 0.1% Ca). There is no evidence of altered vitamin  $\text{D}_3$  metabolism in mice that delivered preterm.

The FGF23/Klotho axis may play a role in calcium/phosphate homeostasis in pregnancy as there is a PTH-independent rise in  $1,25(\text{OH})_2\text{D}_3$  in pregnancy, with no known mechanism. Increased Fgf23 protein in pregnancy would inhibit renal *Cyp27b1* expression and PTH production. Therefore, if FGF23/Klotho levels were low in pregnancy, they would facilitate increased *Cyp27b1* expression. In addition, Klotho (Kuro-o, Matsumura et al. 1997), *Napi2a* and *Napi2c* (Nishimura and Naito 2008), but not FGF23 (Zhong, Wang et al. 2006) are expressed in the placenta. To determine whether FGF23/Klotho axis was altered by pregnancy and diet we examined reporter genes, the sodium phosphate transporters *Napi2a* and *Napi2c*. There was no effect of pregnancy or diet on expression of *Napi2a* or *Napi2c*,



indicating that the FGF23/Klotho axis is not involved in the increase of 1,25(OH)<sub>2</sub>D<sub>3</sub> production in pregnancy. A recent study has discovered that circulating FGF23 is increased 2-fold in pregnant mice, as compared to non-pregnant mice (Kirby, Ma et al. 2013). This data indicates that the FGF23/Klotho is not playing a major role in the up regulation of circulating 1,25(OH)<sub>2</sub>D<sub>3</sub>.

Interestingly, mice that delivered preterm had a significant reduction in both *Napi2a* and *Napi2c* expression compared to pregnant and non-pregnant mice. This may be due to these genes being under the influence of PTH, which increases at post-partum, as these kidneys were harvested at post-mortem after delivery. This decreased expression is unusual though, as previous reports have found that most regulation of these transporters is at the protein level by glycosylation (Forster, Hernando et al. 2013). As the FGF23/Klotho axis has only recently been discovered (Yamashita, Yoshioka et al. 2000), we will surely have more insight into its function in pregnancy in years to come.

### **2.5.6 Strengths and limitations**

Our study is strengthened by examining the role of both calcium and vitamin D<sub>3</sub> as this has allowed us to better tease out their roles, finding that many changes in gene expression and physiology could be attributed to dietary calcium. We were limited in that we had serum measurements for only the first half of our mice and that the levels of blood calcium and 25(OH)D<sub>3</sub> were lower than expected. This could be attributed to the interference of the high albumin levels in these mice, which is why we then corrected calcium for albumin levels. Our study could have been further strengthened by having blood pressure data, as well as 1,25D and renal enzyme activity measures as this would have given us further insight into the physiology of pregnant vitamin D<sub>3</sub> deficient mice.

### 2.5.7 Conclusion

In conclusion, dietary calcium and vitamin D<sub>3</sub> restriction together alters vitamin D<sub>3</sub> metabolism in pregnancy. This is indicated by altered kidney gene expression of *Cyp27b1* and *Cyp24a1*, as well as reduced levels of 25(OH)D<sub>3</sub>. Dietary calcium restriction, regardless of dietary vitamin D<sub>3</sub> altered fetal weight and kidney gene expression. Restriction of vitamin D<sub>3</sub> and calcium also increased the incidence of PTB, with the most deficient mice having a 60% PTB rate. This study emphasizes the need to examine the contribution of both calcium and vitamin D<sub>3</sub> in pregnancy.

# Statement of Authorship

Title of Paper	Chapter 3 - Dietary vitamin D and calcium deficiency alter placental development and gene expression
Publication Status	<input type="radio"/> Published, <input type="radio"/> Accepted for Publication, <input type="radio"/> Submitted for Publication, <input checked="" type="radio"/> Publication style
Publication Details	Written in publication style, currently being prepared for publication.

## Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Jessica Laurence	
Contribution to the Paper	Experimental design Experimental work - mouse, PCR, immunohistochemistry Data analysis Manuscript preparation	
Signature		Date 23/10/14

Name of Co-Author	Tina Bianco-Miotto	
Contribution to the Paper	Experimental troubleshooting - PCR Analysis of data Critical comments on manuscript	
Signature		Date 17/10/14

Name of Co-Author	Paul Anderson	
Contribution to the Paper	Experimental design Interpretation of data Critical comments on manuscript	
Signature		Date 22/10/14

Name of Co-Author	Claire Roberts	
Contribution to the Paper	Experimental design Interpretation of data Critical comments on manuscript	
Signature		Date 23.10.14

## Chapter 3: Dietary vitamin D<sub>3</sub> and calcium deficiency alter placental development and gene expression

### 3.1 Abstract

Vitamin D<sub>3</sub> deficiency has been associated with a wide range of pregnancy complications that feature poor placental development. Vitamin D<sub>3</sub> is best known for its role in calcium homeostasis; however, it also regulates 3% of the genome through the vitamin D receptor (VDR). Although VDR and vitamin D<sub>3</sub> metabolism enzymes are expressed in the placenta, little is known about the role of vitamin D<sub>3</sub> in placental development. This study examines the effects of dietary vitamin D<sub>3</sub> and calcium deficiency on placental morphogenesis during murine pregnancy. We hypothesised that fetal and placental weights will be reduced, with altered placental morphology in response to vitamin D<sub>3</sub> and calcium deficiency.

Three week old female C57Bl6 mice were fed one of 4 diets: normal or vitamin D-deficient and normal or low-calcium (1000 IU/kg or 0 IU/kg vitamin D<sub>3</sub>, 1% or 0.1% calcium) through mating at 13 weeks old. These mice were mated, then sacrificed at day 18.5 of pregnancy. The fetal and placental weights were recorded, with two placentas per litter fixed for histology. Placental histology and gene expression were examined.

Fetuses and placentas were weighted, with no change in placental weight between diets. Surprisingly, fetuses from dams fed calcium deficient diets, were 6% heavier than those on calcium sufficient diets ( $P=0.006$ ), regardless of the level of dietary vitamin D<sub>3</sub>. Fetal to placental weight ratios were also higher ( $P=0.018$ ) suggesting that mice on calcium deficient diets have more efficient placentas. The placental labyrinth (LZ) to junctional zone (JZ) ratio was used to determine the

relative feto-maternal exchange area in each dietary group. The LZ:JZ ratio in mice fed low calcium or vitamin D<sub>3</sub> was 10% lower (P=0.019; trend, P=0.054) than when compared to control mice (1000 IU/kg + 1% Ca) indicating that placentas from mice fed the vitamin D-deficient and/or low calcium diets had proportionally less junctional zone area for feto-maternal exchange.

Morphometry of the labyrinth revealed that vitamin D-deficient-fed mice had increased maternal blood space and fetal capillary density (P<0.01). Whereas mice fed the low calcium diets had reduced fetal capillary volume density, with a thinner trophoblast layer (P<0.001), resulting in reduced trophoblast barrier thickness across which feto-maternal exchange occur. These findings together suggest that placentas from deficient diets adapt by altering labyrinth composition and by reducing the distance for feto-maternal exchange, thus optimizing feto-maternal nutrient transfer, which may result in augmented fetal growth.

Placental gene expression was altered in mice consuming low calcium diets, with increased *Cyp24a1* (P=0.005) and *Vdr* (P=0.042) compared to mice consuming normal calcium, likely to be due to the effect of the low calcium diet raising circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels.

In conclusion, there was an effect of dietary calcium on placental morphometry and vitamin D<sub>3</sub> pathway genes, with little effect of dietary vitamin D<sub>3</sub>. This resulted in deficient mice having a more efficient placenta, with gene expression data suggested compensatory changes in expression. This demonstrates potential interactions between dietary calcium and vitamin D<sub>3</sub> intake in pregnancy and strengthens our knowledge of vitamin D<sub>3</sub>'s actions in pregnancy.

## 3.2 Introduction

Vitamin D<sub>3</sub> deficiency in pregnancy has been associated with a range of pregnancy disorders from infertility (Lerchbaum and Obermayer-Pietsch 2012) to complications such as preeclampsia (Bodnar, Catov et al. 2007, Robinson, Alanis et al. 2010, Nassar, Halligan et al. 2011, Woodham, Brittain et al. 2011, Aghajafari, Nagulesapillai et al. 2013) and pre-term birth (PTB) (Shibata, Suzuki et al. 2011). While an important role for vitamin D<sub>3</sub> is maintaining calcium homeostasis, the role of dietary calcium consumption in these studies has been largely ignored. A Cochrane review of dietary calcium supplementation found that  $\geq 1000$   $\mu\text{g}$  of calcium per day reduced overall blood pressure, as well as the risk of preeclampsia in women with low calcium consumption (Hofmeyr, Atallah et al. 2006). Dietary vitamin D<sub>3</sub> supplementation studies have had mixed results, with the pre-2010 RDI (Recommended Daily Intake) dose of 400 IU/day having no effect on the incidence of pregnancy complications (De-Regil, Palacios et al. 2012). However a high dose of 4000 IU/day resulted in a decreased risk of hypertensive disorders including preeclampsia (Hollis and Wagner 2013). As vitamin D<sub>3</sub> assists in dietary calcium absorption, we need to examine the combined roles of vitamin D<sub>3</sub> and dietary calcium in pregnancy.

The primary role for vitamin D<sub>3</sub> in the body is to maintain calcium homeostasis. Vitamin D<sub>3</sub> production predominantly occurs through the conversion of 7-dehydrocholesterol in the skin by UV-B followed by subsequent hydroxylations in the liver and kidney. The first hydroxylation is catalysed by the liver, cytochrome P450 enzyme, CYP2R1, producing 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) which is the main circulating metabolite of vitamin D<sub>3</sub> and is itself inactive. The second hydroxylation is catalysed by the renal enzyme, CYP27B1, which produces 1 $\alpha$ ,25-

dihydroxy vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) when required and is the biologically active form of vitamin D<sub>3</sub>. This activation of 1,25(OH)<sub>2</sub>D<sub>3</sub> is under the tight control of parathyroid hormone (PTH) which stimulates CYP27B1 gene expression, usually in response to low circulating calcium. Fibroblast growth factor 23 (FGF23) is also known to suppress CYP27B1 activity in response to high circulating phosphate levels. During pregnancy, however, serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels are markedly higher despite low levels of serum PTH (Ardawi, Nasrat et al. 1997), suggesting that either low FGF23 is responsible for high 1,25(OH)<sub>2</sub>D<sub>3</sub> levels or there are alternate regulatory systems of 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis and metabolism during pregnancy. A major role for 1,25(OH)<sub>2</sub>D<sub>3</sub> is to maintain serum calcium levels by both directly stimulating intestinal calcium absorption and stimulating bone mineral catabolism to liberate calcium stores when 1,25(OH)<sub>2</sub>D<sub>3</sub> is sufficiently high. However, 1,25(OH)<sub>2</sub>D<sub>3</sub> has been shown to play important roles in other physiological systems (Bouillon, Carmeliet et al. 2008).

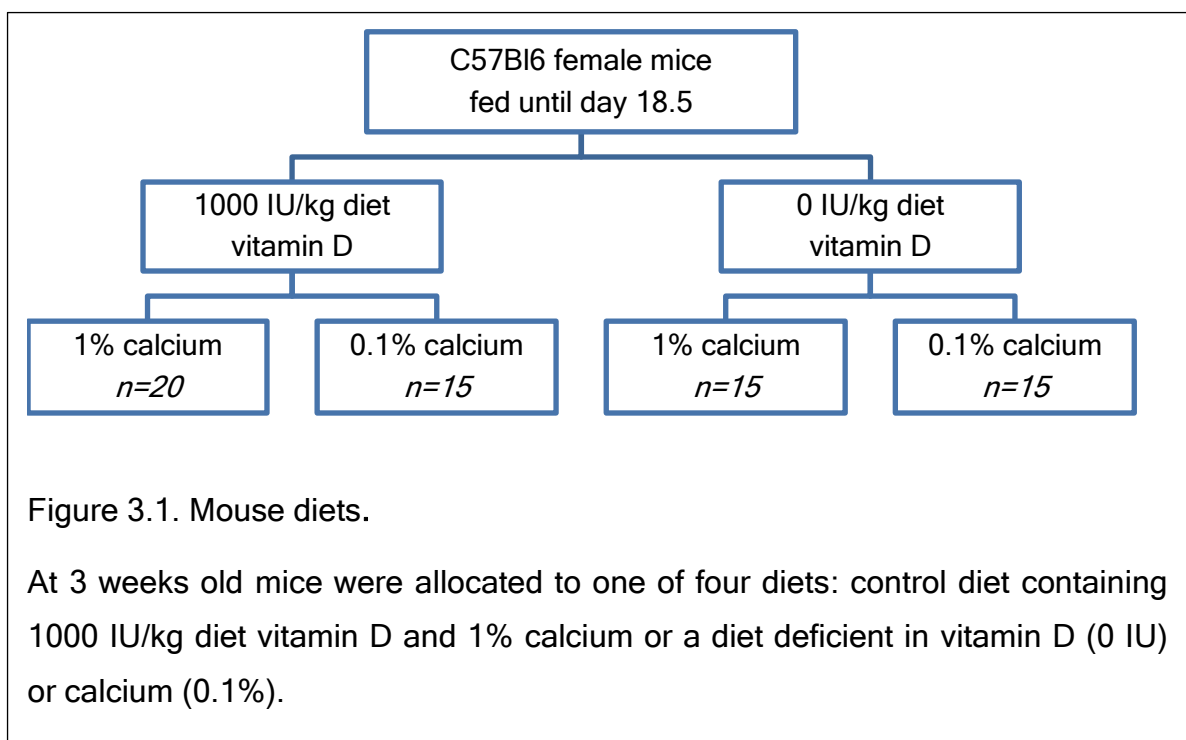
1,25(OH)<sub>2</sub>D<sub>3</sub>, as well as 25(OH)D<sub>3</sub>, are synthesized by the placenta. While the placenta was one of the first organs to demonstrated to synthesise 1,25(OH)<sub>2</sub>D<sub>3</sub> outside of the kidney (Tanaka, Halloran et al. 1979), the role for placental vitamin D<sub>3</sub> metabolism remains largely unclear. The gene for enzyme responsible for 1,25(OH)<sub>2</sub>D<sub>3</sub> catabolism, CYP24A1, has been reported to be heavily methylated in human placenta, suggesting that the placenta is responsible for 1,25(OH)<sub>2</sub>D<sub>3</sub> production and less so about regulating its activity through catabolism. In mice, however, the CYP24A1 gene is not methylated to the same extent (Novakovic, Sibson et al. 2009). The placenta expresses vitamin D receptor (VDR) (Ross, Florer et al. 1989), which suggests that vitamin D<sub>3</sub> metabolism within the placenta may have autocrine or paracrine activities. This is yet to be conclusively shown however. As yet, we know little of the control of vitamin D<sub>3</sub>

metabolism during pregnancy, both from a renal and local placental function, or its primary roles and as well as how vitamin D<sub>3</sub> activity during pregnancy is affected by vitamin D<sub>3</sub> deficiency. To investigate this, we have altered dietary vitamin D<sub>3</sub> and calcium conditions during murine pregnancy in order to modulate vitamin D<sub>3</sub> status and investigate the effects placental composition and gene expression as well as on fetal weight.

### 3.3 Methods

#### 3.3.1 Mice

Mice used for this study are the same as those used in Chapter 2. Briefly, dual ethics approval was obtained from the University of Adelaide Animal Ethics Committee and the IMVS/CNAHS Animal Ethics Committee with animal work complying with the Australian Code of practice for the care and use of animals. C57Bl6 female mice were obtained at 3 weeks old and maintained at a 12h/12h light/dark cycle. Mice were fed *ad libitum* either a standard diet of 1000 IU vitamin





D<sub>3</sub>/kg food and 1% calcium (control AIN93G (Reeves, Nielsen et al. 1993)), or diets deficient in vitamin D<sub>3</sub> (0 IU/kg) or calcium (0.1%), with 15 mice per diet (20 mice on control diet; diets from Specialty Feeds, Australia; Figure 3.1). Mice consumed their allocated diet from 3 weeks old through mating at 13 weeks and post-mortem at day 18.5 post-coitus. Mating was performed with C57Bl6 males maintained on chow, with day 0.5 post-coitus indicated by the presence of a vaginal plug. At post-mortem fetal and placental wet weights were obtained, with placentas fixed for histology or snap-frozen for gene expression. Mice that had an unknown date of conception, did not fall pregnant, or delivered before post-mortem were excluded from the placental analyses.

### **3.3.2 Placental histology**

Two placentas per litter were fixed in 4% (w/v) paraformaldehyde with 2.5 per cent (w/v) polyvinylpyrrolidone in 70 mM phosphate buffer. Sections were immersed overnight then bisected mid-sagittally and fixed for a further 3 hours. Sections were washed four times in phosphate buffered saline (PBS, pH 7.4) over 2 days and stored in 70% ethanol. Tissues were processed and then embedded cut side down into paraffin blocks. Tissue sections (5 µm) were stained with Masson's Trichrome (2 placentas per litter) or by immunohistochemistry (1-2 placentas per litter).

### **3.3.3 Immunohistochemistry**

Immunohistochemistry was conducted on one placenta per litter, with 2 per litter from the most deficient group. Sections were labelled with mouse anti-vimentin (Clone Vim 3B4, Dako) and mouse anti-cytokeratin (AE1/AE3, Chemicon International) with the aid of a Mouse on Mouse IHC Kit (Abcam) to eliminate non-

specific staining. Antigen retrieval was performed by incubation at 37°C for 15 min with Pronase (P8811, Sigma-Aldrich) at 0.3 mg/mL diluted in PBS. Anti-vimentin was diluted 1/10 in diluent (1 per cent porcine serum (Veterinary Services, IMVS, Adelaide), 1 per cent bovine serum albumin (Sigma-Aldrich) (w/v) in PBS) and incubated overnight at room temperature. Anti-cytokeratin was diluted to 1/100 in diluent and incubated for an hour at room temperature. The chromogen diaminobenzidine (Sigma-Aldrich) in PBS was added to the secondary antibody supplied in the kit with 2% nickel II sulphate (Sigma-Aldrich) to form a black precipitate for identification of anti-vimentin, or on its own to form a brown precipitate to identify anti-cytokeratin. Tissue sections were counterstained with Haematoxylin (Sigma-Aldrich) and Eosin (Sigma-Aldrich) to define nuclei and maternal erythrocytes, respectively.

### **3.3.4 Placental morphometry**

All sections were photographed with a NanoZoomer Digital Pathology scanner (Model C9600 v1.2, Hamamatsu Photonics K.K.) and analysed with NDP Scan software (v2.2, Hamamatsu Photonics K.K.). The analysis was performed while blinded to the dietary group of the sample. Masson's Trichrome staining was analysed with NDP View software (v1.2.25) to quantify the junctional zone and labyrinth areas (Figure 1.6), ratios of labyrinth and junctional areas, placental height and diameter, cross sectional area and labyrinth weight. Double-labelled sections were analysed with Video Pro 32 software (Leading Edge, Australia). Placental labyrinth was analysed by randomly choosing a starting field and systematically choosing 10 adjacent fields 500 µm apart for analysis with an isotropic L-36 Merz grid. Morphometric techniques were used to estimate placental composition, as reported previously (Roberts, Sohlstrom et al. 2001). Estimates of

labyrinth components (maternal blood space, trophoblast and fetal capillaries) were determined. Volume density ( $V_d = P_a / P_T$ ) was calculated with  $P_a$  being the number of points counted for a component and  $P_T$  being the total points applied to the section. Volume ( $V = V_d \times \text{labyrinth weight}$ ) was calculated, with volume assuming that  $1\text{cm}^3$  is equivalent to 1 g of labyrinth. Trophoblast surface density ( $S_v = 2 \times I_a / L_T$ ) was calculated as double total number of intersections of the test line with the maternal face of the trophoblast ( $I_a$ ), divided by the length of the test line ( $L_T$ , calculated by the total number of points applied and dimensions of the semi-circular line). Trophoblast surface area ( $SA = V \times S_v$ ) was calculated as the product of  $S_v$  and labyrinth volume ( $V$ ). Mean arithmetic barrier thickness ( $B_T = V_d / S_v$ ) was calculated as the trophoblast  $V_d$  divided by  $S_v$ . Error rate, calculated by counting a field 5 times, was less than 5%.

### 3.3.5 Gene expression

Gene expression was performed on median weight placentas ( $n=25$ ), which were bisected midsagittally, with RNA extracted by the TRIzol® (Invitrogen) method. Briefly, tissues were snap frozen in liquid nitrogen at post-mortem and stored at  $-80^\circ\text{C}$ . Tissue was rapidly bisected over ice without thawing and immersed in TRIzol. Tissue was homogenised in TRIzol by a Powerlyser (MoBio) with ceramic beads (CK14, Geneworks), with RNA extracted as per TRIzol manufacturer's instructions. Rigorous DNase treatment was performed using a TURBO DNase kit (Ambion). Briefly, 2  $\mu\text{g}$  of RNA was incubated for an hour at  $37^\circ\text{C}$  with 0.1 volume of TURBO DNase and of 10x TURBO DNase buffer, with another 0.1 volume of TURBO DNase added after 30 min, followed by quenching of the reaction with 0.2 volumes of DNase Inactivation Reagent. RNA integrity was assessed by Experion Automated Electrophoresis System (BioRad)

with a RQI of >4 accepted for real time quantitative PCR (qPCR) (Fleige and Pfaffl 2006). Absence of genomic DNA was assessed by PCR of DNase-treated RNA with genomic DNA primers (F: 5'-GGCACTGACTGAGGTCAAAC-3'; R: 5'-GTCACAATCACAGAGACTTTGA-3'; amplicon length 120 bp, annealing temperature 60°C, Geneworks). 500 ng of RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad) as per manufacturer's instructions. cDNA was diluted 1/10 and stored at -20°C.

Real time qPCR was set up using a QIAgility (QIAGEN) liquid handling robot and run on a CFX-384 Real Time PCR machine (BioRad). 10 µL PCR reactions were run containing 5 µL TaqMan® Gene Expression Master Mix (Applied Biosystems), 0.5 µL TaqMan® Assay, with 2 µL of diluted cDNA. Cycling conditions were 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. PCR efficiency was determined using a five point standard curve of maternal kidney cDNA and gel electrophoresis was used to confirm amplicon size. TaqMan® Gene Expression Assays (Applied Biosystems) used were for vitamin D<sub>3</sub> metabolism - *Vdr* (Mm00437297\_m1), *Cyp2r1* (Mm00616774\_g1), *Cyp27b1* (Mm01165918\_g1) and *Cyp24a1* (Mm00487244\_m1); for vitamin D<sub>3</sub> responsive genes - *Hif1a* (Mm00468869\_m1), *Vegfa* (Mm01281449\_m1), *Hsd17b2* (Mm00500430\_m1); for FGF23/Klotho action *Napi2a* (*Slc34a1*, Mm00441450\_m1) and *Napi2c* (*Slc34a3*, Mm00551746\_m1); and for reference genes *Gusb* (Mm06003537\_s1), *Hmbs* (Mm01143545\_m1), *Gapdh* (Mm99999915\_g1) and *Hprt1* (Mm00446968\_m1).

Reference gene suitability was analysed by GeNorm (qBasePlus v2, Biogazelle) (Vandesompele, De Preter et al. 2002), with *Gapdh* and *Hmbs* being the optimum genes for normalization of expression (M=0.31, V<0.1). PCR data

was analysed using BioRad CFX Manager (BioRad), which normalised expression of the genes of interest to the geometric mean of the reference genes.

### **3.3.6 Statistics**

Fetal and placental measurements were analysed by a Linear Mixed Model, Repeated Measures, with fetal and placental measurements as repeated measures of the mother and Sidak post-hoc. Gene expression was corrected for litter size and RQI and were analysed by ANCOVA or by non-parametric tests (Kruskal-Wallis or Mann-Whitney U) as appropriate. Analyses were run using IBM SPSS Statistics (v 19, IBM), with significance accepted at  $P < 0.05$ . Results are expressed as mean  $\pm$  SEM.

## **3.4 Results**

### **3.4.1 Pregnancy rate**

Only mice that underwent post-mortem at d18.5 of pregnancy were included in placental histology investigations. Due to early deliveries and mice that did not proceed to pregnancy after mating, the most deficient group (0 IU/kg D<sub>3</sub> + 0.1% Ca) generated 4 pregnant mice at post-mortem. There was no difference in pregnancy rate between diet groups (Table 2.1).

### **3.4.2 Fetal and placental weights**

At post-mortem, there was no difference in the number of viable fetuses per litter (litter size) between diets (Table 2.1), although there was a non-significant 37% increase in resorptions in mice consuming low calcium diets ( $P = 0.439$ ). Placental weight was not significantly altered by changes to dietary vitamin D<sub>3</sub> or calcium. Fetal weight was significantly affected by diet ( $P = 0.001$ , Table 2.2).

Surprisingly, mice fed low calcium had a 6% increase in fetal weight compared to mice fed normal calcium ( $P=0.006$ ). This was reflected in the fetal to placental weight ratios which are a surrogate of placental efficiency, with high ratios indicating more efficient placentas, as they produce a heavier fetus per gram of placenta (Fowden, Sferruzzi-Perri et al. 2009). Thus, fetal to placental weight ratios were altered by diet ( $P<0.001$ ), with low calcium consuming mice having a 7% higher ratio ( $P=0.018$ ). There was a significant interaction between litter size and both fetal weight and fetal to placental weight ratios ( $P<0.05$ ). Fetal and placental weights were non-significantly correlated ( $P=0.057$ ,  $R=0.152$ ). As expected, fetal to placental weight ratios were highly correlated with fetal and placental weights ( $P<0.001$ ).

### 3.4.3 Placental composition

The areas of the placental compartments, the junctional zone (stem cell area; JZ) and labyrinth zone (feto-maternal exchange; LZ), were quantified following Masson's Trichrome staining. While cross-sectional area was not significantly affected by diet ( $P=0.128$ ), both the labyrinth and junctional zone areas were altered by diet ( $P<0.001$ ; Table 3.1, Figure 3.2). Mice consuming low calcium had a 10% reduction in labyrinth to junctional zone ratio (LZ:JZ ratio;  $P=0.019$ ), with a non-significant 11% decrease in those consuming vitamin D-deficient diets ( $P=0.054$ ). This indicates that mice fed the vitamin D-deficient and low calcium diets have proportionally less labyrinth area and hence, less area for feto-maternal exchange. Labyrinth to junctional area ratios were positively correlated with fetal weight ( $P=0.044$ ,  $R=0.321$ ; Figure 3.3A) and fetal to placental weight ratio ( $P=0.010$ ,  $R=0.401$ ), while junctional zone area was positively correlated with placental weight ( $P=0.033$ ,  $R=0.339$ ) and negatively correlated with

fetal to placental weight ratio ( $P=0.019$ ,  $R=0.368$ ; Figure 3.3B). As expected labyrinth to junctional area ratio was positively correlated with labyrinth area and negatively correlated with junctional zone area, while placental weight was positively correlated with both labyrinth and junctional zone areas ( $P<0.05$ , data not shown).

Placental dimensions were also measured in Masson's Trichrome stained sections. As murine placentas are discoid, placental height and diameter were histologically measured. Placental height was altered by diet ( $P=0.043$ ) and was reduced by 2% in mice consuming vitamin D-deficient diets ( $P=0.004$ ), while diameter was also altered by diet ( $P<0.001$ ), with a 5% decrease in vitamin D-deficient diets ( $P=0.005$ ) and 2% increase in mice fed low calcium diets ( $P<0.001$ ). As these changes were small they had no effect on overall placental weights and

Diet	1000 IU + 1% Ca	0 IU + 1% Ca	1000 IU + 0.1% Ca	0 IU + 0.1% Ca	P-value
n (litters / placentas)	6 (11)	7 (12)	6 (11)	3 (6)	
Labyrinth Area (mm <sup>2</sup> )	4.798 ± 0.181 <sup>a</sup>	4.887 ± 0.051 <sup>ab</sup>	4.068 ± 0.007 <sup>ac</sup>	4.554 ± 0.173 <sup>a</sup>	<0.001
Junctional Area (mm <sup>2</sup> )	2.766 ± 0.041 <sup>a</sup>	2.669 ± 0.001 <sup>ab</sup>	3.077 ± 0.022 <sup>c</sup>	3.119 ± 0.001 <sup>ac</sup>	<0.001
Labyrinth:Junctional Area Ratio	1.543 ± 0.023 <sup>ab</sup>	1.477 ± 0.043 <sup>b</sup>	1.434 ± 0.056 <sup>b</sup>	1.360 ± 0.009 <sup>bc</sup>	0.096
Total Cross-sectional Area (mm <sup>2</sup> )	7.282 ± 0.28 <sup>a</sup>	7.816 ± 0.103 <sup>ab</sup>	7.062 ± 0.031 <sup>ac</sup>	7.898 ± 0.395 <sup>a</sup>	0.128
Placental height (mm)	1.431 ± 0.025 <sup>a</sup>	1.437 ± 0.018 <sup>a</sup>	1.492 ± 0.046 <sup>a</sup>	1.418 ± 0.046 <sup>a</sup>	0.043
Placental diameter (mm)	5.920 ± 0.001 <sup>a</sup>	5.582 ± 0.100 <sup>a</sup>	5.700 ± 0.147 <sup>a</sup>	6.158 ± 0.001 <sup>b</sup>	<0.001

Note: mean ± SEM, corrected for litter size, different superscripts denote significant differences

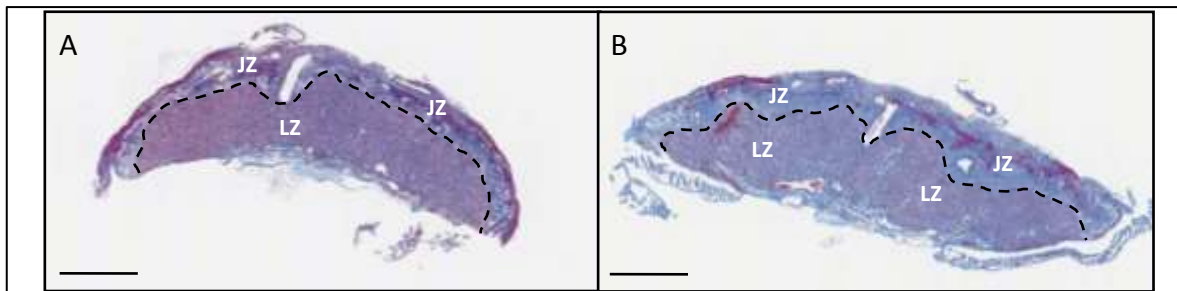


Figure 3.2. Representative images of Masson's Trichrome stained placentas .

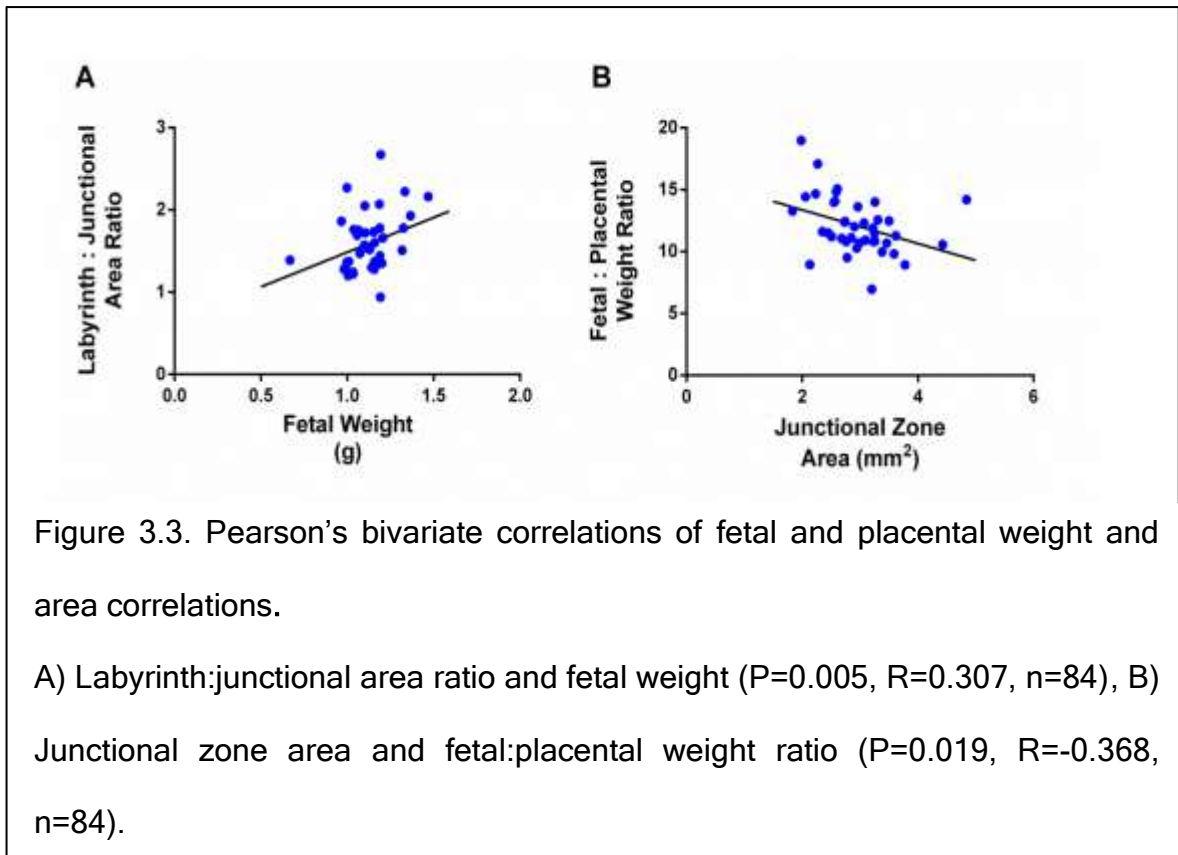
A) control diet consuming mice (1000 IU/kg D<sub>3</sub> + 1% Ca); B) mice consuming the vitamin D- and calcium-deficient diet (0 IU/kg D<sub>3</sub> + 0.1% Ca). LZ - labyrinth zone, JZ - junctional zone, scale bar is 1 mm, 1.25x magnification

would have little effect on placental cross-sectional areas. As expected, labyrinth area was correlated with placental diameter, height and area, while junctional zone area was correlated with diameter and area ( $P < 0.05$ , data not shown).

### 3.4.4 Morphometry

Morphometry was used to estimate composition of the labyrinth area of one placenta per litter (two placentas for the most deficient group due to low numbers) see Figure 3.4. The labyrinth was analysed for volume density and estimated volume of trophoblast, maternal blood space and fetal capillaries. Volume density was altered by diet for trophoblast, maternal blood space and fetal capillaries ( $P < 0.05$ , Table 3.2). Vitamin D<sub>3</sub> also affected volume density, with mice fed a vitamin D-deficient diet observed to increase maternal blood space density by 5% ( $P < 0.001$ ) and decrease fetal capillary density by 1.6% ( $P = 0.010$ ; Table 3.2). Contrary to this, mice on low diets had significantly lower fetal capillary volume density (14%,  $P < 0.001$ ). Volume density of trophoblast was negatively correlated with volume density of both maternal blood space ( $P < 0.001$ ,  $R = 0.866$ ) and fetal capillaries ( $P = 0.038$ ,  $R = 0.417$ ; Table 3.2). Fetal capillary volume density was negatively associated with placental diameter ( $P = 0.007$ ,  $R = 0.533$ ) but not





correlated with fetal weight ( $P=0.059$ ,  $R=0.382$ ). Maternal blood space and fetal capillary volume density were not correlated.

Estimated volume of trophoblast was not altered by dietary levels of vitamin D<sub>3</sub> or calcium. Fetal capillary volume was altered by both diet ( $P=0.042$ ; Table 3.2) and vitamin D<sub>3</sub> levels, with mice fed the vitamin D-deficient diets having increased the volume of fetal capillaries (12.5%,  $P<0.001$ ), with no change in placental weight between diets. Trophoblast volume was positively correlated with placental weight ( $P=0.001$ ,  $R=0.622$ ) and labyrinth to junctional area ratio ( $P=0.010$ ,  $R=0.505$ ) and negatively correlated with maternal blood space volume density ( $P<0.001$ ,  $R=0.659$ ; Figure 3.5). Maternal blood space volume was also negatively correlated with trophoblast volume density ( $P<0.001$ ,  $R=0.768$ ). As expected, volume densities and volumes were positively correlated (trophoblast  $P<0.001$ ,  $R=0.740$ ; maternal blood space  $P<0.001$ ,  $R=0.907$ ; fetal capillaries  $P<0.001$ ,  $R=0.854$ ).

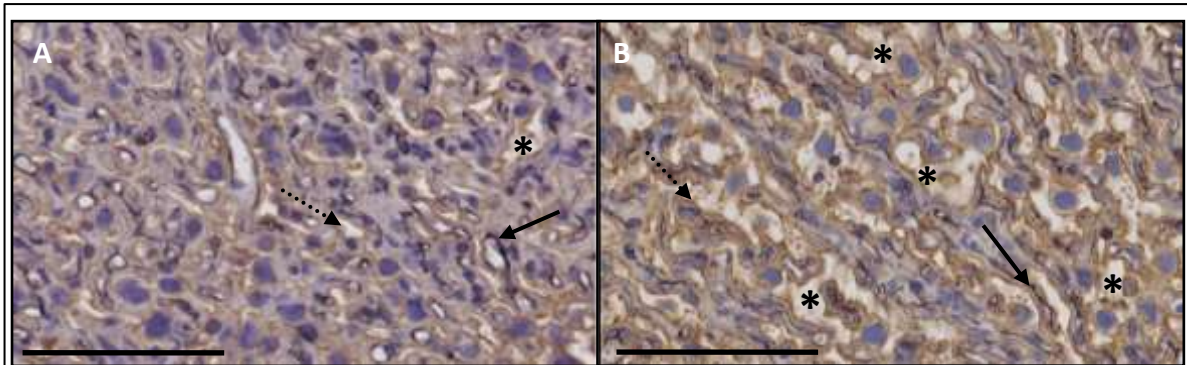


Figure 3.4. Immunohistochemical double-labelling of mouse labyrinth.

Mouse placental labyrinth double labelled with anti-cytokeratin (brown) for trophoblast and anti-vimentin (black) for fetal capillaries, counterstained with haematoxylin and eosin. Representative images from A) control and B) deficient (0 IU + 0.1% Ca). Proportion of maternal blood space is increased and fetal capillaries are decreased ( $P < 0.001$ ). Dashed arrow - anti-cytokeratin; solid arrow - anti-vimentin; star - maternal blood space; scale bar is 100  $\mu\text{m}$ , 40x magnification

Trophoblast surface density was altered by diet ( $P = 0.006$ ), with a trend toward higher density displayed by the vitamin D-deficient diet fed groups (19%,  $P = 0.084$ , Table 3.2). Surface area of trophoblast was also altered by diet ( $P < 0.001$ ), with control-fed mice exhibiting significantly lower (21-25%) surface area than 1000 IU/kg + 0.1% calcium or 0 IU/kg + 1% calcium diets ( $P < 0.05$ ) and a 25% decrease in 0 IU/kg + 0.1% calcium fed mice which did not reach statistical significance ( $P = 0.170$ ). Feto-maternal barrier was altered by diet ( $P < 0.05$ ) and was 35% thinner in mice on the most deficient diet as compared to control ( $P = 0.044$ ). There was also an interaction between litter size with barrier thickness and surface area ( $P = 0.005$ ). Surface density was negatively correlated with labyrinth zone area ( $P = 0.022$ ,  $R = 0.455$ ) and trophoblast volume ( $P = 0.006$ ,  $R = 0.536$ , Figure 3.6). Barrier thickness was positively associated with trophoblast volume ( $P = 0.002$ ,

Table 3.2. Estimated placental labyrinth composition by morphometry.

Diet	1000 IU + 1% Ca	0 IU + 1% Ca	1000 IU + 0.1% Ca	0 IU + 0.1% Ca	P-value
n (litters, placentas)	6 (6)	7 (7)	6 (6)	3 (6)	
Vd - Trophoblast	0.718 ± 0.007 <sup>a</sup>	0.675 ± 0.005 <sup>b</sup>	0.654 ± 0.010 <sup>b</sup>	0.707 ± 0.004 <sup>a</sup>	<b>0.037</b>
Vd - Maternal blood space	0.150 ± 5.0e-6 <sup>a</sup>	0.177 ± 0.005 <sup>b</sup>	0.203 ± 0.012 <sup>b</sup>	0.190 ± 6.0e-6 <sup>b</sup>	<b>&lt; 0.001</b>
Vd - Fetal Capillaries	0.121 ± 0.014 <sup>a</sup>	0.125 ± 1.53e-8 <sup>a</sup>	0.110 ± 3.51e-7 <sup>b</sup>	0.111 ± 0.010 <sup>b</sup>	<b>&lt; 0.001</b>
V - Trophoblast (g)	0.041 ± 0.001 <sup>a</sup>	0.040 ± 4.8e-7 <sup>a</sup>	0.037 ± 0.002 <sup>a</sup>	0.039 ± 4.0e-4 <sup>a</sup>	0.135
V - Maternal blood space (g)	0.010 ± 4.0e-4 <sup>a</sup>	0.011 ± 3.0e-4 <sup>b</sup>	0.012 ± 1.0e-6 <sup>c</sup>	0.010 ± 2.0e-4 <sup>a</sup>	<b>&lt; 0.001</b>
V - Fetal Capillaries (g)	0.006 ± 3.41e-94 <sup>a</sup>	0.008 ± 9.28e-11 <sup>b</sup>	0.005 ± 8.46e-8 <sup>c</sup>	0.006 ± 3.40e-9 <sup>a</sup>	<b>0.042</b>
Sv - Trophoblast	440.8 ± 24.1 <sup>a</sup>	579.8 ± 0.0002 <sup>a</sup>	601.4 ± 12.3 <sup>a</sup>	642.8 ± 7.89 <sup>a</sup>	<b>0.006</b>
SA - Trophoblast (mm <sup>2</sup> )	27.4 ± 0.6 <sup>a</sup>	34.1 ± 0.6 <sup>b</sup>	33.2 ± 0.9 <sup>b</sup>	33.2 ± 5.0e-5 <sup>ab</sup>	<b>&lt; 0.001</b>
Barrier Thickness (µm)	16.976 ± 0.671 <sup>a</sup>	12.720 ± 0.293 <sup>b</sup>	11.954 ± 0.409 <sup>b</sup>	10.743 ± 2.0e-6 <sup>b</sup>	0.067

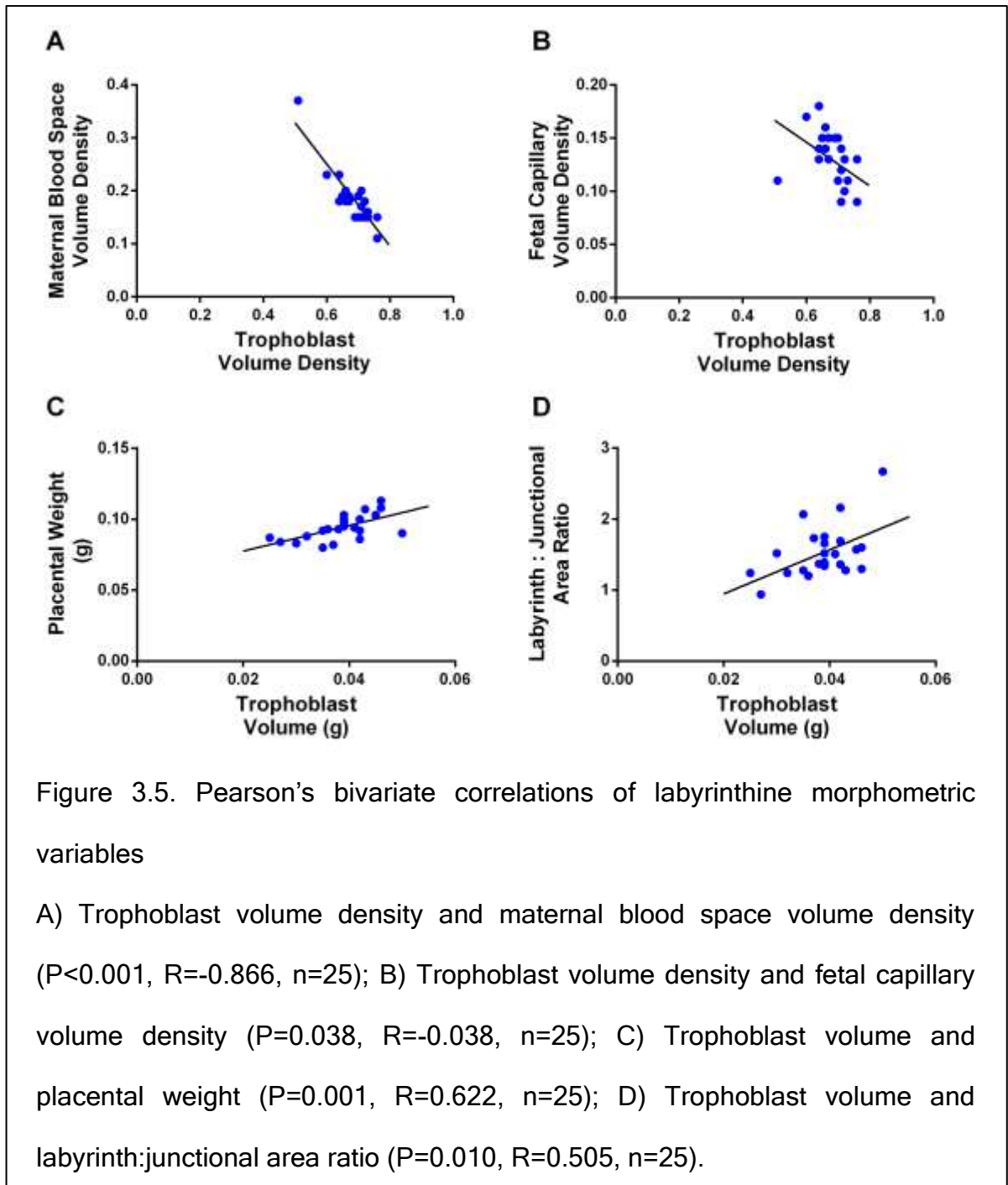
Vd - volume density, V - volume, Sv - surface density, SA - surface area, Mean ± SEM, P<0.05 is significant, different superscripts denote significant differences.

R=0.585) and volume density (P=0.004, R=0.555) and negatively correlated with maternal blood space volume density (P=0.005, R=0.548). As expected, surface density, surface area and barrier thickness were all highly correlated (P<0.001, data not shown).

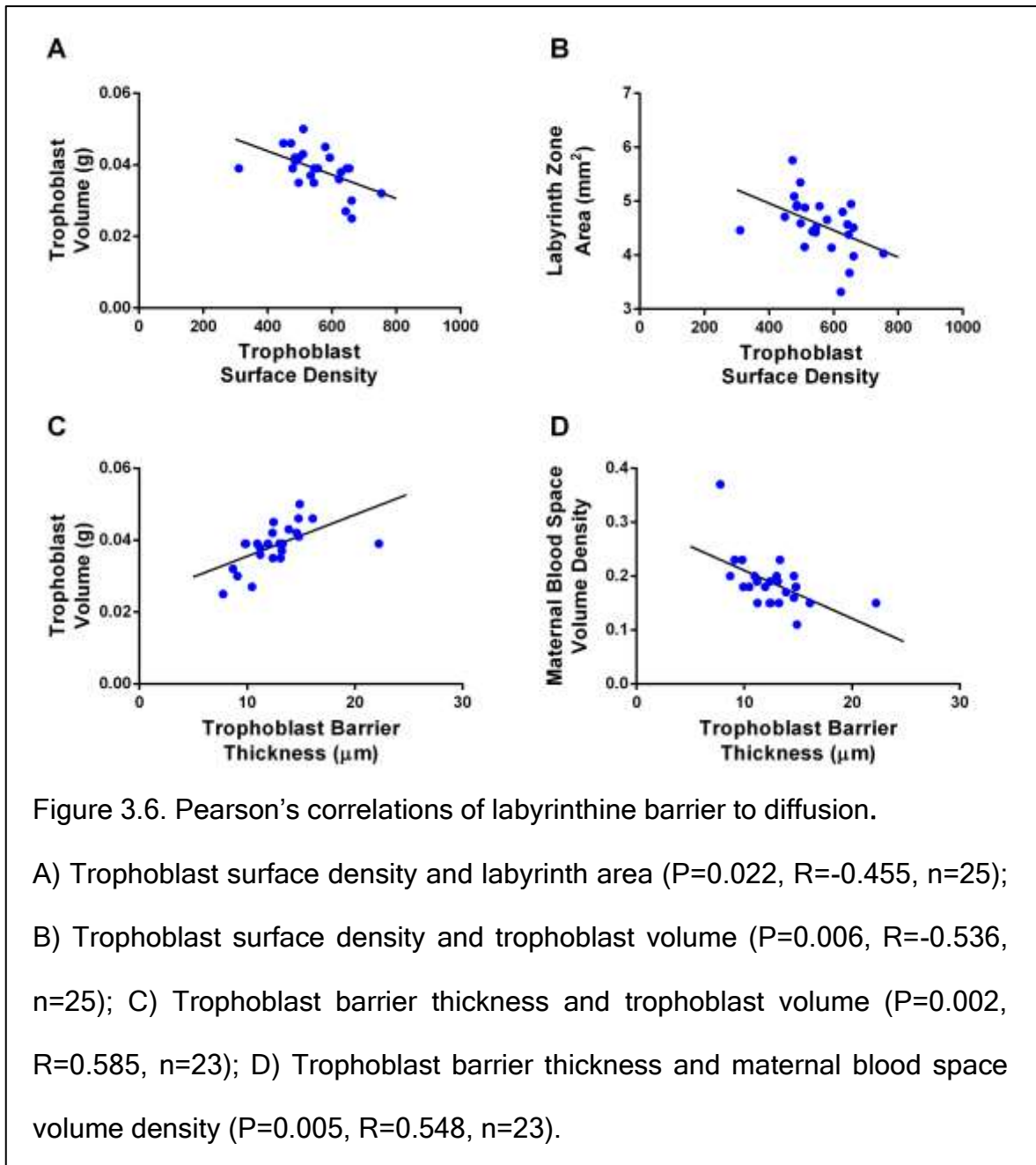
### 3.4.5 Placental gene expression

Placental expression of *Vdr* was 2-fold higher in mice fed low calcium diets compared to normal calcium diets (P=0.042, Figure 3.7), although this failed to reach significance when examining overall dietary vitamin D<sub>3</sub> and calcium.

Placental *Cyp24a1* expression was significantly altered by diet (P=0.036), with expression increased by 6-fold in mice fed low calcium diets when compared to placental levels in mice consuming normal calcium diets (P=0.005). *Cyp27b1*



expression was not altered by diet ( $P = 0.374$ ), although mice consuming 0 IU/kg + 1% Ca diets had on average 3.4-fold higher expression than in controls. *Cyp2r1* expression was unchanged across dietary groups. Expression levels of *Vdr* and *Cyp24a1* were highly positively correlated, regardless of diet ( $P < 0.001$ ,  $R = 0.794$ ; Figure 3.9A), suggesting that this is due to the action of  $1,25(\text{OH})_2\text{D}_3$  and *Vdr* protein up regulating vitamin D responsive genes. Placental expression of



*Cyp27b1* and *Cyp2r1* were similar between diets.

Expression of vitamin D responsive genes was also investigated, through selection of three placentally expressed genes: *Hif1a*, *Hsd17b2*, and *Vegfa*. There was no change in mRNA levels of either of these genes due to diet ( $P=0.364$ ,  $0.858$  and  $0.579$ , respectively, Figure 3.8). Despite this, *Hsd17b2* expression was negatively associated with fetal weight ( $P=0.004$ ,  $R=0.578$ , Figure 3.9C), while

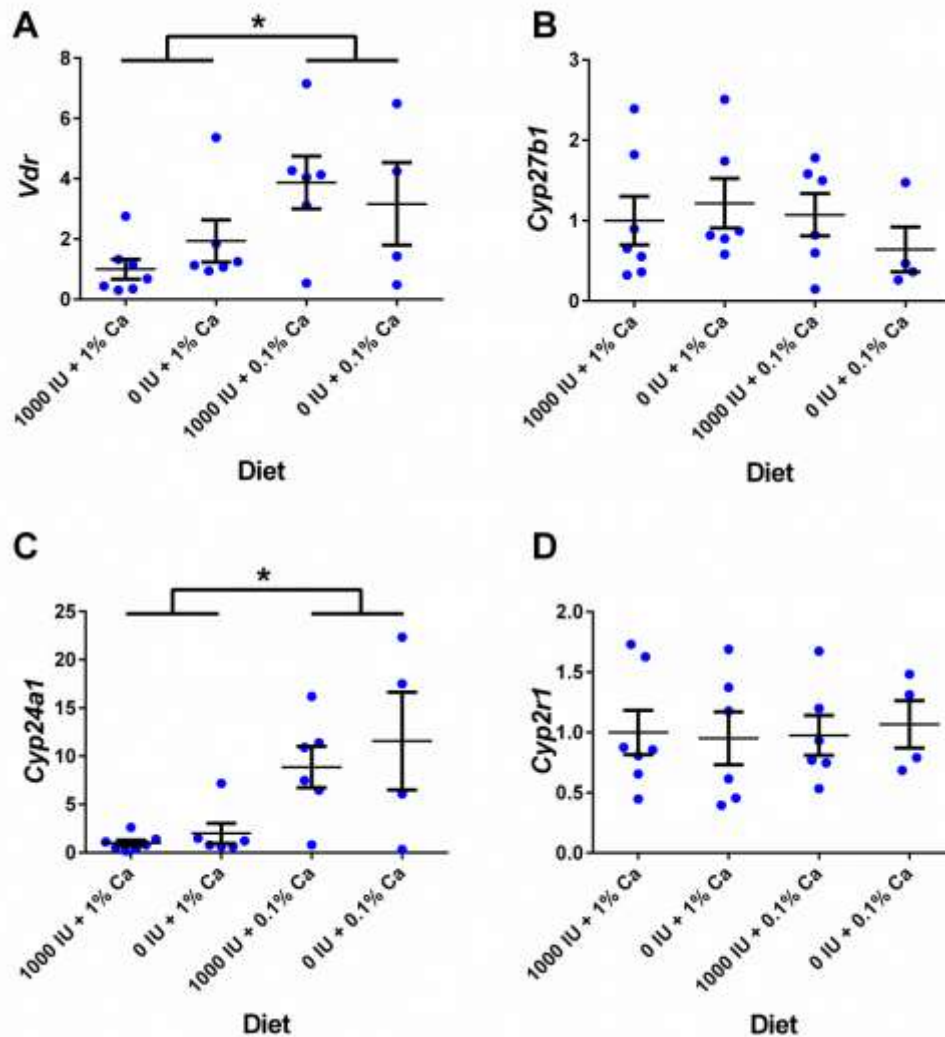
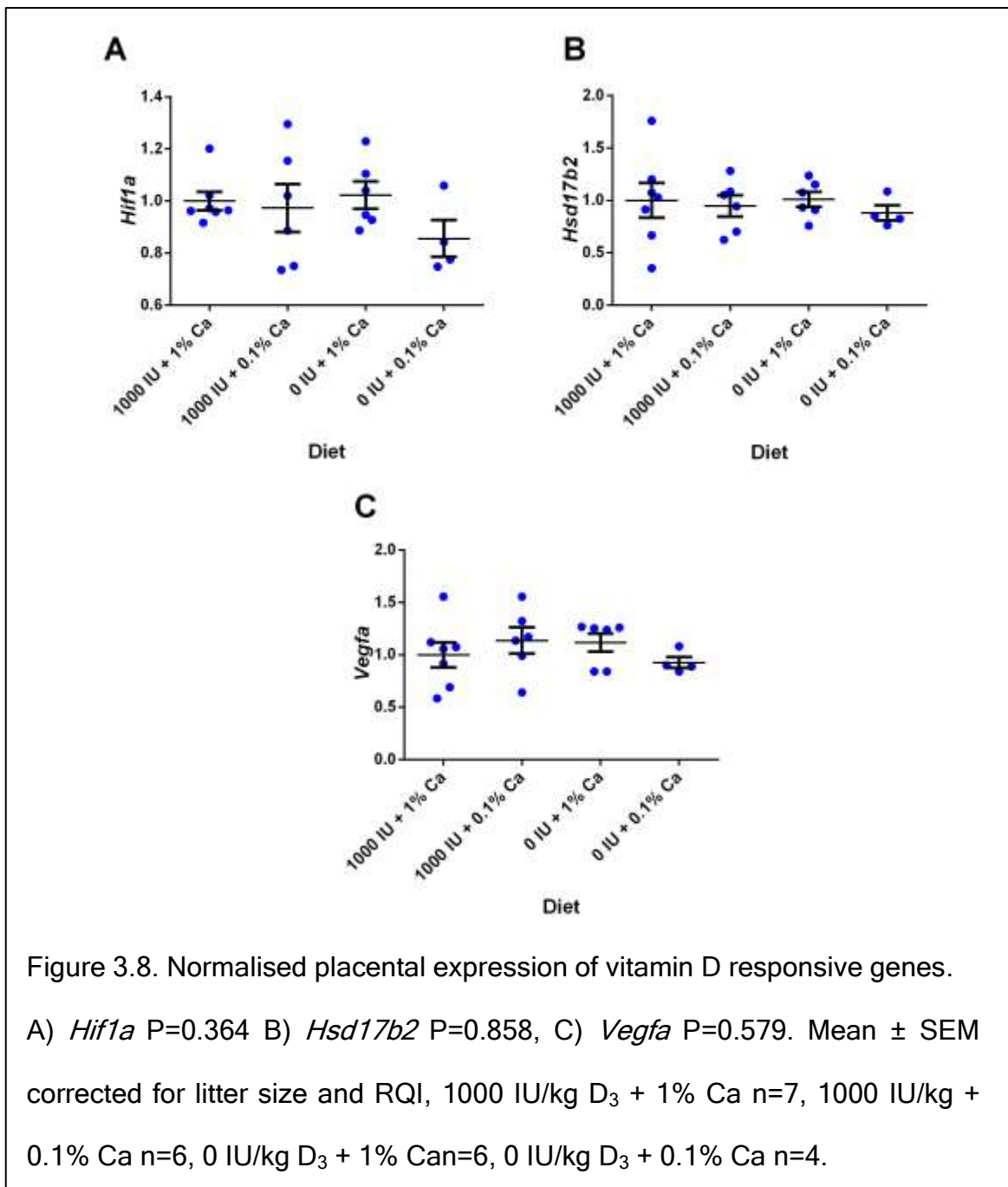


Figure 3.7. Normalised placental expression of vitamin D metabolism genes. *Vdr* P=0.106, B) *Cyp24a1* P=0.036, C) *Cyp27b1* P=0.374, D) *Cyp2r1* P=0.990. Mean ± SEM, 1000 IU/kg D<sub>3</sub> + 1% Ca n=7, 1000 IU/kg + 0.1% Ca n=6, 0 IU/kg D<sub>3</sub> + 1% Ca n=6, 0 IU/kg D<sub>3</sub> + 0.1% Ca n=4.25 placentas from 4-7 mothers per diet.

*Hif1a* and *Vegfa* had positively correlated expression (P<0.001, R=0.696, Figure 3.9B).

Expression of the FGF23/Klotho reporter genes *Napi2a* and *Napi2c* were also quantified in the placenta. *Napi2a* was unaffected by diet (P=0.999, data not shown), while *Napi2c* showed a non-significant 40% increased expression in mice on deficient diets compared to control due to high variation (P=0.529, data not



shown). *Napi2a* and *Napi2c* are highly expressed in the kidney; placental levels are significantly lower than kidney levels. *Napi2c* expression was positively correlated with *Vdr* ( $P < 0.001$ ,  $R = 0.683$ ), *Cyp24a1* ( $P = 0.006$ ,  $R = 0.538$ ), *Hsd17b2* ( $P = 0.012$ ,  $R = 0.493$ ) and *Vegfa* ( $P = 0.015$ ,  $R = 0.480$ ; Figure 3.10).



## 3.5 Discussion

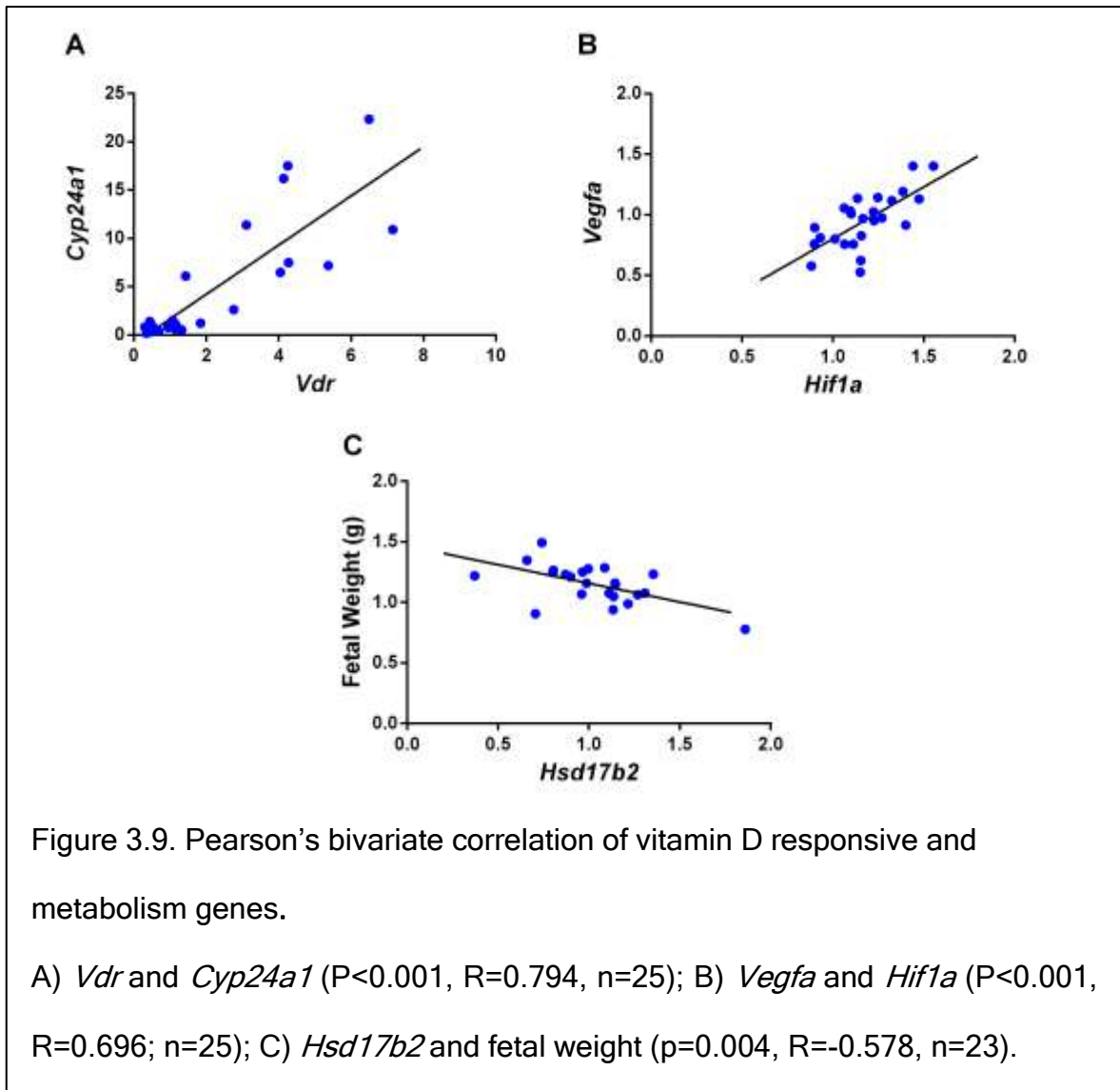
### 3.5.1 Fetal and Placental Weights

Although fertility is reduced in *Vdr* knockout (*Vdr* null) mice and improved by dietary calcium supplementation (Johnson and DeLuca 2001), there was no evidence of reduced fertility in our mice, with no difference in pregnancy rate or litter size. Although there was a 37% increase in resorptions in controls compared to the most deficient group (0 IU/kg + 0.1% Ca), there was no significant change across all diets. This may be due to the relatively mild phenotype induced by our diets, as our lowest 25(OH)D<sub>3</sub> serum measurement was 10 nmol/L with no difference in serum calcium between diets (see Chapter 2). Fetal weights were expected to decrease in those mice on the most deficient diets, but we found that fetal weights were increased on diets with low calcium. As there were no effects of diet on placental weight, this was also reflected in the fetal to placental weight ratios, which are a surrogate of placental efficiency. This is contrary to what has been found in *Vdr* null mice, with a 9% decrease in fetal weight and no difference in placental weight (Rummens, van Cromphaut et al. 2003, Kovacs, Woodland et al. 2005). This indicates that there were placental and/or maternal adaptations to the low calcium environment which compensated for the nutritional deficit, as seen in other mild nutritional deficiencies such as protein deficiency (Fowden, Sferruzzi-Perri et al. 2009).

### 3.5.2 Morphometry

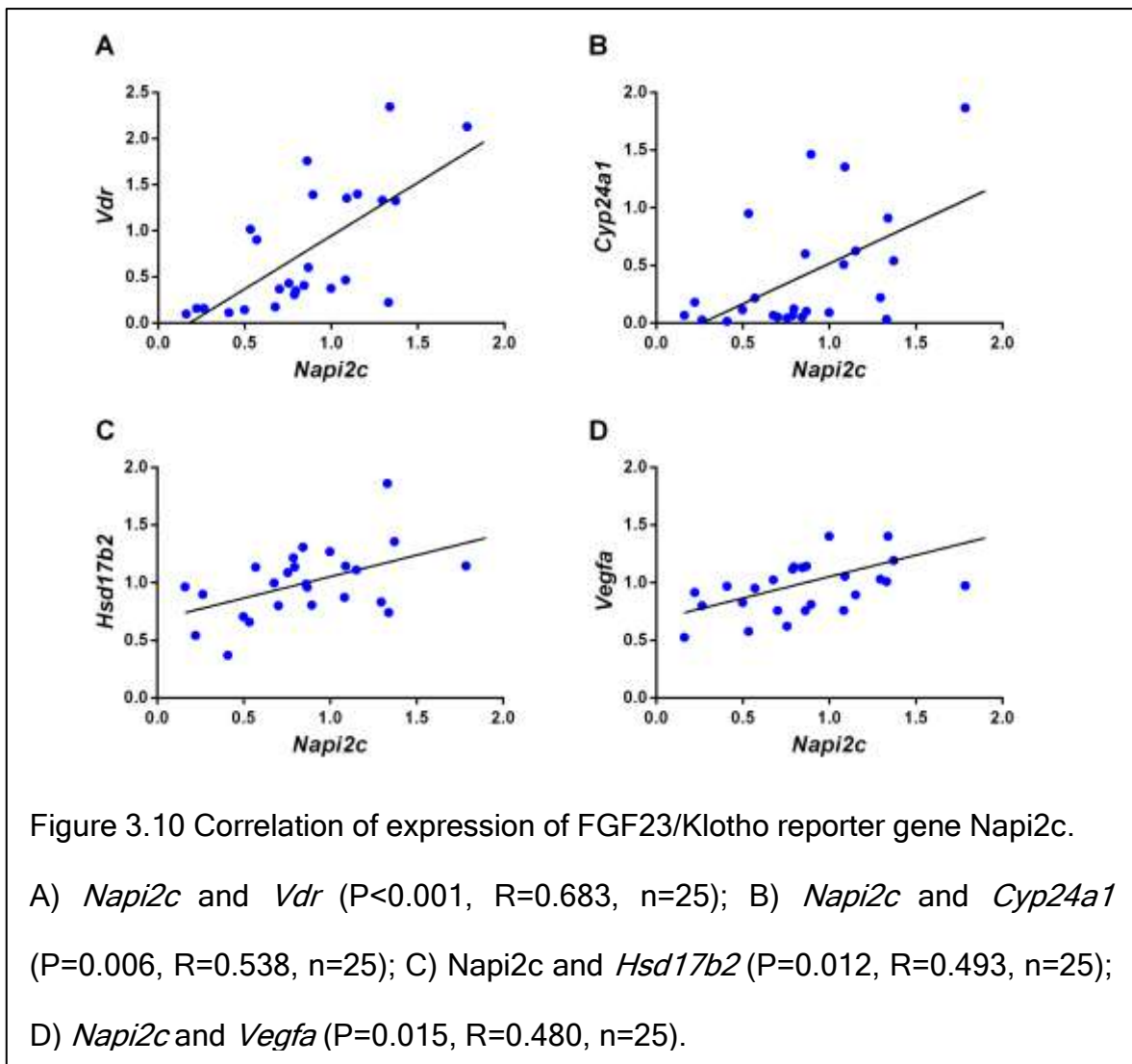
Labyrinth to junctional area ratio was decreased in mice that fed low calcium diets. This was expected, as the offspring were also expected to have lower fetal weights, as does the *Vdr* null mouse. Instead, the fetuses were heavier





with no change in placental weight and instead had changes in placental morphometry.

Morphometry revealed no alteration in trophoblast volume due to diet, although maternal blood space was increased and fetal capillary density was decreased in more deficient mice. Low dietary vitamin D<sub>3</sub> was associated with both the increase in blood space and the decrease in capillaries. Increased maternal blood space could be due to increased blood pressure in the deficient mice. Vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) is a negative regulator of renin-dependent hypertension, by reducing renin expression and angiotensinogen II levels (Li, Kong et al. 2002). During pregnancy, vitamin D<sub>3</sub> deficiency induces increased blood pressure which



is normalized by re-introducing dietary vitamin D<sub>3</sub> (Liu, Ouyang et al. 2013). Modest increases in blood pressure are observed in normal pregnancies to increase placental perfusion, but major increases are a feature of preeclampsia (Stegers, von Dadelszen et al. 2010). Through this increased placental perfusion, the deficient placenta is able to maintain fetal growth and exceed that of the control fetuses.

Trophoblast surface area and barrier thickness were also investigated to determine if there was an alteration in the exchange capacity of the fetomaternal barrier. Mice on the control diet had lower surface density and surface area, with a thicker trophoblast barrier compared to deficient mice. This indicates that the

placentas from “deficient” mice had a more optimal area for exchange, although they also had lower density of fetal capillaries. This reduced barrier thickness may be due to increased perfusion pressure due to increased maternal blood pressure, or local hormonal changes as vitamin D<sub>3</sub> stimulates production of estradiol and human placental lactogen among others (Stephanou, Ross et al. 1994, Barrera, Avila et al. 2007). A subset of mice had serum 25(OH)D<sub>3</sub> levels measured, with the mice on the control diet having a mean concentration of 40 nmol/L and mice on deficient diets having 17 nmol/L (see Chapter 2). This is similar to the distribution of barrier thicknesses as those mice in deficient groups had both low 25(OH)D<sub>3</sub> and thinner barriers, whereas control mice had higher 25(OH)D<sub>3</sub> levels and thicker barriers, suggesting that 25(OH)D<sub>3</sub> deficiency is associated with reduced barrier thickness. Although 25(OH)D<sub>3</sub> appears to modulate placental cellular composition, we saw no observable effects on fetal skeletal mineralisation (see Chapter 2). This corresponds with other studies in vitamin D<sub>3</sub> deficient rats (Brommage and DeLuca 1984, Glazier, Mawer et al. 1995), but not with *Vdr* null mice (Rummens, van Cromphaut et al. 2003, Kovacs, Woodland et al. 2005), which had reduced placental calbindin-9k and fetal calcium content.

### 3.5.3 Gene expression

Expression of vitamin D<sub>3</sub> metabolism genes in the placenta has been previously characterized in normal pregnant mice, but not in response to dietary calcium and vitamin D<sub>3</sub> deficiency. Low calcium diets induced a 2-fold increase in placental *Vdr* expression and a 6-fold increase in *Cyp24a1* expression. There was also a strong positive correlation between *Vdr* and *Cyp24a1* expression. As *Cyp24a1* is directly up-regulated by *Vdr* protein in response to high 1,25(OH)<sub>2</sub>D<sub>3</sub> levels, *Cyp24a1* expression is a marker of circulating 1,25(OH)<sub>2</sub>D<sub>3</sub>. Mice on low

calcium diets had increased circulating  $1,25(\text{OH})_2\text{D}_3$ . This is due to the high calcium requirements in pregnancy; circulating  $1,25(\text{OH})_2\text{D}_3$  levels are increased in normal pregnancy (Ardawi, Nasrat et al. 1997), but more so in animals on low calcium diets. This allows  $1,25(\text{OH})_2\text{D}_3$  to promote intestinal calcium absorption and resorption of calcium from the maternal skeleton. Surprisingly, *Cyp24a1* was up-regulated in association with increased renal  $1,25(\text{OH})_2\text{D}_3$  synthesis, despite previous reports indicating that *Cyp24a1* is significantly methylated in the mouse placenta and thus silenced (Novakovic, Sibson et al. 2009).

There was no effect of diet on either *Cyp27b1* or *Cyp2r1* expression. As *Cyp2r1* is the main enzyme converting vitamin  $\text{D}_3$  to  $25(\text{OH})\text{D}_3$ , this indicates that there is no drive for the placenta to increase production of  $25(\text{OH})\text{D}_3$  in response to deficient diets. *Cyp27b1* expression was highly variable within dietary groups, but was not significantly altered by diet, although expression was 3.4-fold higher in the most deficient diet (0 IU/kg  $\text{D}_3$ + 0.1% Ca) compared to the controls. As placental *Cyp27b1* expression was unaffected by diet, in contrast to *Cyp24a1* and *Vdr*, we can assume that the placenta is not responsible for the increase in circulating  $1,25(\text{OH})_2\text{D}_3$  found in pregnancy. This has been supported by work in the kidney in our lab (see Chapter 2) as well as recent work by Kirby *et al.* (Kirby, Ma et al. 2013).

Vitamin  $\text{D}_3$  responsive genes have the vitamin  $\text{D}_3$  responsive element (VDRE) in their promoter region and are up or down regulated by  $1,25(\text{OH})_2\text{D}_3$  and *Vdr* binding. As *Vdr* and *Cyp24a1* are up-regulated through this process under the influence of  $1,25(\text{OH})_2\text{D}_3$ , we investigated whether other genes with identified VDRE's expressed in the mouse placenta were also responsive to high circulating levels of  $1,25(\text{OH})_2\text{D}_3$  in low calcium consuming mice. We examined three VDRE genes: *Hsd17b2* - a steroid metabolism enzyme responsible for producing

estradiol and estrone; *Vegfa* - vascular endothelial growth factor, involved in angiogenesis and *Hif1a* - hypoxia inducible factor 1a, transcription factor involved in oxygen-mediated gene expression such as *Vegfa* (Wang, Tavera-Mendoza et al. 2005). There was no association between changes in the synthesis of renal 1,25(OH)<sub>2</sub>D<sub>3</sub> levels due to diet and altered levels of mRNA for these genes, indicating that they are not readily regulated by changes to circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels. Whether 1,25(OH)<sub>2</sub>D<sub>3</sub> has no effect at d18.5 perhaps due to *Cyp24a1* protein induced catabolism, or that these genes are simply not vitamin D<sub>3</sub> responsive in mice, remains to be determined.

Expression of phosphate transporters *Napi2a* and *Napi2c* were quantified to determine whether FGF23/Klotho affects expression of placental vitamin D<sub>3</sub> metabolism genes. FGF23 is released from osteocytes within bone into the circulation in response to high serum phosphorus levels and, in combination with its cofactor Klotho, increases transcription of phosphate transporters in target tissues, such as the kidney. As the placenta has been shown to express *Napi2a*, *Napi2c*, *Klotho* and FGF23/Klotho receptors (Kuro-o, Matsumura et al. 1997, Wu, Orozco et al. 2009), but not *Fgf23* (Kirby, Ma et al. 2013) we examined reporter genes of their activity. There was no change in expression of either *Napi2a* or *Napi2c* in response to diet, although *Napi2c* was non-significantly increased in mice on deficient diets compared to control. Although *Napi2c* was not altered by diet, it was highly correlated with genes associated with vitamin D<sub>3</sub> activity and metabolism (*Vdr*, *Cyp24a1*) and genes with known VDREs (*Hsd17b2*, *Vegfa*). This indicates that there may be an influence of FGF23 which is not detected when analysing by diet and warrants further investigation. Recently, a study demonstrated that there is a 2.25-fold increase in *Fgf23* serum levels during pregnancy in wild type mice, this was not influenced by 1,25(OH)<sub>2</sub>D<sub>3</sub> although in

non-pregnant mice it would suppress  $1,25(\text{OH})_2\text{D}_3$  synthesis (Kirby, Ma et al. 2013).

### 3.5.4 Strengths and limitations

The strength of our study lies in our investigating both the effects of vitamin  $\text{D}_3$  and calcium, as dietary vitamin  $\text{D}_3$  and  $25(\text{OH})\text{D}_3$  levels were found to affect placental morphometry, while dietary calcium and inferred  $1,25(\text{OH})_2\text{D}_3$  levels affected gene expression. This sets this study apart, as many previous studies examine only the effect of vitamin  $\text{D}_3$  on pregnancy, while calcium has a leading role. The study was limited in that the pregnancy rate in our mice was only 60%, reducing the number of litters and placentas available for analysis. We determined that levels of serum total calcium, corrected total calcium and inorganic phosphorous were unaffected by diet (data not shown). This demonstrates that we have induced mild vitamin  $\text{D}_3$  deficiency, without causing hypocalcaemia, at a similar level to that seen in vitamin  $\text{D}_3$  deficiency in human pregnancy.

### 3.5.5 Conclusion

In conclusion, mild vitamin  $\text{D}_3$  and calcium deficiency had multiple effects on the fetus and placenta. Increased fetal weight was observed from low calcium consuming mice explained by placental adaptations including increased maternal blood space and reduced placental barrier thickness to increase the opportunity for feto-maternal exchange. Gene expression was also altered, with increased *Cyp24a1* and *Vdr* expression indicating high levels of circulating  $1,25(\text{OH})_2\text{D}_3$  in low calcium consuming mice. FGF23/Klotho reporter *Napi2c* was also found to be correlated with *Cyp24a1* and *Vdr*, indicating that there may be an influence of Fgf23 on placental gene expression. Taken together, these findings increase our

understanding of the effect of vitamin D<sub>3</sub> and calcium deficiency on placental development and local vitamin D<sub>3</sub> metabolism. This knowledge will aid our understanding of placental vitamin D<sub>3</sub> metabolism as well as inform us of vitamin D<sub>3</sub>'s interaction with the placenta, which can then be applied to pregnancy complications.

# Statement of Authorship

Title of Paper	Chapter 4 – Human placental expression of vitamin D metabolism components across gestation and in complications [proposed title]
Publication Status	<input type="radio"/> Published, <input type="radio"/> Accepted for Publication, <input type="radio"/> Submitted for Publication, <input checked="" type="radio"/> Publication style
Publication Details	Written in publication style, currently being prepared for publication.

## Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Jessica Laurence		
Contribution to the Paper	Experimental design Experimental work - PCR, Immunohistochemistry Analysis of data Interpretation of results Manuscript preparation		
Signature		Date	23/10/14

Name of Co-Author	Tina Blanco-Miotto		
Contribution to the Paper	Experimental design Experimental troubleshooting - PCR Critical comments on manuscript		
Signature		Date	17.10.14

Name of Co-Author	Prabha Andrewera		
Contribution to the Paper	Assistance with experimental work and study design		
Signature		Date	22.10.14

Name of Co-Author	Paul Anderson		
Contribution to the Paper	Data interpretation Critical comments on manuscript		
Signature		Date	22/10/14



# Statement of Authorship

Title of Paper	Chapter 4 – Human placental expression of vitamin D metabolism components across gestation and in complications [proposed title]
Publication Status	<input type="radio"/> Published, <input type="radio"/> Accepted for Publication, <input type="radio"/> Submitted for Publication, <input checked="" type="radio"/> Publication style
Publication Details	Written in publication style, currently being prepared for publication.

## Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Gustaff Dekker		
Contribution to the Paper	Critical comments on manuscript		
Signature		Date	13.10.14

**PROFESSOR GUS DEKKER**  
**Medical Co-Director**  
**Women & Children's Division**  
**Northern Adelaide Local Health Network**

Name of Co-Author	Claire Roberts		
Contribution to the Paper	Experimental design Immunohistochemistry troubleshooting Critical comments on manuscript		
Signature		Date	23.10.14

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

# Chapter 4: Human placental expression of vitamin D<sub>3</sub> metabolism and insulin-like growth factor components across gestation and in disease

## 4.1 Abstract

Pregnancy complications affect 25% of ongoing pregnancies and are associated with placental insufficiency. As vitamin D and the insulin-like growth factor (IGF) pathways interact, we hypothesise that placental vitamin D and IGF-related pathways are differentially expressed across gestation and in complications.

Placentas were collected from elective first trimester pregnancy terminations (n=17), uncomplicated term (n=30) and complicated pregnancies (preeclampsia (PE, n=19), spontaneous preterm birth (sPTB, n=14), gestational hypertension (GH, n=14), small for gestational age (SGA, n=14), gestational diabetes mellitus (GDM, n=6)). Vitamin D and IGF pathway components were examined by qPCR and immunohistochemistry.

*VDR* and *CYP2R1* mRNA were expressed highly in uncomplicated term placentas, whereas *IGF2* and *IGF1R* expression were lowest at term, with *VDR* and *IGF2* positively correlated across gestation ( $P < 0.05$ ). Immunohistochemistry revealed intense localisation of CYP27B1, VDR and IGF2 in early gestation, compared to uncomplicated term placentas. At term, increased *VDR* mRNA with low protein may indicate high turnover.

Analysis of complicated pregnancies revealed reduced VDR mRNA in PTB and SGA, whereas IGF1 was increased in PTB ( $P < 0.05$ ). This suggests placental vitamin D and IGF pathways are affected in SGA and sPTB pregnancies.

Differential placental gene and protein expression was found across gestation in both vitamin D and IGF pathways. Placentas complicated with SGA and sPTB also had altered expression of VDR and IGF1. These data support a role for vitamin D in placental development and pregnancy complications, as well as suggesting potential interactions between IGFs and vitamin D in the placenta.

## 4.2 Introduction

Vitamin D<sub>3</sub> deficiency during pregnancy has been associated with pregnancy complications, such as preeclampsia (PE), preterm birth (PTB), small-for-gestational age (SGA) babies and gestational diabetes mellitus (GDM) (Wei, Qi et al. 2013). As these complications are also associated with poor placental development (Khong, De Wolf et al. 1986, Khong, Liddell et al. 1987, Morgan, Tolosa et al. 2013), discerning the role of vitamin D<sub>3</sub> in pregnancy and in the placenta may provide insight into pregnancy complications.

Vitamin D<sub>3</sub> is produced in the skin through the action of UV-B, which breaks down 7-dehydrocholesterol into vitamin D<sub>3</sub>. This is then hydroxylated in the liver by 25-hydroxylase (encoded by CYP2R1) to 25(OH)D<sub>3</sub>, which is measured clinically to determine vitamin D<sub>3</sub> status. This is further hydroxylated by 1 $\alpha$ -hydroxylase (encoded by CYP27B1) to the active form of vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), which can bind the nuclear transcription factor vitamin D<sub>3</sub> receptor (encoded by VDR). Active vitamin D<sub>3</sub> is then metabolized through further hydroxylation by 24-hydroxylase (encoded by CYP24A1) to inactive metabolites (Jones, Prosser et al. 2014).

Circulating active 1,25(OH)<sub>2</sub>D<sub>3</sub> in the mother increases across gestation, reaching 3-5 fold higher levels by term compared to the non-pregnant state (Ardawi, Nasrat et al. 1997). Vitamin D<sub>3</sub> in the placenta was originally thought to contribute to calcium transfer but this has been found to be a vitamin D<sub>3</sub>-

independent mechanism (Kovacs, Woodland et al. 2005). Active  $1,25(\text{OH})_2\text{D}_3$  in the placenta up regulates gene expression through VDR of many placenta- and pregnancy-related factors, including components of the insulin-like growth factor (IGF) family.

The IGF family of molecules includes IGF1 and IGF2 (encoded by IGF1 and IGF2), their receptors (IGF1R and IGF2R) and binding proteins (IGFBP1-7). Both IGF1 and IGF2 are essential for fetal growth. Mice that have either IGF1 or IGF2 ablated have 45% and 60% lower birth weights, respectively, than wild type (DeChiara, Efstratiadis et al. 1990, Liu, Baker et al. 1993). While IGF2 has a role in placental differentiation, IGF1 is involved in modulating maternal adaptation to pregnancy and increasing placental nutrient transport (Roberts, Owens et al. 2008). Circulating levels of IGF1 initially decrease in early pregnancy, while in third trimester levels increase by 33% of non-pregnant levels (Halhali, Villa et al. 2004, Moller, Streym et al. 2013).

There is some evidence of biologically significant interactions between vitamin  $\text{D}_3$  and IGF pathways. Addition of IGF1 to trophoblast cultures induced  $1,25(\text{OH})_2\text{D}_3$  production, although this failed to occur in the presence of a protein synthesis inhibitor, cyclohexamide, suggesting that increased CYP27B1 was responsible for increased  $1,25(\text{OH})_2\text{D}_3$  production (Halhali, Diaz et al. 1999). Although there are potential interactions between vitamin  $\text{D}_3$  and IGF pathways, there has been no investigation into changes in IGF gene expression on addition of  $1,25(\text{OH})_2\text{D}_3$  in the placenta. In other tissues, addition of  $1,25(\text{OH})_2\text{D}_3$  in vitro increased expression of IGF1 and IGFBP-3 (mRNA and protein) in chondrocytes (Fernandez-Cancio, Audi et al. 2009), increased IGF1R protein in cancer cells (Liu, Guo et al. 2011) and IGFBP-1 protein in ovarian cells (Parikh, Varadinova et al.

2010). Interestingly, addition of  $1,25(\text{OH})_2\text{D}_3$  to calvaria cells increased IGF2, but inhibited IGF1 protein secretion (Linkhart and Keffer 1991).

There is evidence for the proposed interaction between vitamin  $\text{D}_3$  and IGFs in pregnancy, with altered levels of IGF and vitamin  $\text{D}_3$  pathway components across gestation. Halhali et al. showed that at delivery maternal and fetal circulating concentrations of  $1,25(\text{OH})_2\text{D}_3$ , IGF1 and calcium are lower in PE affected compared to control pregnancies (Halhali, Tovar et al. 2000, Halhali, Diaz et al. 2014). Circulating  $1,25(\text{OH})_2\text{D}_3$  and IGF1 levels are correlated in uncomplicated pregnancies in late gestation, with this relationship lost in preeclamptic pregnancies (Halhali, Villa et al. 2004). Placental homogenates from preeclamptic pregnancies that were treated with  $25(\text{OH})\text{D}_3$  convert less  $25(\text{OH})\text{D}_3$  to  $1,25(\text{OH})_2\text{D}_3$ , have lower IGF1 protein levels (Halhali, Diaz et al. 2014). Furthermore, when primary placental cultures from preeclamptic pregnancies were treated with IGF1 their rates of production of  $1,25(\text{OH})_2\text{D}_3$  were lower than normal placentas (Diaz, Arranz et al. 2002). This demonstrates that there is a relationship between IGF1 and  $1,25(\text{OH})_2\text{D}_3$  in the placenta.

As both maternal circulating  $1,25(\text{OH})_2\text{D}_3$  and IGF1 levels are highest at term, have interacting pathways and are both associated with pregnancy complications, we hypothesise that expression in these gene families is altered between early gestation and term, as well as between term uncomplicated and complicated placentas.

## **4.3 Methods**

### **4.3.1 Recruitment**

Human placental tissue from first trimester elective terminations of pregnancy was collected from the Women's and Children's Hospital, Adelaide. The study was approved by the Children Youth and Women's Health Service Research Ethics Committee (Approval #REC1835/8/09). Term placental samples for RNA extraction were collected from women enrolled in the Screening for Pregnancy Endpoints (SCOPE) and Prediction of Adverse Pregnancy Outcomes (PAPO) studies at the Lyell McEwin Health Service, Elizabeth Vale and the Women's and Children's Hospital, Adelaide with approval from the Central Northern Adelaide Health Service Ethics of Human Research Committee and the Women's and Children's Health Network (WCHN) Human Research Ethics Committee (Approval# 2005082, REC# 1481/6/09). Term placenta samples for immunohistochemistry were also collected from the Lyell McEwin Health Service (Approval #HREC/12/TQEHLMH/16). All samples were obtained with written informed patient consent.

### **4.3.2 Classification of placental samples**

First trimester placenta samples were classified by gestational age, as estimated by the last menstrual period (LMP) and/or ultrasound by the attending clinician. Samples were grouped into early first trimester (6-8 weeks' gestation) and late first trimester (10-12 weeks' gestation). Extravillous cytotrophoblasts occlude the uterine spiral arterioles until 8 weeks' gestation, preventing maternal blood flow to the placenta. The plugs are dislodged over the next few weeks (Burton, Jauniaux et al. 1999), with maternal blood flow to the placenta occurring in

most placentas by 12 weeks' gestation (Jauniaux et al. 2003). Comparisons were made between early first trimester placentas (n=9, 7.0 ± 0.3 weeks' gestation), late first trimester placentas (n=8, 10.4 ± 0.2 weeks' gestation) and term controls (n=18, 40.5 ± 0.2 weeks' gestation).

For term placenta samples, pregnancy complications were classified by an obstetrician as described previously (Andraweera, Dekker et al. 2012), with patient demographics listed in Table 4.1. Gestational hypertension (GH) was defined as blood pressure of ≥140 mmHg systolic or ≥ 90 mmHg diastolic on at least two occasions more than 4 h apart after 20 weeks' gestation. Spontaneous preterm birth (sPTB) was defined as spontaneous preterm labour or premature rupture of membranes resulting in birth at less than 37 completed weeks of gestation. Normotensive small for gestational age (SGA) was defined as a birth weight <10<sup>th</sup> centile using customized centiles adjusting for maternal height, weight, parity and ethnicity, fetal sex, birth weight and gestational age ([www.gestation.net](http://www.gestation.net)). Preeclampsia (PE) was defined as gestational hypertension with proteinuria and/or any multi-system complication, including SGA (Tranquilli, Dekker et al. 2014). Gestational diabetes mellitus (GDM) was diagnosed by a glucose tolerance test (GTT) with a loading dose of 75g followed by a fasting glucose ≥ 5.5 mmol/L or a 2 h glucose ≥ 8.0 mmol/L. Uncomplicated pregnancies were defined as pregnancies with no antenatal medical or obstetric complications, resulting in the delivery of an appropriately grown healthy infant at or after 37 weeks of gestation. In the few cases with multiple complications in a pregnancy (n=8), the following hierarchy was used to classify cases: PE>sPTB>GH>SGA>GDM.

Table 4.1. Maternal characteristics associated with term placental samples for gene expression.

Characteristic	Control N=30	PE N=19	GH N=14	sPTB N=14	SGA N=14	GDM N=6
Maternal Age (years)	25.4 ± 1.1	26.5 ± 1.5	25.5 ± 1.8	25.6 ± 1.6	23.1 ± 1.6	28.7 ± 2.5
Gravidity	1.8 ± 0.2	1.7 ± 0.2	1.6 ± 0.3	2.6 ± 0.3	1.3 ± 0.3	1.7 ± 0.5
Parity	0.5 ± 0.1	0.2 ± 0.1	0.1 ± 0.1 <sup>a</sup>	0.4 ± 0.2	0.1 ± 0.2	0.3 ± 0.3
BMI (kg/m <sup>2</sup> )	25.4 ± 1.1	29.4 ± 1.5	27.8 ± 1.3	25.2 ± 1.6	25.5 ± 1.5	31.5 ± 2.6
Gestation at delivery (weeks)	40.2 ± 0.3 <sup>a</sup>	39.0 ± 0.4 <sup>a</sup>	39.4 ± 0.3 <sup>a</sup>	34.4 ± 0.5 <sup>b</sup>	39.5 ± 0.5 <sup>a</sup>	38.9 ± 0.7 <sup>a</sup>
Birth weight (kg)	3.6 ± 0.1 <sup>a</sup>	3.0 ± 0.1 <sup>a</sup>	3.4 ± 0.1 <sup>a</sup>	2.5 ± 0.1 <sup>b</sup>	2.6 ± 0.1 <sup>b</sup>	3.3 ± 0.2 <sup>a</sup>
Placental weight (g)	610 ± 29 <sup>a</sup>	527 ± 30 <sup>a</sup>	520 ± 22 <sup>a</sup>	497 ± 40 <sup>a</sup>	461 ± 37 <sup>b</sup>	580 ± 56 <sup>a</sup>
Fetal : placental weight ratio	6.3 ± 0.2 <sup>a</sup>	5.9 ± 0.3 <sup>a</sup>	6.6 ± 0.3 <sup>a</sup>	5.3 ± 0.3 <sup>b</sup>	5.8 ± 0.3 <sup>a</sup>	6.5 ± 0.2 <sup>a</sup>
Fetal sex (% male)	43 ± 0.1	47 ± 0.1	64 ± 0.1	50 ± 0.1	50 ± 0.1	50 ± 0.2

Note: Placental weight was recorded for 85% of samples (Control 23/30, PE 16/19, PTB 12/14, NSGA 14/14, GH 11/14, GDM 6/6); PE - preeclampsia, GH - gestational hypertension, sPTB - spontaneous preterm birth, SGA - small for gestational age, GDM - gestational diabetes mellitus; Mean ± SEM; different superscripts denote significant differences (P<0.05).

### 4.3.3 Placental tissue sampling

First trimester placental and decidual tissues were collected after elective pregnancy termination surgery, washed briefly to remove blood (PBS, pH 7.4) and were either snap-frozen in liquid nitrogen then stored at -80°C until RNA extraction or were fixed for histology. Full thickness term placental tissue was collected at delivery and stored in RNAlater® (Ambion, Life Technologies, CA, USA) for 24 h at 4°C then transferred to -80°C until RNA extraction. Tissue collected for histology



was fixed after surgery or delivery (term placenta, first trimester placenta and decidua) and processed into paraffin wax.

#### 4.3.4 RNA Extraction

RNA was extracted from villous tissue by the TRIzol method (Invitrogen, Life Technologies, CA, USA). Briefly, 100 mg of frozen villous tissue was added to 1 mL of TRIzol. Tissue was homogenised in TRIzol by a Powerlyser (MoBio, CA, USA) with ceramic beads (CK14; Geneworks, SA, AU) followed by RNA extraction according to the manufacturer's instructions. RNA was reconstituted in nuclease-free water (Ambion, Life Technologies, CA, USA) and subjected to rigorous DNase treatment using a TURBO *DNase-free*<sup>™</sup> kit (Ambion, Life Technologies, CA, USA) according to the manufacturer's instructions

RNA integrity was assessed by Experion (BioRad, CA, USA) with RQI >5 acceptable quality for qPCR, although small amplicons (<250 bp) can be considered independent of RQI (Fleige and Pfaffl 2006). Absence of genomic DNA was confirmed by PCR of DNase-treated RNA with genomic DNA primers(F-GCCTCTTCTTGTTAATTTCCCTGTT; R - TTCAGTTTCTCCACAGACATTCAA; amplicon length 95 bp, annealing temperature 60°C, Geneworks), with lack of amplification indicating absence of gDNA. RNA (500 ng) was reverse transcribed using iScript cDNA Synthesis Kit (BioRad, CA, USA) following the manufacturer's instructions. cDNA was diluted 1/10 and stored at -20°C until required.

Human kidney RNA (First Choice Human Total RNA Survey Panel, Life Technologies, CA, USA) was used as a positive control for *CYP27B1* expression as this gene is highly expressed in the kidney (Lawson, Fraser et al. 1971). RNA was reverse transcribed and diluted as above and analysed alongside placental cDNA to compare cycle of amplification.

Table 4.2. Custom primer sequences for gene expression

Gene	Primer Sequence	Amplicon length	Annealing Temp
<i>IGF1</i>	F - ATGCTCTTCAGTTCGTGTGT R - AGCTGACTTGGCAGGCTTGA	176 bp	63°C
<i>IGF2</i>	F - CCCCTCCGACCGTGCT R - TGGACTGCTTCCAGGTGTCAT	90 bp	60°C
<i>IGF1R</i>	F - CTTCTCCTAAAAACCTTCGCCTCAT R - GTACATTTCCCTGCTTTGATGGT	142 bp	60°C
<i>IGF2R</i>	F - GCAGAAGCTGGGTGTCATAGG R - CACGGAGGATGCGGTCTTAT	88 bp	60°C
<i>YWHAZ</i>	F - TCCGGACACAGAACATCCAG R - AGAAGATTCCTCTCCTCATTGGATAAT	153 bp	60°C
<i>GUSB</i>	F - CGTCCCACCTAGAATCTGCT R - TTGCTCACAAAGGTCACAGG	94 bp	63°C
<i>HPRT1</i>	F - GTTATGGCGACCCGACG R - ACCCTTTCAAATCCTCAGC	107 bp	60°C
<i>RPL32</i>	F - TTCCTGGTCCACAACGTCAAG R - TTGTGAGCGATCTCGGCAC	77 bp	60°C
<i>HBMS</i>	F - CCACACACAGCCTACTTTCCAA R - TTTCTTCCGCCGTTGCA	70 bp	60°C

#### 4.3.5 Quantitative PCR (qPCR)

Quantitative PCR was performed on a CFX-384 real-time PCR machine (BioRad, CA, USA). All reactions were in 10  $\mu$ L total volumes, composed of 5  $\mu$ L Master Mix, 2  $\mu$ L cDNA (diluted 1/10 in nuclease free water), 0.5  $\mu$ L TaqMan Assay or 0.25  $\mu$ L each of forward and reverse primers at 10  $\mu$ M and 2.5  $\mu$ L nuclease-free water. Reactions were set up in triplicate using a QIAgility liquid handling system (QIAGEN, Hilden, DE). PCR reactions were comprised of either TaqMan Gene Expression Master Mix and TaqMan Assays (Invitrogen, Life Technologies, CA, USA; *VDR* - Hs01045840\_m1; *CYP24A1* - Hs00167999\_m1; *CYP2R1* - Hs01379776\_m1; *CYP27B1* - Hs01096154\_m1) or FastStart Universal SYBR Master Mix (Roche) and custom primers (Geneworks, SA, AU; Table 4.2).

GeNorm algorithms embedded in the CFX Manager Software (version 3.0, BioRad) were used to determine the optimal combination of reference genes for normalization (Hellemans, Mortier et al. 2007). PCR data were normalized to the geometric mean of the reference genes using CFX Manager Software. The optimal

reference genes had the lowest coefficient of variance (CV) and variation score (M) calculated by GeNorm, with the optimal reference genes being *RPL32* and *YWHAZ* for first trimester using (CV - 0.2152, M - 0.6160) and term complication (CV -0.2541, M - 0.7241) gene expression, while *GUSB*, *HMBS* and *HPRT1* were less stable.

#### 4.3.6 Histology

Placenta samples were fixed in 10% neutral buffered formalin (Australian Biostain, NSW, AU) for 24 h, then washed three times in PBS and stored in 70% ethanol. Samples were then processed through 70%, 80%, 90% ethanol and three changes of absolute ethanol, respectively, for 1 h each for term placenta or 20 min each for first trimester placenta and decidua. Samples were then incubated in two changes of paraffin wax at 60°C for 2 h each for term placenta or 40 min each for first trimester and decidua. Samples were then embedded in paraffin wax before being cut into 5 µm sections.

#### 4.3.7 Immunohistochemistry

Sections were immunohistochemically labelled for the vitamin D<sub>3</sub> metabolic proteins VDR, CYP27B1, CYP24A1 (Santa Cruz Biotechnology, TX, USA) and IGF2 (R&D Systems, Minneapolis, USA) (see Table 4.3 for details). After dewaxing and re-hydration, sections were subjected to antigen retrieval by incubation with 0.3 mg/mL Pronase (P8811, Sigma-Aldrich, MO, USA) diluted in PBS for 5 min at 37°C.

Primary antibody was diluted in PBS with 10% serum (VDR and CYP24A1 - goat serum (Gibco, Life Technologies) CYP27B1 and IGF2 - rabbit serum (Veterinary Services, IMVS, SA, AU)) and 1% bovine serum albumin (BSA, Sigma-

Table 4.3. Primary and secondary antibody dilutions for immunohistochemistry, with corresponding serum used to dilute them.

1° Antibody (cat No.)	Dilution	2° Antibody (Catalogue No.)	Dilution
VDR D-6 (sc-13133)	1:200 - Early 1:50 - Term	Goat anti-mouse (E0433)	1:400
CYP24A1 H-87 (sc-66851)	1:100 - Early 1:100 - Term	Goat anti-rabbit (E0432)	1:800
CYP27B1 D-20 (sc-49643)	1:100 - Early 1:25 - Term	Rabbit anti-goat (E0466)	1:800
IGF2	1:50 - Early 1:50 - Term		1:800

2°Antibodies were purchased from Dako, Glostrup, Germany.

Aldrich, MO, USA) and was incubated overnight at room temperature. Secondary antibody was diluted in 1% BSA in PBS and incubated for 1 h. The conjugate streptavidin horseradish peroxidase (Rockland, PA, USA) was diluted 1:250 in PBS then incubated for an hour. Sections were incubated for 5 min with the chromogen diaminobenzidine (DAB, Sigma-Aldrich, MO, USA) in PBS to form a brown precipitate to identify the site of antibody binding. Tissue sections were counterstained with haematoxylin and eosin (Sigma-Aldrich) to label nuclei and cytoplasm, respectively. Sections were imaged using the NanoZoomer Digital Pathology scanner (Model C9600 v1.2, Hamamatsu Photonics K.K., JP) and visualized with NDP Scan software (v2.2, Hamamatsu Photonics K.K., JP).

#### 4.3.8 Statistics

Maternal characteristics were analysed by ANOVA with Sidak post-hoc testing. Outliers of greater than 2 standard deviations in placental gene expression data were excluded. Mann-Whitney U and Kruskal-Wallis tests were performed as relative gene expression was not normally distributed. Pearson's Bivariate Correlation tests were undertaken to determine correlations between gene expression. All analyses were performed using PASW Statistics 18 (SPSS,

Chicago, IL, USA). Results are presented as mean  $\pm$  standard error of the mean (SEM). Probabilities of  $P < 0.05$  were considered statistically significant.

## 4.4 Results

### 4.4.1 Gene expression across gestation: vitamin D<sub>3</sub> pathway

Expression of vitamin D<sub>3</sub> metabolism genes was analysed in early (6-8 weeks') and late (10-12 weeks') first trimester placenta samples and compared to term placenta from uncomplicated pregnancies. Samples had RQI  $> 5$  (RQI range: early first trimester, 5.2-8.5, late first trimester, 6.5-7.8 and term control placenta 6.3-9.0), which is acceptable for qPCR analysis (Fleige and Pfaffl 2006).

*CYP2R1* expression was highest in term placenta, with a 2-fold increase compared to first trimester ( $P < 0.001$ , figure 4.1A). There was also a 1.8-fold increase in *CYP2R1* expression from early to late first trimester ( $P = 0.020$ ). *VDR* was highly expressed in term placenta samples, with a 12-fold increase in expression between first trimester and term ( $P < 0.001$ , Figure 4.1B). *CYP24A1* was non-significantly higher in first trimester compared to term placentas, although term expression was 60% lower than in late first trimester placenta which approached statistical significance ( $P = 0.058$ , Figure 4.1C).

*CYP27B1* was expressed at a very low level, with some samples not amplifying in 40 cycles of PCR and therefore was considered not detectable. In the samples that had detectable *CYP27B1* ( $n = 21/34$  total) expression appeared to be higher in early first trimester than in term samples, as expected. As there were not enough samples to be adequately powered, there was no significant difference between early and late gestation ( $P = 0.335$ ). Human kidney RNA was used as a

positive control for *CYP27B1* expression, with kidney amplifying at cycle 29, compared to cycle 36-40 for placenta samples.

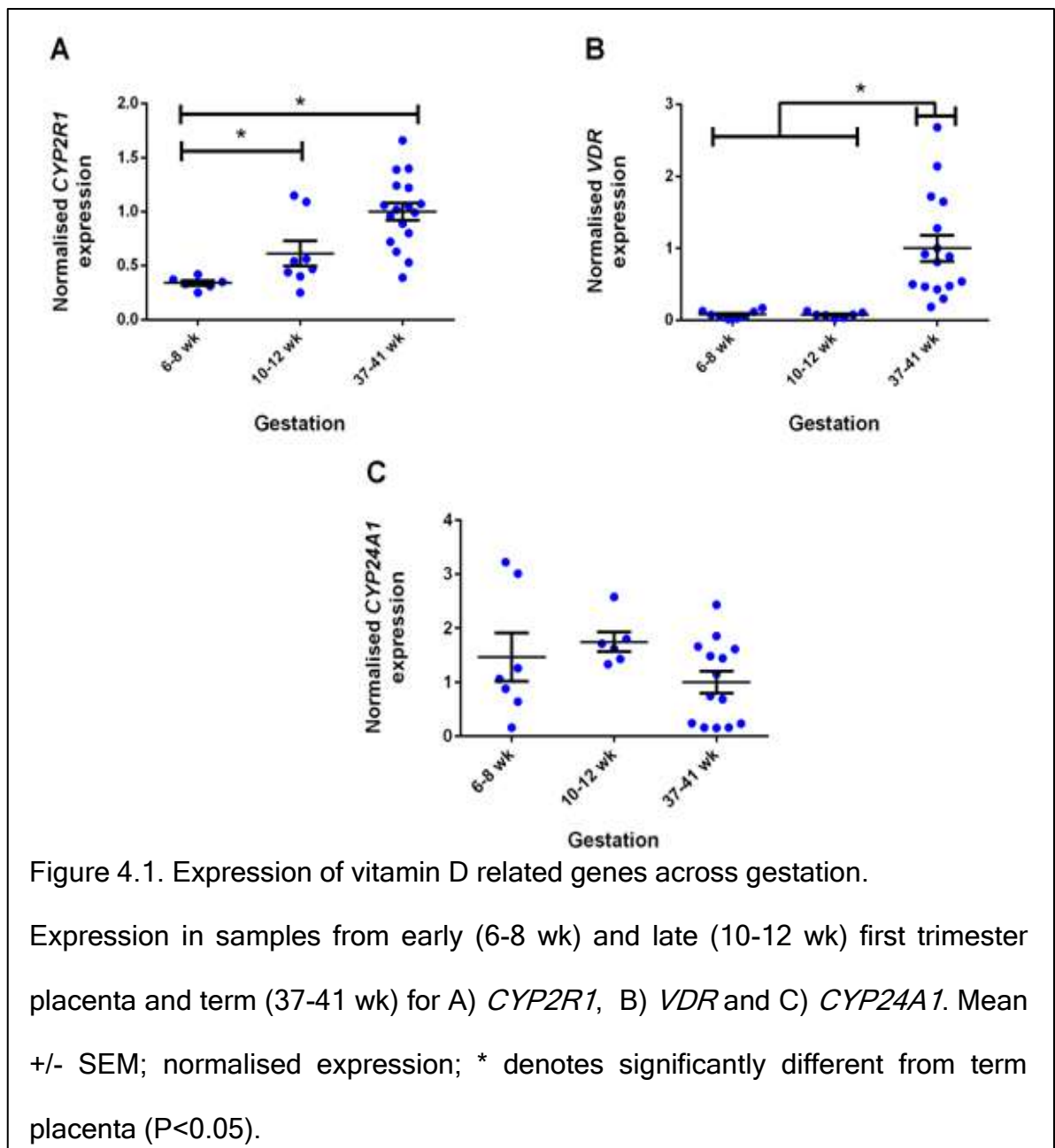


Figure 4.1. Expression of vitamin D related genes across gestation.

Expression in samples from early (6-8 wk) and late (10-12 wk) first trimester placenta and term (37-41 wk) for A) *CYP2R1*, B) *VDR* and C) *CYP24A1*. Mean +/- SEM; normalised expression; \* denotes significantly different from term placenta (P<0.05).

#### 4.4.2 Gene expression across gestation: IGF family

Placental expression of IGF family genes was much higher than vitamin D<sub>3</sub> metabolism genes, with *IGF2* having the highest expression (amplification at cycle 21-26) and *CYP27B1* having the lowest (cycle 36-40). *IGF2* expression was highest in first trimester (P=0.007, Figure 4.2B), with early first trimester placenta

having 3-fold higher expression ( $P=0.004$ ) and late first trimester having 70% higher expression ( $P=0.038$ ) than in term placentas. *IGF1R* expression was highest in first trimester, with expression 57% higher than at term ( $P<0.01$ ; Figure 4.2C). However, there was no change in expression of *IGF1* ( $P=0.284$ , Figure 4.2A) or *IGF2R* ( $P=0.267$ , Figure 4.2D) across gestation.

#### 4.4.3 Correlations of vitamin D<sub>3</sub> pathway and IGF family genes

Correlation analyses revealed several significant associations between genes, some of which were maintained across gestation. Placental *VDR* was positively associated with *IGF2* in both first trimester ( $P=0.007$ ,  $R=0.665$ , Figure 4.3A) and at term ( $P=0.001$ ,  $R=0.764$ , Figure 4.3B) and with *IGF2R* in first trimester ( $P<0.001$ ,  $R=0.857$ , Figure 4.3C) and at term ( $P=0.013$ ,  $R=0.603$ , Figure 4.3D). Correlations between *VDR* and *IGF2* in placenta have not been reported previously.

There were also gene expression correlations which were not present in both first trimester and term placentas. In first trimester placentas, *IGF1* and *CYP24A1* ( $P=0.008$ ,  $R=0.724$ ; Figure 4.3E) expression was positively correlated, but was not at term. However, *IGF1* and *IGF2R* ( $P=0.036$ ,  $R=-0.564$ , Figure 4.3F) were negatively correlated at term, but not in first trimester. *IGF2* and *IGF2R* were positively correlated in term placenta ( $P=0.005$ ,  $R=0.660$ , Figure 4.3H). *VDR* was positively correlated with *IGF1* in late first trimester placenta ( $P=0.035$ ,  $R=0.842$ , Figure 4.3G) but not in early first trimester or term placentas.

#### 4.4.4 Protein localization across gestation

*VDR* encodes a nuclear transcription factor which binds active vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) to alter gene expression. *VDR* protein expression was altered across gestation, with higher expression in first trimester than at term, (detected at 1:200 1°Ab dilution in first trimester but 1:50 at term, Figure 4.4). Expression was highly variable at term, ranging from nearly negative to strong staining. Across first trimester there was no difference in *VDR* staining intensity. *VDR* was strongly

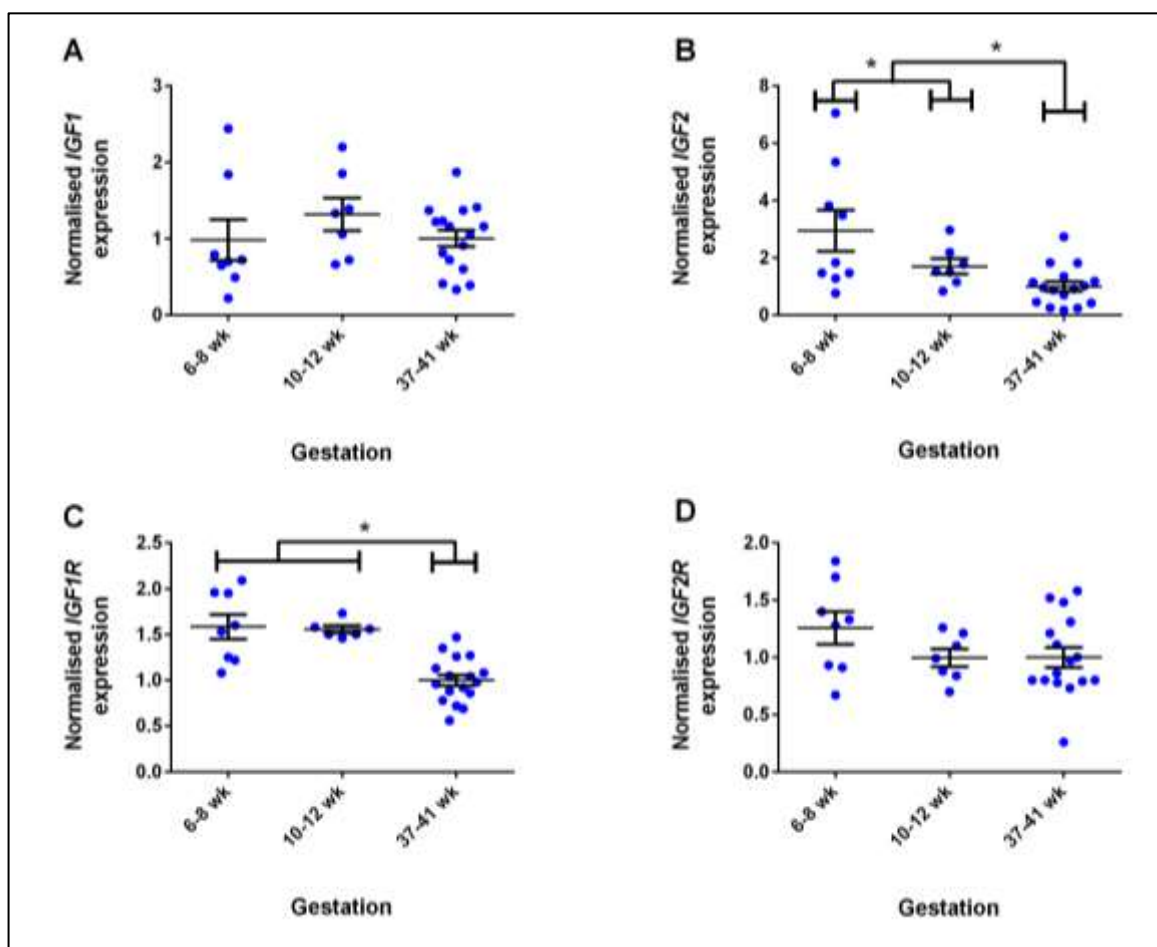
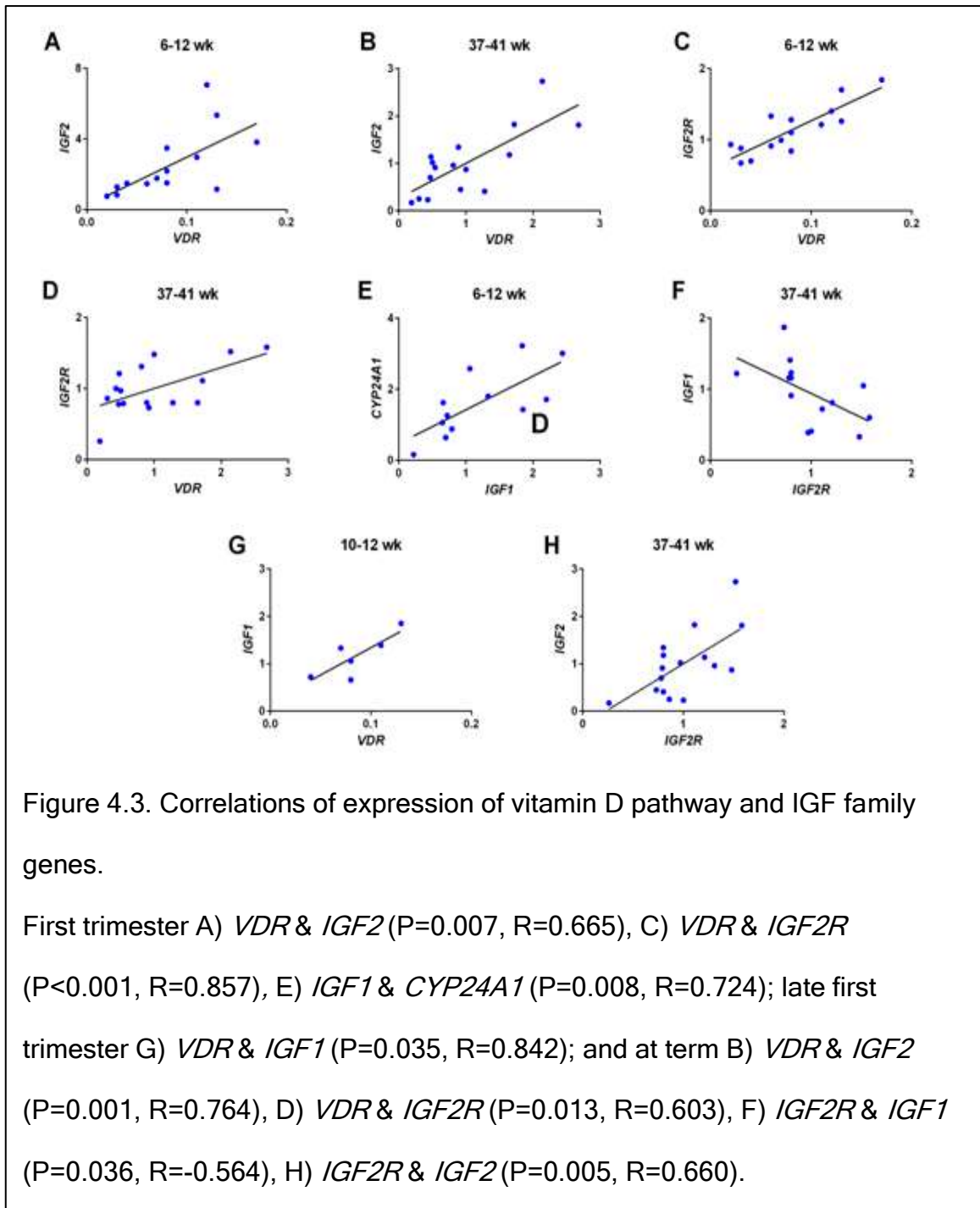


Figure 4.2. Expression of insulin-like growth factor genes across gestation.

Expression of samples from early (6-8 wk) and late (10-12 wk) first trimester placenta and term (37-41 wk) for A) *IGF1*, B) *IGF2*, C) *IGF1R* and D) *IGF2R*; Mean +/- SEM; normalised expression; \* denotes significantly different from term placenta (P<0.05).





localized to the syncytiotrophoblast and cytotrophoblast layers across gestation, with weaker staining observed in the fetal endothelium at term. The antibody has been previously validated in *VDR* null mouse skeletal muscle (Girgis, Mokbel et al. 2014) As *VDR* expression is under complex regulation, serum  $1,25(\text{OH})_2\text{D}_3$  levels, which are increased at term, may be one factor which alters placental *VDR* expression and may account for variability between samples.

CYP27B1 is a mitochondrial membrane protein which converts inactive vitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) to active vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>). Across gestation there was variation in staining intensity (Figure 4.5), with the most intense staining in first trimester (primary antibody dilution 1:25), compared to term placenta (detected at 1:10). Although CYP27B1 protein was found to be hypoxia responsive in primary cultures of term placenta (Ma, Gu et al. 2012), the change in CYP27B1 localisation does not correspond with the onset of placental blood flow and, instead, may be driven by decreased levels of circulating IGF1 (Kumar, Leverence et al. 2012) and increased 1,25(OH)<sub>2</sub>D<sub>3</sub> (Ardawi, Nasrat et al. 1997) across gestation. Staining

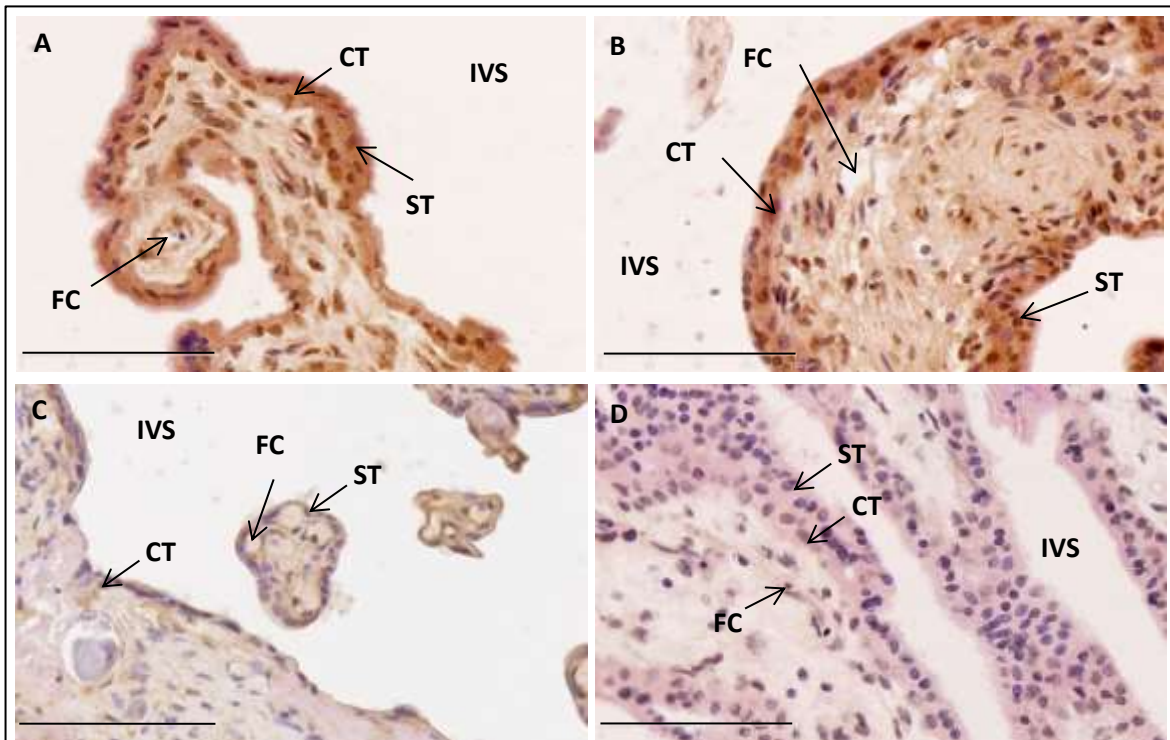


Figure 4.4. Localisation of VDR protein in first trimester and term placenta.

A) Early first trimester, B) late first trimester C) term control, D) negative.

Primary antibody was diluted 1:200 for both first trimester groups and 1:50 for term placenta scale line is 100µm; magnification is 40x; ST - syncytiotrophoblast, CT - cytotrophoblast, FC - fetal capillary, IVS - intervillous space.

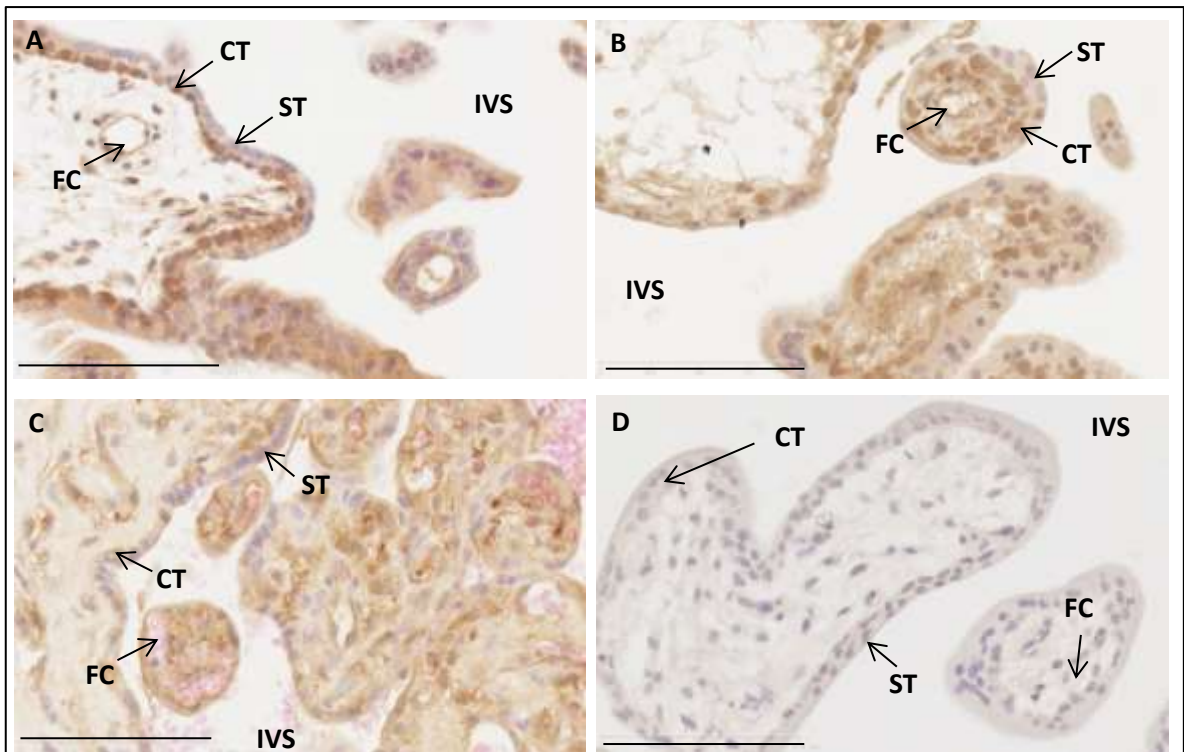


Figure 4.5. Localization of CYP27B1 protein in first trimester and term placenta.

A) Early first trimester placenta, B) late first trimester placenta, C) term control placenta, D) negative. Primary antibody was diluted 1:25 for both first trimester groups and 1:10 for term placenta; scale line is 100 $\mu$ m; magnification is 40x; ST - syncytiotrophoblast, CT - cytotrophoblast, FC - fetal capillary, IVS - intervillous space.

intensity was variable at all gestational ages, with two early gestation placentas and two term placentas having intense staining. In first trimester CYP27B1 staining was localized to the syncytiotrophoblast, cytotrophoblast and endothelium of villi, with weak staining in the villous extracellular matrix. At term, staining was localised to the basal syncytiotrophoblast and cytotrophoblast membranes and fetal endothelium. Some early gestation placentas also had cytotrophoblast nuclear localisation of CYP27B1.

CYP24A1 is also a mitochondrial membrane protein which catabolizes 1,25(OH)<sub>2</sub>D<sub>3</sub> to inactive metabolites. Across gestation there was less variation than that found for CYP27B1 staining. CYP24A1 staining was of similar intensity across gestation (Figure 4.6). In first trimester CYP24A1 was strongly localized to the cytotrophoblast layer, labelling both the cytoplasm and nucleus, while in term placenta CYP24A1 was localized to the cytoplasm. Syncytiotrophoblast cytoplasm also labelled. The fetal endothelium also expressed CYP24A1, with some expression in the villous ECM. In term placentas, expression was localized to the fetal endothelium, with patchy expression in the syncytiotrophoblast. Although high

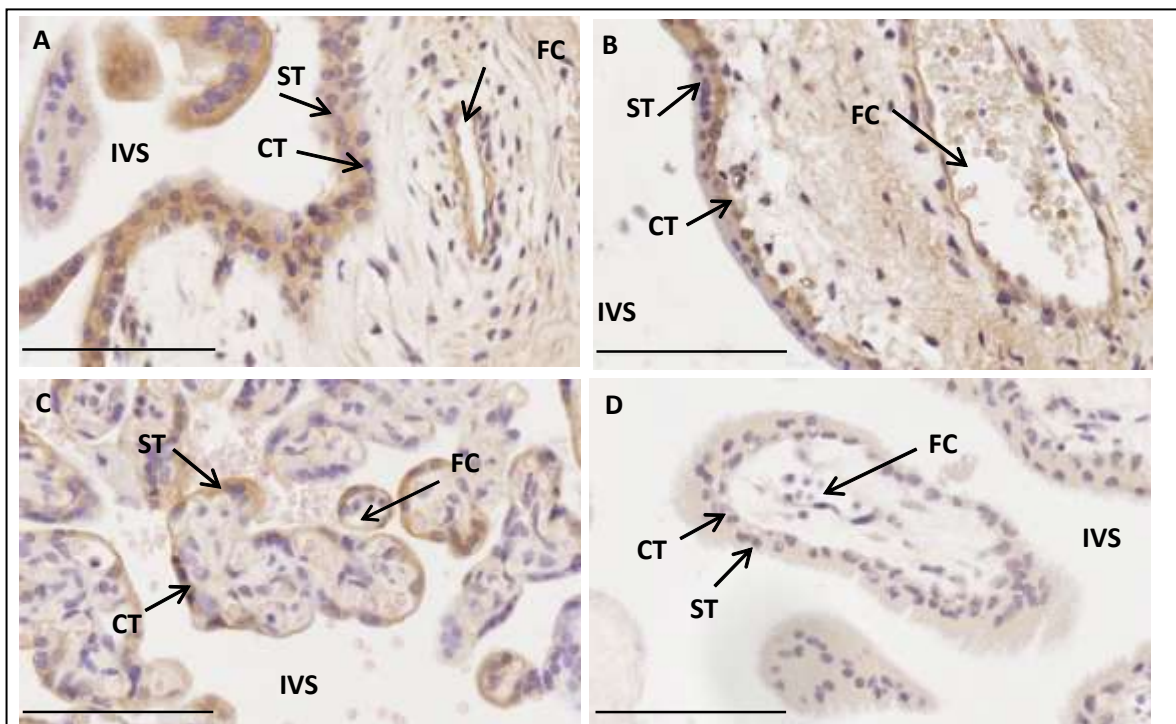


Figure 4.6. Localization of CYP24A1 protein in first trimester and term placenta.

A) early first trimester, B) late first trimester, C) term control, D) negative. ST - syncytiotrophoblast, CT - cytotrophoblast, FC - fetal capillary, IVS - intervillous space; primary antibody was diluted 1/100 for samples; scale line is 100µm; magnification is 40x; ST - syncytiotrophoblast, CT - cytotrophoblast, FC - fetal capillary, IVS - intervillous space.

circulating  $1,25(\text{OH})_2\text{D}_3$  levels should up regulate CYP24A1 expression, localisation and abundance was similar across gestation.

IGF2 is an insulin-like growth factor that mediates placental development and has key roles in first trimester. IGF2 staining was strongest in early first trimester, localising mainly to the syncytiotrophoblast and cytotrophoblast cytoplasm, with some nuclear staining evident (Figure 4.7). In late first trimester, expression is decreased with weak staining observed in the syncytiotrophoblast and moderate staining in the cytotrophoblasts, with some intervillous cells also staining, possibly immune cells. At term IGF2 staining was weak and localised to the syncytiotrophoblast and endothelium.

First trimester decidua was also immunolabelled, although decidua was not collected at term (Figure 4.8). VDR, CYP27B1 and CYP24A1 were all localised to the first trimester decidua, with stronger staining in decidual glands than in villous or non-glandular decidual tissue. CYP24A1 nuclear staining was observed in glandular and villous tissue, as well as some isolated decidual cells, possibly lymphocytes, while nuclear VDR was observed only in villous tissue and occasional isolated cells. CYP27B1 also had nuclear localization, with most isolated lymphocyte-like cells and some glandular cells demonstrating nuclear localization. As the decidua expresses all components of the vitamin  $\text{D}_3$  metabolic pathway, this could be a source of vitamin  $\text{D}_3$  in early gestation.

Decidual CYP27B1 was localized to the ECM, cytoplasm of endometrial cells, and glandular tissue, with the latter staining strongly (Figure 4.8). Immune cells were mostly unstained, although a few expressed CYP27B1. There was



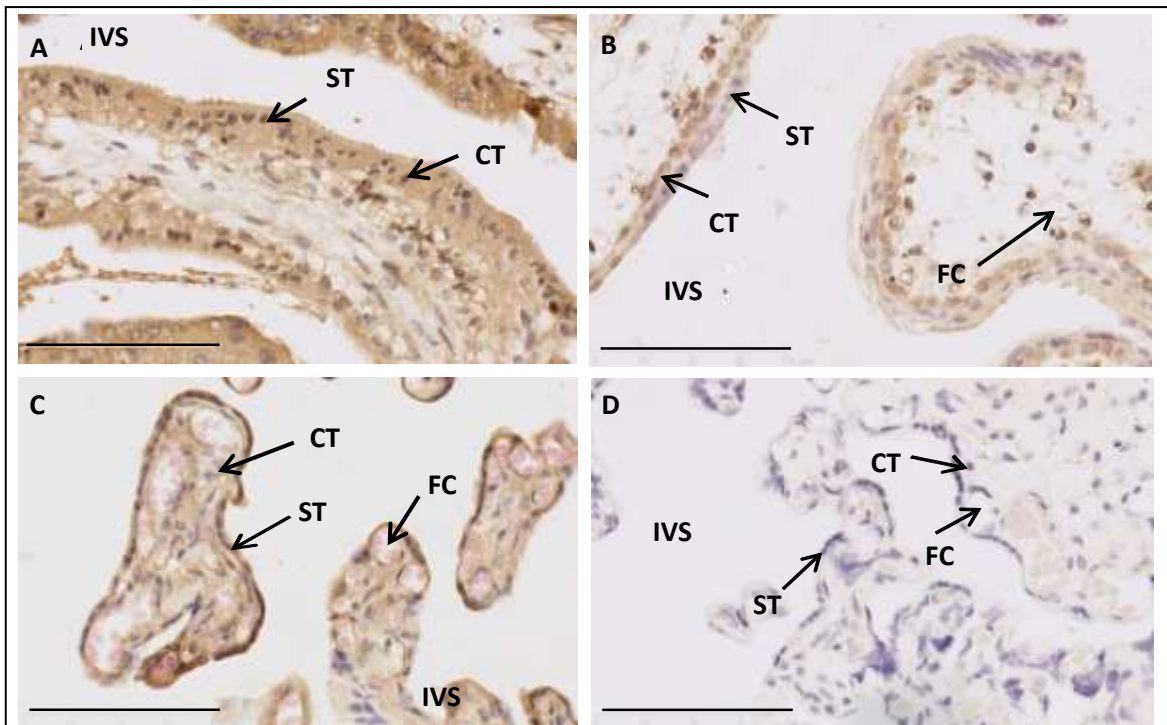


Figure 4.7. Localization of IGF2 protein in first trimester vs. term control placenta.

A) Early first trimester, B) late first trimester, C) term control, D) negative. ST - syncytiotrophoblast, CT - cytotrophoblast, FC - fetal capillary, IVS - intervillous space; primary antibody was diluted 1/50; scale line is 100µm; magnification is 40x; ST - syncytiotrophoblast, CT - cytotrophoblast, FC - fetal capillary, IVS - intervillous space.

considerable variation between samples, with no difference in staining intensity between early and late first trimester.

CYP24A1 labelling was evident in the decidua in both the cytoplasm and nucleus of endometrial cells (Figure 4.8). Endometrial glands stained strongly for CYP24A1, with both nuclear and cytoplasmic localization. Decidual ECM labelled weakly, while cells labelled strongly, with some nuclear staining. Immune cells did not label with CYP24A1. Endometrial staining was of a similar intensity to that of syncytial staining, although glandular staining was much more intense. Again,

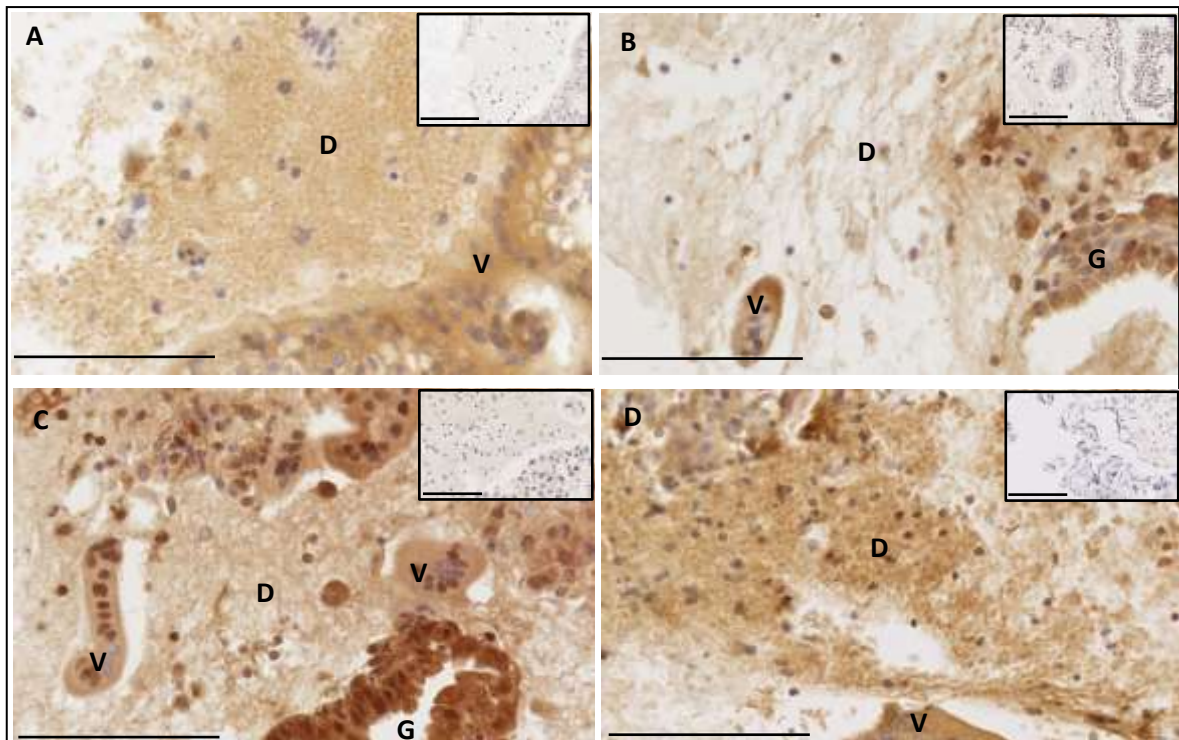


Figure 4.8. Representative images of immunohistochemically stained first trimester decidua.

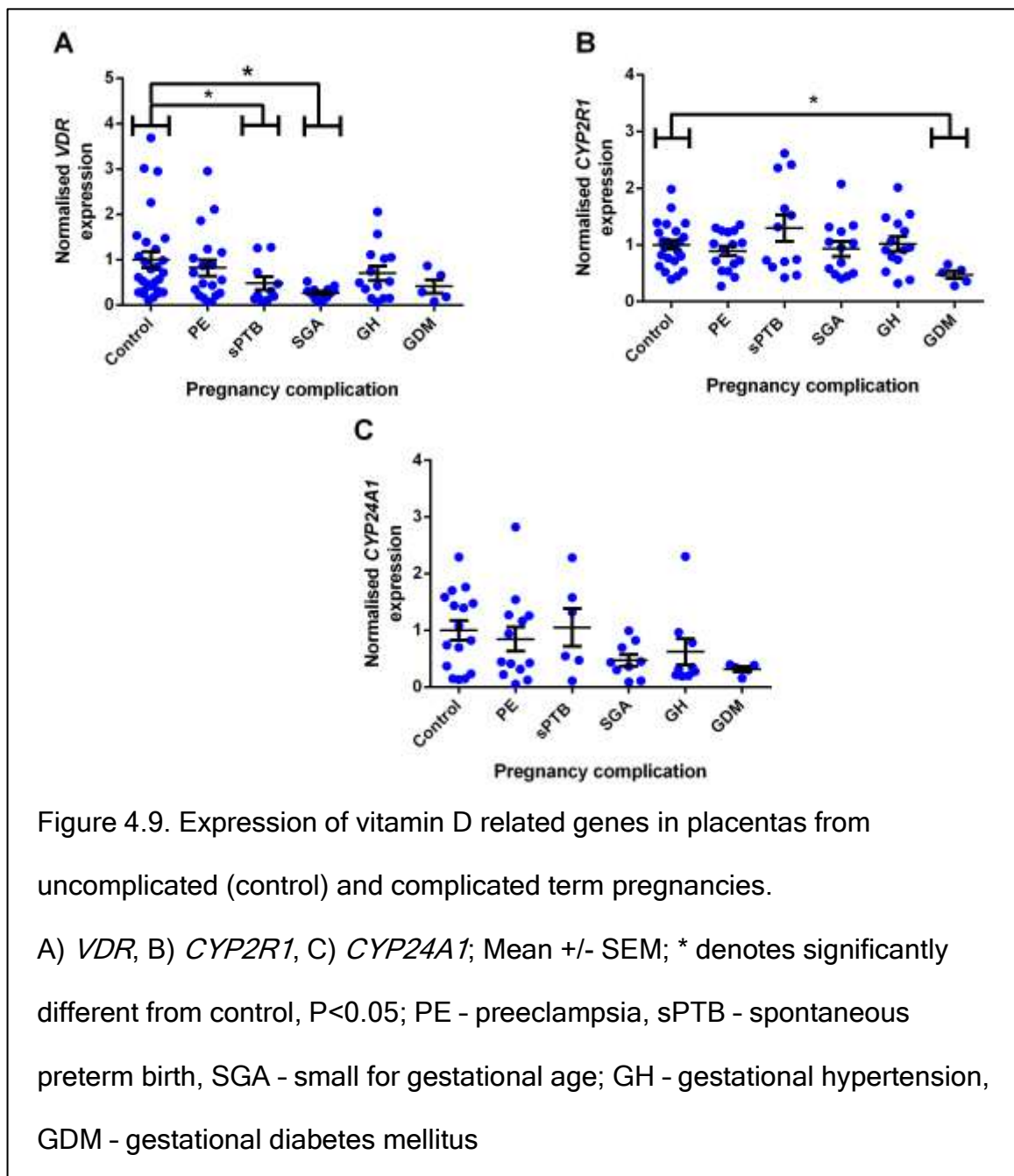
Decidua was stained with VDR, CYP27B1 and CYP24A1. A) VDR, B) CYP27B1, C) CYP24A1, D) IGF2; inset is negative staining. Scale is 100um; 40x magnification; D - decidua, V - villi, G - endometrial gland.

staining intensity was variable, with some first trimester decidual samples having highly intense staining, and others having weak, almost negative staining. The level of CYP24A1 protein is known to be dependent on circulating  $1,25(\text{OH})_2\text{D}_3$  in most tissue types and so may indicate that there was great variation of  $1,25(\text{OH})_2\text{D}_3$  levels between women.

#### 4.4.5 Maternal characteristics: complicated and uncomplicated pregnancies

Maternal and neonatal data was collected for the pregnancies of the placentas that were used in this study. There were no significant differences between maternal age, BMI, gravidity, parity or fetal sex between complications

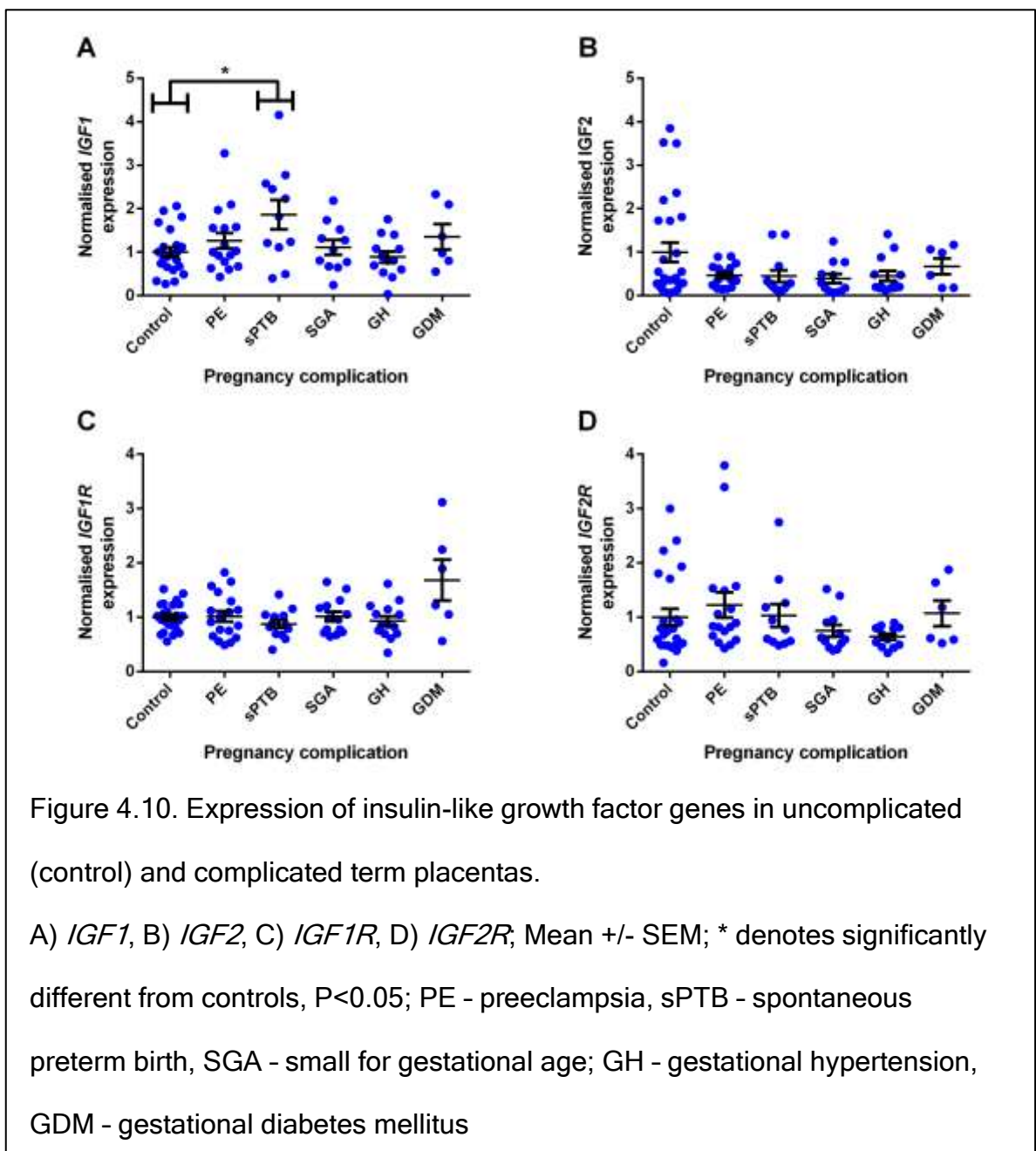
(Table 4.1). As expected length of gestation was shorter in PE and PTB pregnancies compared to controls ( $P=0.063$  and  $P<0.001$ , respectively). Birth weight was also reduced in PE, PTB and SGA pregnancies compared to controls ( $P=0.012$ ,  $P<0.001$  and  $P<0.001$ , respectively); with a trend for reduced placental weight in SGA ( $P=0.061$ ). There was also a trend for reduced birth to placental weight ratio compared to controls ( $P=0.066$ ).





#### 4.4.6 Gene expression: uncomplicated and complicated pregnancies

Placental sample collection was performed by midwives attending the delivery and so there was some variation from time to delivery to placental sampling and immersion in RNA Later; therefore, term placental samples were analysed for RQI, with samples with a RQI from 3 to 9.5 used for analysis. RQI range was similar between groups: controls 3.6-9.0, PE 3.3-8.6, GH 3.6-9.3, sPTB 3.3-8.6, SGA 3-9.5, GDM 3.5-9.4. After removal of outliers from gene expression



data (>2 standard deviations from the mean), RQI was found not to affect expression of these genes (ANOVA,  $P>0.05$ ).

Placental expression of vitamin D<sub>3</sub> metabolism genes was altered in pregnancy complications. Placental *VDR* expression was significantly different across groups ( $P=0.025$ ), with PTB pregnancies having 52% less expression and SGA placentas having a 73% reduction in expression ( $P=0.043$  and  $P=0.001$ , respectively; Figure 4.9A). *CYP2R1* was reduced by 52% in GDM ( $P=0.005$ ; Figure 4.9B). There was no significant change in *CYP24A1* expression, as there was much variation between samples, possibly reflecting local circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels.

*CYP27B1* expression was undetectable in many placental samples, with numbers for analysis subsequently reduced. Those that did amplify did so late (>35 cycles), rendering the results unreliable due to high variation. As a positive control for *CYP27B1* expression, a composite human kidney sample was analysed by PCR. This revealed that term uncomplicated placentas amplified approximately 10 cycles later than the human kidney samples, resulting in approximately 1000-fold reduction of *CYP27B1* expression between non-pregnant human kidney and control term placenta.

IGF family gene expression was altered to a lesser extent than was vitamin D<sub>3</sub>. *IGF1* expression was increased by 90% in sPTB placentas compared to controls ( $P=0.013$ , Figure 4.10A). *IGF1R* expression was non-significantly decreased in GDM affected placentas compared to controls ( $P=0.087$ , Figure 4.10C). There was no alteration in *IGF2* or *IGF2R* expression between samples from different pregnancy complications as these had highly variable expression.

Correlations between expression of genes were examined in placentas from both control and complicated pregnancies (Table 4.4). Control placentas had

Table 4.4. Summary of correlations of placental expression of vitamin D and IGF family genes in uncomplicated (control) compared to complicated pregnancies.

Correlation	Control	PE	sPTB	GH	SGA	GDM
<i>VDR</i> and <i>IGF2</i>	<b>P=0.004</b> R=0.543	P=0.839 R=0.069	<b>P=0.001</b> R=0.904	<b>P=0.048</b> R=0.485	<b>P=0.009</b> R=0.740	P=0.118 R=0.783
<i>VDR</i> and <i>IGF2R</i>	<b>P=0.030</b> R=0.452	<b>P=0.036</b> R=0.510	P=0.449 R=0.290	<b>P=0.023</b> R=0.564	<b>P=0.033</b> R=0.642	<b>P=0.013</b> R=0.950
<i>IGF2</i> and <i>IGF2R</i>	<b>P&lt;0.001</b> R=0.842	P=0.106 R=0.514	<b>P=0.027</b> R=0.692	<b>P=0.026</b> R=0.571	<b>P&lt;0.001</b> R=0.882	<b>P=0.005</b> R=0.941

Note: Only significant correlations of placental gene expression from uncomplicated term pregnancies and the corresponding correlations in complicated pregnancies are shown (bold values are significant;  $P < 0.05$ ); PE - preeclampsia, sPTB - spontaneous preterm birth, SGA - small for gestational age; GH - gestational hypertension, GDM - gestational diabetes mellitus

positive correlations between *VDR* and *IGF2* ( $P=0.030$   $R=0.452$ ), *VDR* and *IGF2R* ( $P=0.004$ ,  $R=0.543$ ), while *IGF2* and *IGF2R* expression were also positively correlated ( $P < 0.001$ ,  $R=0.0842$ ). Complicated placentas displayed a variety of correlations, with the positive correlation between *VDR* and *IGF2* maintained in placentas from sPTB and SGA pregnancies ( $P=0.001$ ,  $R=0.904$ ;  $P=0.009$ ,  $R=0.740$ ; respectively), but lost in PE, GH and GDM pregnancies. The positive correlation between *VDR* and *IGF2R* were also maintained in placentas from PE, SGA and GDM pregnancies ( $P=0.036$ ,  $R=0.510$ ;  $P=0.033$ ,  $R=0.642$ ;  $P=0.023$ ,  $R=0.564$ ; respectively). *IGF2* and *IGF2R* correlation were maintained in placentas from sPTB, SGA and GDM pregnancies ( $P=0.027$ ,  $R=0.692$ ;  $P < 0.001$ ,  $R=0.882$ ;  $P=0.005$ ,  $R=0.941$ ; respectively).

## 4.5 Discussion

This is, to our knowledge, the first study to examine the expression of vitamin D<sub>3</sub> metabolism and insulin like growth factor genes in placentas from a range of pregnancy complications. While other papers have focused on preeclampsia, we have also examined placentas from other pregnancy complications, including sPTB, SGA and GDM. Also, as these pregnancy complications have origins in first trimester placental development, we examined gene and protein expression in first trimester.

### 4.5.1 First trimester placenta

Maternal blood flow into the placenta is absent in early first trimester, with blood flow initiated in late first trimester (Burton, Jauniaux et al. 1999), affecting expression of hypoxia-responsive genes and their downstream targets. Our data show differential expression of *VDR*, *CYP2R1*, *IGF2* and *IGF1R* across gestation, with *IGF2* and *CYP2R1* expression also likely to be affected by the initiation of maternal blood flow into the placenta, as expression was altered between early and late first trimester placentas. This change in expression is overlooked by most studies which focus on the clinical timeframes of first and second trimester, rather than focusing on the period around the establishment of placental blood flow. Other studies have found similar differences between first trimester and term gene expression, including changes in both *VDR* and *CYP27B1* expression across gestation (Zehnder, Evans et al. 2002). When primary placental cultures were exposed to hypoxia by CoCl<sub>2</sub> treatment, protein expression of CYP2R1 and VDR was decreased while CYP27B1 protein levels were increased suggesting the expression is hypoxia-regulated (Ma, Gu et al. 2012). However, two large studies examined hypoxia-responsive elements in genes and found that VDR, CYP27B1,

CYP24A1 and CYP2R1 do not have hypoxia responsive elements (Tanimoto, Tsuchihara et al. 2010, Schodel, Oikonomopoulos et al. 2011), suggesting that the response to oxygen may be a downstream effect or through another mechanism. These alterations in gene expression suggest a role for these genes in early gestation.

Expression and localization of vitamin D<sub>3</sub> pathway proteins was also different across gestation. While *VDR* gene expression was increased in term placenta, levels of VDR protein (by immunohistochemistry) were reduced in term placenta, compared to first trimester placenta. VDR regulation is complex and incompletely understood, with many factors regulating its gene expression including growth factors, 1,25(OH)<sub>2</sub>D<sub>3</sub> and post-translational modifications (Pike, Meyer et al. 2011). The change in VDR protein levels may be due to the high turnover of VDR protein in an environment of high 1,25(OH)<sub>2</sub>D<sub>3</sub>, levels of which in the maternal circulation are increased 3-fold by term (Ardawi, Nasrat et al. 1997). The VDR antibody (D-6) has been previously validated in VDR knockout (*Vdr* null) mouse skeletal muscle samples and is the only VDR antibody currently that does not stain *Vdr* null tissues (Girgis, Mokbel et al. 2014). CYP24A1 protein levels were stable across gestation, as were mRNA levels. The increased concentration of circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> would be assumed to enhance placental *CYP24A1* expression, although due to low VDR protein levels at term may indicate that the increased circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations may not have opportunities to act through VDR in the placenta.

Although the expression of *CYP27B1* could not be quantified due to low expression levels, other studies have reported increased expression in first trimester in placenta and decidua compared to term (Zehnder, Evans et al. 2002). The reported high expression levels of *CYP27B1* in first trimester may be

stimulated by parathyroid hormone (PTH) which increases renal CYP27B1 levels outside of pregnancy, but as PTH decreases over pregnancy, this may be stimulated by other factors (Kovacs 2014). We also observed changes in localization of CYP27B1 and VDR across gestation as reported previously (Ma, Gu et al. 2012). This demonstrates that while the structure and gene expression pattern of the placenta dynamically change across gestation, so too does protein localization of vitamin D<sub>3</sub> pathway proteins, although these don't necessarily correlate.

The IGF family of proteins is essential for placental development, with IGF1 modifying the maternal environment, while IGF2 promotes placental differentiation (Roberts, Owens et al. 2008). IGF1 acts through binding IGF1R, although this receptor can also bind IGF2, while the latter also acts through IGF2R. *IGF2* expression decreased across gestation in our study, as did *IGF1R*, highlighting their importance in early placental development. It has been found previously that circulating IGF1 increases at term (Moller, Streym et al. 2013), which may be associated with the increase in circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in late gestation.

Our work is in line with previous reports of interactions between vitamin D<sub>3</sub> and IGF family members (Figure 4.11). Interactions may include an IGF1-mediated increase of 1,25(OH)<sub>2</sub>D<sub>3</sub> production through stimulation of CYP27B1 transcription (Halhali, Diaz et al. 1999). Transcription of IGF family genes can also be affected by 1,25(OH)<sub>2</sub>D<sub>3</sub> in other tissues, acting through VDR to increase expression of IGFBP-1 protein in the ovary and IGFBP-3 mRNA in a squamous tumour cell line (Wang, Tavera-Mendoza et al. 2005, Parikh, Varadinova et al. 2010, Baxter 2013). There is also evidence for IGFBP-3 inhibiting the action of VDR and its cofactor retinoic acid receptor (RXR) in cultured myeloid cells (Ikezoe, Tanosaki et al. 2004). Interestingly, we found a correlation between *IGF2* and *VDR* gene

expression in the placenta, which has not previously been described. Since CHIP analysis has not revealed a vitamin D<sub>3</sub> responsive element (VDRE) in the gene *IGF2* it may be that both VDR protein activity and IGF2 free circulating levels are regulated by IGFBP-3 which controls the free circulating levels of IGF1 and IGF2 proteins as well as inhibiting VDR transcriptional activity (Baxter 2013). VDR mRNA and protein are controlled by multiple regulatory mechanisms including

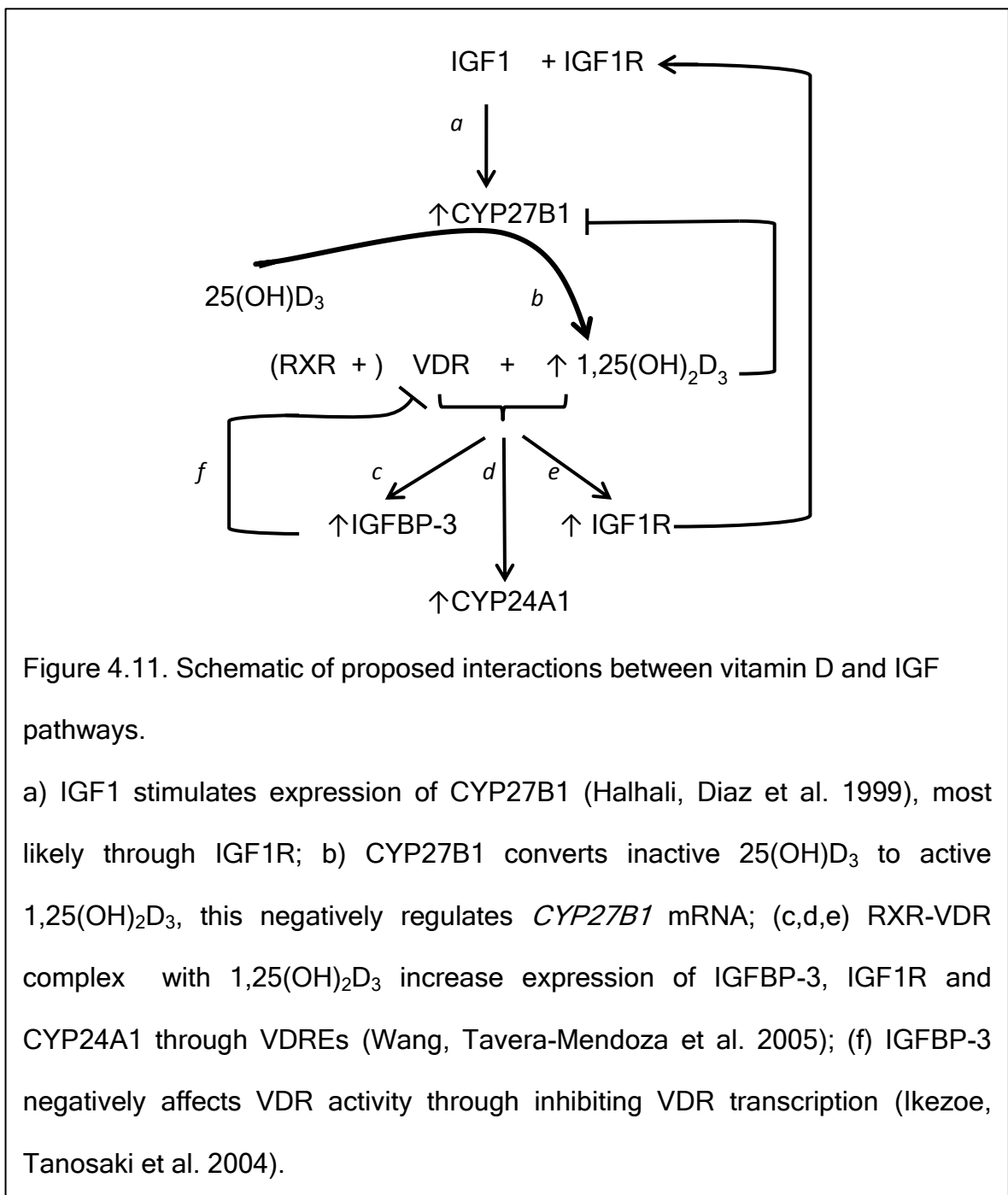


Figure 4.11. Schematic of proposed interactions between vitamin D and IGF pathways.

a) IGF1 stimulates expression of CYP27B1 (Halhali, Diaz et al. 1999), most likely through IGF1R; b) CYP27B1 converts inactive 25(OH)D<sub>3</sub> to active 1,25(OH)<sub>2</sub>D<sub>3</sub>, this negatively regulates *CYP27B1* mRNA; (c,d,e) RXR-VDR complex with 1,25(OH)<sub>2</sub>D<sub>3</sub> increase expression of IGFBP-3, IGF1R and CYP24A1 through VDREs (Wang, Tavera-Mendoza et al. 2005); (f) IGFBP-3 negatively affects VDR activity through inhibiting VDR transcription (Ikezoe, Tanosaki et al. 2004).

regulation by peptides, phosphorus and calcium as well as homoregulation by  $1,25(\text{OH})_2\text{D}_3$  (Pike, Meyer et al. 2011).

#### **4.5.2 The role of vitamin D<sub>3</sub> in placental differentiation**

The role of vitamin D<sub>3</sub> in placental development is currently under investigation by a number of research groups. From research on vitamin D<sub>3</sub> in other tissues, we know that vitamin D<sub>3</sub> reduces proliferation and induces differentiation in some cells and tissues, such as cancers, but we know little about its actions in the placenta.  $1,25(\text{OH})_2\text{D}_3$  has been shown to alter expression of key placental genes (Barrera, Avila et al. 2007, Barrera, Avila et al. 2008), modify placental structure (Chapter 3), as well as influence pregnancy outcomes. A possible role for vitamin D<sub>3</sub> in early gestation is to protect the placenta from infection. Vitamin D<sub>3</sub> down regulates cytokine production and upregulates cathelicidin, an antimicrobial protein which is found only in the placenta (Liu, Kaplan et al. 2009). Vitamin D has also been shown, in the placenta, to down regulate pro-inflammatory cytokines (IL-6, IFN- $\gamma$ , TNF- $\alpha$  and may up regulate anti-inflammatory cytokines and induce immune tolerance (Evans, Bulmer et al. 2004, Diaz, Noyola-Martinez et al. 2009, Shin, Choi et al. 2010). Therefore, there are many possible roles for vitamin D<sub>3</sub> in early gestation in both protecting the placenta and aiding in differentiation.

#### **4.5.3 Placentas from control vs complicated pregnancies**

Gene expression analyses revealed differences in expression of key vitamin D<sub>3</sub> pathway genes between term control placentas and those from complicated pregnancies. SGA affected placentas had significantly reduced *VDR* expression compared to control, which has not been shown previously, although low



circulating 25(OH)D<sub>3</sub> levels have been associated with SGA (Wei, Qi et al. 2013). We found placentas from sPTB affected pregnancies had decreased *VDR* expression, although another study earlier reported increased *VDR* and *CYP27B1* and decreased *CYP24A1* expression (Fischer, Schroer et al. 2007). As neither study reported vitamin D status, this may indicate that these pregnancies have lower circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> as *VDR* is self-regulated following binding with 1,25(OH)<sub>2</sub>D<sub>3</sub>, as well as other complex regulators (Pike, Meyer et al. 2011).

Placentas affected by GDM also had significantly reduced *CYP2R1* expression, which has not been reported previously. A recent study did, however report increased levels of *CYP24A1* protein and mRNA expression (Cho, Hong et al. 2013). Although the authors suggested that vitamin D<sub>3</sub> deficiency may be a result of increased placental *CYP24A1* expression, it is more likely that low 25(OH)D<sub>3</sub> would contribute to the aetiology of GDM, as vitamin D-deficiency has been negatively associated with markers of glucose homeostasis, with the mechanism suggested to be poor calcium motility in pancreatic cells (Alzaim and Wood 2013)

The conflicting results may be due to differences in either the mode of delivery of the baby (which was not reported), circulating 25(OH)D<sub>3</sub> levels, or the method of normalization of gene expression with only one reference gene. Although we found no difference in PE or GH affected placental gene expression, other studies have found PE placentas to have higher *CYP27B1* and lower *CYP24A1* expression levels compared to those from uncomplicated pregnancies (Fischer, Schroer et al. 2007). Placental explants from preeclamptic pregnancies have previously been cultured and shown to have reduced expression of *CYP27B1* compared to controls (Diaz, Arranz et al. 2002, Halhali, Diaz et al. 2014). Hence, there is considerable variation in results between studies of gene

expression at term, with differences in gestation, delivery methods and analytic methods potentially confounding the data.

Expression of IGF family genes was also examined in complicated placentas in the present study. Although there was no overall difference between those in placentas from complicated or control pregnancies, *IGF1* expression was significantly increased in sPTB affected placentas. This directly contradicts a recent paper, which showed decreased *IGF1* expression in PTB placentas compared to those from uncomplicated term pregnancies (Demendi, Borzsonyi et al. 2012). This may be due to *IGF1* expression decreasing across gestation (Kumar, Leverence et al. 2012), although the gestational ages of our study and Demendi *et al.* were similar. Alternatively, the difference may be due to different normalization methods, with Demendi *et al.* using normalization to  $\beta$ -actin using the delta-delta-CT method of normalization rather than the more robust normalization to the geometric mean of optimal reference genes. In growth restricted pregnancies, increased placental *IGF2R*, *IGF2* and *IGFBP3* expression have also been reported (Borzsonyi, Demendi et al. 2011, Kumar, Leverence et al. 2012). While we did not find this differential expression in growth restricted pregnancies, the difference may have been in the definition of growth restriction, the severity of SGA, the method of delivery of the placenta or other factors between the studies.

Vitamin D<sub>3</sub> levels are measured in many studies and related to outcomes, without speculation as to how vitamin D<sub>3</sub> alters the fetomaternal environment and affects placental development. Concentrations of both IGFs and vitamin D<sub>3</sub> in the placenta and maternal circulation change across gestation, with circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels increasing 3-5 fold across gestation, while both circulating levels of IGF1 and IGF2 lower than non-pregnant levels at 15 weeks' gestation and

IGF1 concentrations are reported to increase late in gestation (Moller, Streym et al. 2013). In preeclamptic pregnancies, circulating IGF1 levels are significantly lower than in controls in later pregnancy, suggesting alterations in IGF pathways in complicated pregnancies (Halhali, Villa et al. 2004). These studies have failed to investigate placental changes that may be associated with pregnancy complications, so it is difficult to determine the roles of circulating metabolites and the placenta in pregnancy complications.

#### 4.5.4 Conclusion

Although many studies have examined the roles of the vitamin D<sub>3</sub> and IGF families in pregnancy, most have only looked at circulating levels within each family, failed to consider interactions and have not examined complicated pregnancies. We aimed to study vitamin D<sub>3</sub> metabolism and IGF families together to start to elucidate the complex changes that occur in the placenta across pregnancy and identify possible relationships between the two pathways. We have shown that there are differences in gene expression of vitamin D<sub>3</sub> and IGF family genes in placentas across gestation and in complicated pregnancies. We have revealed novel gene expression interactions between the families which require further investigation, such as the correlations between *IGF2* and *VDR*. We have also localized key vitamin D<sub>3</sub> metabolism proteins in the placenta and decidua. These investigations will contribute to our understanding of the role of vitamin D<sub>3</sub> role in the placenta and how vitamin D<sub>3</sub> is involved in pregnancy complications.

## Chapter 5: General Discussion

In the last decade, interest in vitamin D<sub>3</sub> has increased dramatically. This is mainly due to resurgence in the incidence of rickets, although research has expanded from bone-related disorders to encompass many diseases from cancers to cardiovascular disease. In parallel there has been an interest in vitamin D<sub>3</sub> in pregnancy. The importance of vitamin D<sub>3</sub> in pregnancy was revealed through three major findings: the association of vitamin D<sub>3</sub> deficiency with pregnancy complications (Aghajafari, Nagulesapillai et al. 2013), increased levels of circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> in late pregnancy (Ardawi, Nasrat et al. 1997) and the selective methylation of *CYP24A1* in the placenta but not in other tissues (Novakovic, Galati et al. 2012). Although there have now been many association studies examining pregnancy complications and vitamin D<sub>3</sub> levels, there are still few studies which examine the placenta and the function of vitamin D<sub>3</sub> within the placenta.

### 5.1 The role of calcium in vitamin D<sub>3</sub> metabolism in pregnancy

The primary role of vitamin D<sub>3</sub> in the body is to maintain calcium homeostasis; therefore, this must be addressed when exploring the role of vitamin D<sub>3</sub> in pregnancy. There is little known about the interactions of vitamin D<sub>3</sub> and calcium in pregnancy as most studies have focused on calcium transfer to the fetus. When it was found that vitamin D<sub>3</sub> is not required for placental transfer of calcium to the fetus (Kovacs, Woodland et al. 2005), other functions of vitamin D<sub>3</sub> in the placenta were then explored. In the non-pregnant state serum calcium is tightly regulated by a number of factors, including PTH, PTHrP, FGF23/Klotho and vitamin D<sub>3</sub> (Kovacs 2008). We found this regulation to be altered in pregnancy, as

diets low in vitamin D<sub>3</sub> and calcium did not affect kidney *CYP27B1* gene expression in non-pregnant mice, whereas pregnant mice had increased renal *CYP27B1* when consuming low calcium diets compared to calcium replete diets (see Chapter 2). This may be due to the high calcium demands in pregnancy, increasing 1,25(OH)<sub>2</sub>D<sub>3</sub> production to increase uptake of dietary calcium.

Alterations in kidney gene expression due to vitamin D and calcium deficiency were reflected in placental gene expression. Kidney gene expression of *Cyp27b1* was increased in mice consuming low calcium diets. This suggests that these mice were trying to increase their 1,25(OH)<sub>2</sub>D<sub>3</sub> production through up regulation of *Cyp27b1*. Alternately in the placenta of mice on low calcium diets there is no change in *Cyp27b1* gene expression, although *Cyp24a1* is increased in the placentas of those mice, suggesting that the placenta may respond to increased circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> levels that are indicated by altered kidney *Cyp27b1* expression. As these analyses were from the dam's kidney and one pup's placenta from her litter, correlations between maternal and placental expression were not performed.

Both calcium and vitamin D<sub>3</sub> intake are known to be important, as a sparing effect exists where adequate calcium intake preserves 25(OH)D<sub>3</sub> stores whereas low calcium intake or absorption causes circulating PTH to increase, increasing conversion of 25(OH)D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub> thereby reducing circulating 25(OH)D<sub>3</sub> (Lips, van Schoor et al. 2014). This was observed in our mice, with non-pregnant mice fed low calcium diets having the lowest 25(OH)D<sub>3</sub> levels, regardless of vitamin D<sub>3</sub> intake (see Chapter 2). In our pregnant mice calcium sparing was not observed, as all mice on diets deficient in vitamin D<sub>3</sub> and/or calcium had similar 25(OH)D<sub>3</sub> levels. This may indicate that pregnant mice have higher vitamin D<sub>3</sub> and calcium requirements than non-pregnant mice, as a low calcium and high dietary

vitamin D<sub>3</sub> diet did not increase circulating 25(OH)D<sub>3</sub> levels. Therefore, it would be reasonable to conclude that adequate intake of both calcium and vitamin D<sub>3</sub> are essential to maintain circulating 25(OH)D<sub>3</sub> levels, especially in pregnancy where 25(OH)D<sub>3</sub> requirements seem to be increased.

Although no other studies have addressed the issue of vitamin D<sub>3</sub> sparing in pregnancy, the phenomenon has been described in non-pregnant animals and humans. In rats, a high calcium diet maintained 25(OH)D<sub>3</sub> stores, whereas these were depleted in rats on low calcium diets (Anderson, Lee et al. 2010). Vitamin D sparing was also identified in rats with a linear relationship between vitamin D<sub>3</sub> half-life and diets containing high or low calcium, suggesting that calcium spares 25(OH)D<sub>3</sub> levels (Clements, Johnson et al. 1987). Patients with partial gastrectomy were found to have increased 25(OH)D<sub>3</sub> half-life when increasing oral calcium intake. (Davies, Heys et al. 1997). While other studies have not examined half-life, but rather efficiency of calcium absorption, participants supplemented with vitamin D<sub>3</sub> (20 µg every second day for 2 weeks, mean serum levels of 86 nmol/L 25(OH)D<sub>3</sub>) had 65% higher calcium absorption than those without supplementation (mean 50 nmol/L 25(OH)D<sub>3</sub>) (Heaney, Dowell et al. 2003). Therefore, high dietary calcium consumption has been shown to spare 25(OH)D<sub>3</sub> serum levels in rats and humans.

## **5.2 Increased production of 1,25(OH)<sub>2</sub>D<sub>3</sub> in pregnancy**

Across gestation maternal circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> increases 3-5 fold above non-pregnant levels (Ardawi, Nasrat et al. 1997). This was initially assumed to be due to increased production by the placenta, as it seemed unlikely that the kidney's tight regulation of vitamin D<sub>3</sub> activation would be altered in pregnancy. The placenta had been confirmed as a site of vitamin D<sub>3</sub> activation as it expresses

*CYP27B1* and cultured placentae can produce  $1,25(\text{OH})_2\text{D}_3$  *in vitro* (Diaz, Arranz et al. 2002). However, a recent study found there is far greater *CYP27B1* expression in the kidney, compared to the placenta, of pregnant mice (Kirby, Ma et al. 2013). We also found a significant difference in *CYP27B1* expression in these organs, with renal expression highest in pregnant compared to non-pregnant mice and in kidney compared to placenta (see Chapter 2 and Chapter 3). This is also likely to be the case in women, as in our human placental studies *CYP27B1* was expressed at a very low level compared to a commercially available human kidney RNA sample and was below the limit of detection in some samples. The level of expression was also orders of magnitude lower than in non-pregnant human kidney mRNA (see Chapter 4). These data indicate that the increased circulating  $1,25(\text{OH})_2\text{D}_3$  in late gestation is not likely to be due to increased placental production, but is much more likely to be a consequence of altered kidney regulation. It would be reasonable to conclude that placental production of  $1,25(\text{OH})_2\text{D}_3$  would influence local concentrations only. Outside of pregnancy renal *CYP27B1* is under the control of PTH. However, circulating levels of PTH are low in pregnancy and instead *CYP27B1* may be stimulated by circulating PTHrP which has been shown to be abundant in pregnancy (Ardawi, Nasrat et al. 1997). Alternatively, the decidua may produce additional circulating  $1,25(\text{OH})_2\text{D}_3$  as it has been shown to express *CYP27B1* highly in early gestation compared to placenta (Zehnder, Evans et al. 2002).

### **5.3 Effect of vitamin D<sub>3</sub> in pregnancy**

Fetal and placental weights were expected to be altered by diet at d18.5 in pregnant mice. Interestingly, there was no change in placental weight but there was an increase in fetal weight in vitamin D<sub>3</sub> and calcium deficient mice (see

Chapter 3). Similar findings have been reported previously, with mild deficiencies in other nutrients such as protein resulting in increased fetal growth, while severe deficiencies decrease fetal growth. This is thought to be due to compensatory changes in placental function which increase the growth of the fetus, such as increased placental labyrinth volume or alterations to its composition or transport capacity (Fowden, Sferruzzi-Perri et al. 2009).

We also examined changes in mouse placental structure in response to diet. We found changes in structural correlates of placental function in response to vitamin D<sub>3</sub> and calcium deficiency (see Chapter 3). Mild vitamin D<sub>3</sub> and calcium deficiency increased the volume of the maternal blood space and thinned the feto-maternal trophoblast barrier, as well as increased trophoblast exchange surface area. This may be due to compensatory increases in maternal blood pressure in deficient mice which has been reported previously in pregnant, vitamin D<sub>3</sub> deficient mice (Liu, Ouyang et al. 2013). That study also observed, but did not quantify, increased maternal blood space in placentas of vitamin D<sub>3</sub> deficient mice. By increasing maternal blood pressure placental perfusion can be maintained or increased. If this is the case, one would expect to see consequent modifications to the placental labyrinth as we observed and could explain the increased fetal weight we measured. Although this phenomenon has not been reported in human studies, it may be an explanation of conflicting results found in initial studies of vitamin D and SGA babies, as mild deficiency may have increased placental efficiency.

## **5.4 The role of vitamin D<sub>3</sub> in the placenta**

The actions of vitamin D<sub>3</sub> in the placenta are poorly defined, although potentially it could have wide-ranging effects as VDR ligation effects transcription of many genes. A study examining vitamin D response elements (VDREs) *in silico*



found over 900 responsive genes in cancer cells (Wang, Tavera-Mendoza et al. 2005). Addition of  $1,25(\text{OH})_2\text{D}_3$  to primary term explant placenta cultures stimulates production of pregnancy hormones such as hCG (Barrera, Avila et al. 2008), estradiol and progesterone (Barrera, Avila et al. 2007), as well as having antimicrobial (Liu, Kaplan et al. 2009, Shuler, Hendrix et al. 2013) and, in mouse placenta, anti-inflammatory (Liu, Kaplan et al. 2011) functions. In our mouse work, we found that although the human genes vascular endothelial growth factor (*VEGFA*), hypoxia inducible factor 1-alpha (*HIF1A*) and hydroxysteroid 17-beta dehydrogenase 2 (*HSD17B2*) genes contain VDREs, as do the mouse counterparts for *Hif1a* and *Hsd17b2* (Wang, Tavera-Mendoza et al. 2005), gene expression in mouse placenta did not significantly differ between diets (see Chapter 3). We have only assessed gene expression at day 18.5 in the mouse placenta. It is possible that effects of deficient diets may have been observed earlier in gestation or there are no effects on expression of these genes.

Placental gene expression of vitamin  $\text{D}_3$  pathway components has also been examined, both in early gestation and term human placental samples. *CYP27B1* was previously reported to be highly expressed in first trimester and reduced with advancing gestation for both RNA (Zehnder, Evans et al. 2002) and protein (Ma, Gu et al. 2012). Our investigations into *CYP27B1* revealed that RNA expression is very low in human placenta in both early and late gestation (see Chapter 4), compared with abundant genes such as the IGFs and also with human kidney *CYP27B1* mRNA. This makes it unlikely that increased vitamin  $\text{D}_3$  production in pregnancy is due to increased placental production.

Conflicting data on human placental *VDR* gene expression have also been reported. We found *VDR* expression was highest in term compared to early gestation placentas (see Chapter 4), although it was previously reported that first

trimester expression was higher than at term (Zehnder, Evans et al. 2002). One difference between the studies was that Zehnder *et al.* did not specify whether the placentas were from laboured or non-laboured deliveries, which affects placental gene expression. We also found evidence of altered protein expression, with our placentas having low VDR expression at term, compared to first trimester (see Chapter 4). This is different to previous findings of consistently expressed VDR across gestation (Ma, Gu et al. 2012) in which VDR localization was achieved with the same antibodies (VDR D-6) and similar protocol to our work. Ma *et al.* found that that VDR protein expression was hypoxia sensitive, with protein levels decreasing in hypoxic environments (Ma, Gu et al. 2012). Differences may arise in both gene and protein expression results when laboured or non-laboured placentas are used. Laboured placentas would be expected to be subject to hypoxia during labour while those from elective Caesarean deliveries would not be so exposed.

## **5.5 Effect of vitamin D<sub>3</sub> and IGFs in pregnancy complications**

Although there was initially controversy about whether vitamin D<sub>3</sub> deficiency in pregnancy was associated with pregnancy complications, recent systematic reviews have concluded that vitamin D<sub>3</sub> deficiency is associated with PE, GDM, SGA and PTB (Aghajafari, Nagulesapillai et al. 2013, Tabesh, Salehi-Abargouei et al. 2013, Wei, Qi et al. 2013). When we examined expression of the vitamin D<sub>3</sub> and IGF family genes in placentas from complicated pregnancies (see Chapter 4), there was a trend toward increased *IGF1* (in PTB) and decreased *VDR* and *CYP24A1* (in SGA) expression in placentas from complicated pregnancies compared to controls. Decreased expression of IGF family members in early pregnancy may impair maternal adaptation to pregnancy and trophoblast invasion

restricting transformation of spiral arteries. Later in gestation, placental *IGF1* mRNA has been shown to decrease from 25 to 40 weeks' gestation (Kumar, Leverence et al. 2012). This is consistent with the increased expression in PTB samples compared to term controls, as these are from an earlier gestation, indicating that this may be a physiological finding rather than a pathological finding. Decreased levels of placental *VDR* in late gestation would indicate a reduced ability of the placenta to mediate  $1,25(\text{OH})_2\text{D}_3$  signalling impacting vitamin D responsive gene expression such as that of 17-beta hydroxy steroid dehydrogenase 2 (*HSD17B2*), a gene which is involved in steroidogenesis. Reduced placental *CYP24A1* expression would suggest that concentrations of  $1,25(\text{OH})_2\text{D}_3$  are at a low level in the placenta, as little  $1,25(\text{OH})_2\text{D}_3$  appears to be synthesised locally in late gestation, due to reduced *CYP27B1* mRNA expression (Zehnder, Evans et al. 2002). This is also supported by our data showing decreased *VDR* mRNA as VDR protein directly up regulates *CYP24A1*.

As VDR regulates the expression of many genes, we propose that vitamin D responsive genes alter placental development and that these pathways are essential for normal pregnancy. Such vitamin D responsive genes include *HIF1A* which is a hypoxia inducible transcription factor that regulates expression of genes involved in trophoblast invasion, differentiation, placental vascularization and nutrient transport (Pringle, Kind et al. 2010), in addition HIF-1 $\alpha$  regulates *VEGF* expression which is involved in angiogenesis and *HSD17B2* which is involved in steroidogenesis (Wang, Tavera-Mendoza et al. 2005). When circulating vitamin  $\text{D}_3$  is deficient, these pathways may be altered, possibly perturbing placental development and contributing to development of pregnancy complications. Further work is required to determine whether these pathways are affected by vitamin  $\text{D}_3$  deficiency in the placenta.

From this work I would suggest that vitamin D status should be tested in all pregnant women on an intention-to-treat basis as two recent meta-analyses concluded that increased vitamin D and calcium intake is protective for pregnancy complications (PE, SGA, PTB, GDM) (Aghajafari, Nagulesapillai et al. 2013, Wei, Qi et al. 2013). Although there is controversy over accuracy of testing methods, this is currently being investigated so that, in the future, the method of assaying vitamin D at different sites will be standardised (Jones 2015).

## 5.6 Conclusions

In conclusion, mice consuming low calcium diets had an increased risk of PTB, while mice consuming diets deficient in vitamin D<sub>3</sub> or calcium had modifications of placental structural correlates of function, contributing to higher fetal weights. Vitamin D<sub>3</sub> metabolism was altered by diet in pregnant and non-pregnant mice, while non-pregnant mice had depleted serum 25(OH)D<sub>3</sub> levels when consuming low calcium diets compared to controls, providing evidence of vitamin D<sub>3</sub> sparing. In human placentas we found altered gene expression of vitamin D<sub>3</sub> and IGF family genes across gestation and in complicated pregnancies, including reduced *VDR* expression in PTB and SGA placentas. These results together indicate that vitamin D<sub>3</sub> metabolism is altered in mice consuming vitamin D-deficient and low calcium diets and also in women with complicated pregnancies. Increasing our understanding of vitamin D<sub>3</sub>'s role in pregnancy will positively impact pregnancy management and reduce the risk of pregnancy complications.

## Chapter 6: References

- (2006). Nutrient Reference Values for Australia and New Zealand. K. Baghurst, National Health and Medical Research Council (NHMRC): 1-333.
- (2011). Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC, Institute of Medicine (IOM), The National Academies Press.
- (2011, 11.6.2011). "Food Standards Australia New Zealand, Standard 2.4.2 - Edible Oil Spreads." Retrieved 7.7.14, 2014, from [www.foodstandards.gov.au](http://www.foodstandards.gov.au).
- (2012). Born Too Soon: The Global Action Report on Preterm Birth. C. P. Howson, M. V. Kinney and J. E. Lawn. Geneva, March of Dimes, PMNCH, Save the Children, WHO.
- (2013). "ACOG committee opinion no. 560: Medically indicated late-preterm and early-term deliveries." *Obstet Gynecol* **121**(4): 908-910.
- (2013). South Australian Paediatric Clinical Guidelines: Vitamin D deficiency. G. o. S. A. Department of Health, South Australian Child Health Clinical Network.
- (2013). Surviving the First Day: State of the World's Mothers 2013, Save the Children.
- Abedi, P., Z. Mohaghegh, P. Afshary and M. Latifi (2014). "The relationship of serum vitamin D with pre-eclampsia in the Iranian women." *Matern Child Nutr* **10**(2): 206-212.
- Abrahams, V. M., P. Bole-Aldo, Y. M. Kim, S. L. Straszewski-Chavez, T. Chaiworapongsa, R. Romero and G. Mor (2004). "Divergent trophoblast responses to bacterial products mediated by TLRs." *J Immunol* **173**(7): 4286-4296.
- Adamson, S. L., Y. Lu, K. J. Whiteley, D. Holmyard, M. Hemberger, C. Pfarrer and J. C. Cross (2002). "Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta." *Dev Biol* **250**(2): 358-373.
- Aghajafari, F., T. Nagulesapillai, P. E. Ronksley, S. C. Tough, M. O'Beirne and D. M. Rabi (2013). "Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies." *BMJ* **346**: f1169.
- Alzaim, M. and R. J. Wood (2013). "Vitamin D and gestational diabetes mellitus." *Nutr Rev* **71**(3): 158-167.
- Anderson, P. H., I. Hendrix, R. K. Sawyer, R. Zarrinkalam, J. Manavis, G. T. Sarvestani, B. K. May and H. A. Morris (2008). "Co-expression of CYP27B1 enzyme with the 1.5kb CYP27B1 promoter-luciferase transgene in the mouse." *Mol Cell Endocrinol* **285**(1-2): 1-9.
- Anderson, P. H., A. M. Lee, S. M. Anderson, R. K. Sawyer, P. D. O'Loughlin and H. A. Morris (2010). "The effect of dietary calcium on 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis and sparing of serum 25(OH)D<sub>3</sub> levels." *J Steroid Biochem Mol Biol* **121**(1-2): 288-292.
- Andraweera, P. H., G. A. Dekker, J. A. Laurence and C. T. Roberts (2012). "Placental expression of VEGF family mRNA in adverse pregnancy outcomes." *Placenta* **33**(6): 467-472.
- Ardawi, M. S., H. A. Nasrat and B. A. A. HS (1997). "Calcium-regulating hormones and parathyroid hormone-related peptide in normal human pregnancy and postpartum: a longitudinal study." *Eur J Endocrinol* **137**(4): 402-409.
- August, P., B. Marcaccio, J. M. Gertner, M. L. Druzin, L. M. Resnick and J. H. Laragh (1992). "Abnormal 1,25-dihydroxyvitamin D metabolism in preeclampsia." *Am J Obstet Gynecol* **166**(4): 1295-1299.
- Avila, E., L. Diaz, A. Halhali and F. Larrea (2004). "Regulation of 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase, 1,25-dihydroxyvitamin D<sub>3</sub> 24-hydroxylase

and vitamin D receptor gene expression by 8-bromo cyclic AMP in cultured human syncytiotrophoblast cells." J Steroid Biochem Mol Biol **89-90**(1-5): 115-119.

Bacq, Y. (2000). The Liver in Normal Pregnancy. Madame Curie Bioscience Database. Austin (TX).

Baker, A. M., S. Haeri, C. A. Camargo, Jr., J. A. Espinola and A. M. Stuebe (2010). "A nested case-control study of midgestation vitamin D deficiency and risk of severe preeclampsia." J Clin Endocrinol Metab **95**(11): 5105-5109.

Baker, A. M., S. Haeri, C. A. Camargo, Jr., A. M. Stuebe and K. A. Boggess (2012). "First-trimester maternal vitamin D status and risk for gestational diabetes (GDM) a nested case-control study." Diabetes Metab Res Rev **28**(2): 164-168.

Barker, D. J. (2001). "The malnourished baby and infant." Br Med Bull **60**: 69-88.

Barrera, D., E. Avila, G. Hernandez, A. Halhali, B. Biruete, F. Larrea and L. Diaz (2007). "Estradiol and progesterone synthesis in human placenta is stimulated by calcitriol." J Steroid Biochem Mol Biol **103**(3-5): 529-532.

Barrera, D., E. Avila, G. Hernandez, I. Mendez, L. Gonzalez, A. Halhali, F. Larrea, A. Morales and L. Diaz (2008). "Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts." Reprod Biol Endocrinol **6**: 3.

Baxter, R. C. (2013). "Insulin-like growth factor binding protein-3 (IGFBP-3): Novel ligands mediate unexpected functions." J Cell Commun Signal **7**(3): 179-189.

Beck, S., D. Wojdyla, L. Say, A. P. Betran, M. Merialdi, J. H. Requejo, C. Rubens, R. Menon and P. F. Van Look (2010). "The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity." Bull World Health Organ **88**(1): 31-38.

Belkacemi, L., D. M. Nelson, M. Desai and M. G. Ross (2010). "Maternal Undernutrition Influences Placental-Fetal Development." Biology of Reproduction **83**(3): 325-331.

Bodnar, L. M., J. M. Catov, J. M. Roberts and H. N. Simhan (2007). "Pregpregnancy obesity predicts poor vitamin D status in mothers and their neonates." J Nutr **137**(11): 2437-2442.

Bodnar, L. M., J. M. Catov, H. N. Simhan, M. F. Holick, R. W. Powers and J. M. Roberts (2007). "Maternal vitamin D deficiency increases the risk of preeclampsia." J Clin Endocrinol Metab **92**(9): 3517-3522.

Bodnar, L. M., J. M. Catov, J. M. Zmuda, M. E. Cooper, M. S. Parrott, J. M. Roberts, M. L. Marazita and H. N. Simhan (2010). "Maternal serum 25-hydroxyvitamin D concentrations are associated with small-for-gestational age births in white women." J Nutr **140**(5): 999-1006.

Bodnar, L. M., H. N. Simhan, R. W. Powers, M. P. Frank, E. Cooperstein and J. M. Roberts (2007). "High prevalence of vitamin D insufficiency in black and white pregnant women residing in the northern United States and their neonates." J Nutr **137**(2): 447-452.

Borzsonyi, B., C. Demendi, Z. Nagy, K. Toth, M. Csanad, A. Pajor, J. Rig, Jr. and J. G. Joo (2011). "Gene expression patterns of insulin-like growth factor 1, insulin-like growth factor 2 and insulin-like growth factor binding protein 3 in human placenta from pregnancies with intrauterine growth restriction." J Perinat Med **39**(6): 701-707.

Botto, L. D., C. A. Moore, M. J. Khoury and J. D. Erickson (1999). "Neural-tube defects." N Engl J Med **341**(20): 1509-1519.

Bouillon, R., G. Carmeliet, L. Verlinden, E. van Etten, A. Verstuyf, H. F. Luderer, L. Lieben, C. Mathieu and M. Demay (2008). "Vitamin D and human health: lessons from vitamin D receptor null mice." Endocr Rev **29**(6): 726-776.

Bowyer, L., C. Catling-Paull, T. Diamond, C. Homer, G. Davis and M. E. Craig (2009). "Vitamin D, PTH and calcium levels in pregnant women and their neonates." Clin Endocrinol (Oxf) **70**(3): 372-377.

Brockman, H. (1936). "Die Osolierung des antirachitischen Vitamins aus Thunfischeleberol." H.-S.Zeit. Physiol. Chem. **241**: 104-115.

Brommage, R. and H. F. DeLuca (1984). "Placental transport of calcium and phosphorus is not regulated by vitamin D." Am J Physiol **246**(4 Pt 2): F526-529.

Brooke, O. G., F. Butters and C. Wood (1981). "Intrauterine vitamin D nutrition and postnatal growth in Asian infants." Br Med J (Clin Res Ed) **283**(6298): 1024.

Burriss, H. H., S. L. Rifas-Shiman, K. Kleinman, A. A. Litonjua, S. Y. Huh, J. W. Rich-Edwards, C. A. Camargo, Jr. and M. W. Gillman (2012). "Vitamin D deficiency in pregnancy and gestational diabetes mellitus." Am J Obstet Gynecol **207**(3): 182 e181-188.

Burton, G. J. and E. Jauniaux (2004). "Placental oxidative stress: from miscarriage to preeclampsia." J Soc Gynecol Investig **11**(6): 342-352.

Burton, G. J., E. Jauniaux and A. L. Watson (1999). "Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited." Am J Obstet Gynecol **181**(3): 718-724.

Burton, G. J., A. W. Woods, E. Jauniaux and J. C. Kingdom (2009). "Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy." Placenta **30**(6): 473-482.

Calvo, M. S., S. J. Whiting and C. N. Barton (2004). "Vitamin D fortification in the United States and Canada: current status and data needs." Am J Clin Nutr **80**(6 Suppl): 1710S-1716S.

Castillo, A. I., A. M. Jimenez-Lara, R. M. Tolon and A. Aranda (1999). "Synergistic activation of the prolactin promoter by vitamin D receptor and GHF-1: role of the coactivators, CREB-binding protein and steroid hormone receptor coactivator-1 (SRC-1)." Mol Endocrinol **13**(7): 1141-1154.

Catalano, P. M., J. P. Kirwan, S. Haugel-de Mouzon and J. King (2003). "Gestational diabetes and insulin resistance: role in short-and long-term implications for mother and fetus." The Journal of nutrition **133**(5): 1674S-1683S.

Chaiworapongsa, T., P. Chaemsaihong, L. Yeo and R. Romero (2014). "Pre-eclampsia part 1: current understanding of its pathophysiology." Nat Rev Nephrol **10**(8): 466-480.

Cheng, J. B., M. A. Levine, N. H. Bell, D. J. Mangelsdorf and D. W. Russell (2004). "Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase." Proc Natl Acad Sci U S A **101**(20): 7711-7715.

Cho, G. J., S. C. Hong, M. J. Oh and H. J. Kim (2013). "Vitamin D deficiency in gestational diabetes mellitus and the role of the placenta." Am J Obstet Gynecol **209**(6): 560 e561-568.

Christakos, S., D. V. Ajibade, P. Dhawan, A. J. Fechner and L. J. Mady (2012). "Vitamin D: metabolism." Rheum Dis Clin North Am **38**(1): 1-11, vii.

Clements, M. R., L. Johnson and D. R. Fraser (1987). "A new mechanism for induced vitamin D deficiency in calcium deprivation." Nature **325**(6099): 62-65.

Clifton-Bligh, R. J., P. McElduff and A. McElduff (2008). "Maternal vitamin D deficiency, ethnicity and gestational diabetes." Diabet Med **25**(6): 678-684.

Coan, P. M., N. Conroy, G. J. Burton and A. C. Ferguson-Smith (2006). "Origin and characteristics of glycogen cells in the developing murine placenta." Dev Dyn **235**(12): 3280-3294.

Coan, P. M., A. C. Ferguson-Smith and G. J. Burton (2004). "Developmental dynamics of the definitive mouse placenta assessed by stereology." Biol Reprod **70**(6): 1806-1813.

Cockburn, F., N. R. Belton, R. J. Purvis, M. M. Giles, J. K. Brown, T. L. Turner, E. M. Wilkinson, J. O. Forfar, W. J. Barrie, G. S. McKay and S. J. Pocock (1980). "Maternal vitamin D intake and mineral metabolism in mothers and their newborn infants." Br Med J **281**(6232): 11-14.

Cooke, P. S. and A. Naaz (2004). "Role of estrogens in adipocyte development and function." Exp Biol Med (Maywood) **229**(11): 1127-1135.

Damm, P., C. Kuhl, A. Bertelsen and L. Molsted-Pedersen (1992). "Predictive factors for the development of diabetes in women with previous gestational diabetes mellitus." Am J Obstet Gynecol **167**(3): 607-616.

Dardenne, O., J. Prud'homme, A. Arabian, F. H. Glorieux and R. St-Arnaud (2001). "Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets." Endocrinology **142**(7): 3135-3141.

David, V. and L. D. Quarles (2011). FGF23/Klotho New Regulators of Vitamin D Metabolism. Vitamin D (Third Edition). F. David, J. W. Pike and J. S. Adams. San Diego, Academic Press. 1: 747-761.

Davies, M., S. E. Heys, P. L. Selby, J. L. Berry and E. B. Mawer (1997). "Increased catabolism of 25-hydroxyvitamin D in patients with partial gastrectomy and elevated 1,25-dihydroxyvitamin D levels. Implications for metabolic bone disease." J Clin Endocrinol Metab **82**(1): 209-212.

Dawodu, A., H. F. Saadi, G. Bekdache, Y. Javed, M. Altaye and B. W. Hollis (2013). "Randomized controlled trial (RCT) of vitamin D supplementation in pregnancy in a population with endemic vitamin D deficiency." J Clin Endocrinol Metab **98**(6): 2337-2346.

De-Regil, L. M., C. Palacios, A. Ansary, R. Kulier and J. P. Pena-Rosas (2012). "Vitamin D supplementation for women during pregnancy." Cochrane Database Syst Rev **2**: CD008873.

De Laine, K. M., G. Matthews and R. M. Grivell (2013). "Prospective audit of vitamin D levels of women presenting for their first antenatal visit at a tertiary centre." Aust N Z J Obstet Gynaecol **53**(4): 353-357.

DeChiara, T. M., A. Efstratiadis and E. J. Robertson (1990). "A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting." Nature **345**(6270): 78-80.

Dekker, G. and P. Y. Robillard (2007). "Pre-eclampsia: Is the immune maladaptation hypothesis still standing? An epidemiological update." J Reprod Immunol **76**(1-2): 8-16.

Demendi, C., B. Borzsonyi, Z. B. Nagy, J. Rigo, Jr., A. Pajor and J. G. Joo (2012). "Gene expression patterns of insulin-like growth factor 1, 2 (IGF-1, IGF-2) and insulin-like growth factor binding protein 3 (IGFBP-3) in human placenta from preterm deliveries: influence of additional factors." Eur J Obstet Gynecol Reprod Biol **160**(1): 40-44.

Desforges, M. and C. P. Sibley (2010). "Placental nutrient supply and fetal growth." Int J Dev Biol **54**(2-3): 377-390.

Diaz, L., C. Arranz, E. Avila, A. Halhali, F. Vilchis and F. Larrea (2002). "Expression and activity of 25-hydroxyvitamin D-1 alpha-hydroxylase are restricted in cultures of human syncytiotrophoblast cells from preeclamptic pregnancies." J Clin Endocrinol Metab **87**(8): 3876-3882.



Diaz, L., N. Noyola-Martinez, D. Barrera, G. Hernandez, E. Avila, A. Halhali and F. Larrea (2009). "Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts." J Reprod Immunol **81**(1): 17-24.

Diaz, L., I. Sanchez, E. Avila, A. Halhali, F. Vilchis and F. Larrea (2000). "Identification of a 25-hydroxyvitamin D3 1alpha-hydroxylase gene transcription product in cultures of human syncytiotrophoblast cells." J Clin Endocrinol Metab **85**(7): 2543-2549.

Duley, L. (2009). "The global impact of pre-eclampsia and eclampsia." Semin Perinatol **33**(3): 130-137.

Edlow, A. G., S. K. Srinivas and M. A. Elovitz (2007). "Second-trimester loss and subsequent pregnancy outcomes: What is the real risk?" American Journal of Obstetrics and Gynecology **197**(6): 581.e581-581.e586.

Elder, C. J. and N. J. Bishop (2014). "Rickets." Lancet **383**(9929): 1665-1676.

Emkey, R. D. and G. R. Emkey (2012). "Calcium metabolism and correcting calcium deficiencies." Endocrinol Metab Clin North Am **41**(3): 527-556.

Equils, O. and M. Hewison (2010). "A role for vitamin D in placental immunology." J Infect Dis **201**(12): 1950-1951; author reply 1951.

Ertl, R., C. K. Yu, R. Samaha, R. Akolekar and K. H. Nicolaides (2012). "Maternal serum vitamin D at 11-13 weeks in pregnancies delivering small for gestational age neonates." Fetal Diagn Ther **31**(2): 103-108.

Evans, K. N., J. N. Bulmer, M. D. Kilby and M. Hewison (2004). "Vitamin D and placental-decidual function." J Soc Gynecol Investig **11**(5): 263-271.

Farrant, H. J., G. V. Krishnaveni, J. C. Hill, B. J. Boucher, D. J. Fisher, K. Noonan, C. Osmond, S. R. Veena and C. H. Fall (2009). "Vitamin D insufficiency is common in Indian mothers but is not associated with gestational diabetes or variation in newborn size." Eur J Clin Nutr **63**(5): 646-652.

Faurschou, A., D. M. Beyer, A. Schmedes, M. K. Bogh, P. A. Philipsen and H. C. Wulf (2012). "The relation between sunscreen layer thickness and vitamin D production after ultraviolet B exposure: a randomized clinical trial." Br J Dermatol **167**(2): 391-395.

Favus, M. J., D. A. Bushinsky and J. Lemann (2006). "Regulation of calcium, magnesium and phosphate metabolism." Primer on the metabolic bone diseases and disorders of mineral metabolism **6**: 76-83.

Fernandez-Cancio, M., L. Audi, A. Carrascosa, N. Toran, P. Andaluz, C. Esteban and M. L. Granada (2009). "Vitamin D and growth hormone regulate growth hormone/insulin-like growth factor (GH-IGF) axis gene expression in human fetal epiphyseal chondrocytes." Growth Horm IGF Res **19**(3): 232-237.

Figueras, F. and J. Gardosi (2009). "Should we customize fetal growth standards?" Fetal Diagn Ther **25**(3): 297-303.

Fischer, D., A. Schroer, D. Ludders, T. Cordes, B. Bucker, J. Reichrath and M. Friedrich (2007). "Metabolism of vitamin D3 in the placental tissue of normal and preeclampsia complicated pregnancies and premature births." Clin Exp Obstet Gynecol **34**(2): 80-84.

Fleige, S. and M. W. Pfaffl (2006). "RNA integrity and the effect on the real-time qRT-PCR performance." Mol Aspects Med **27**(2-3): 126-139.

Fleischman, A. R., J. F. Rosen, J. Cole, C. M. Smith and H. F. Deluca (1980). "Maternal and fetal serum 1,25-dihydroxyvitamin D levels at term." J Pediatr **97**(4): 640-642.

Forster, I. C., N. Hernando, J. Biber and H. Murer (2013). "Phosphate transporters of the SLC20 and SLC34 families." Mol Aspects Med **34**(2-3): 386-395.

Fowden, A. L., A. N. Sferruzzi-Perri, P. M. Coan, M. Constancia and G. J. Burton (2009). "Placental efficiency and adaptation: endocrine regulation." J Physiol **587**(Pt 14): 3459-3472.

Gargosky, S. E., K. J. Moyse, P. E. Walton, J. A. Owens, J. C. Wallace, J. S. Robinson and P. C. Owens (1990). "Circulating levels of insulin-like growth factors increase and molecular forms of their serum binding proteins change with human pregnancy." Biochem Biophys Res Commun **170**(3): 1157-1163.

Georgiades, P., A. C. Ferguson-Smith and G. J. Burton (2002). "Comparative developmental anatomy of the murine and human definitive placentae." Placenta **23**(1): 3-19.

Girgis, C. M., N. Mokbel, K. M. Cha, P. J. Houweling, M. Abboud, D. R. Fraser, R. S. Mason, R. J. Clifton-Bligh and J. E. Gunton (2014). "The vitamin D receptor (VDR) is expressed in skeletal muscle of male mice and modulates 25-hydroxyvitamin D (25OHD) uptake in myofibers." Endocrinology **155**(9): 3227-3237.

Glazier, J. D., E. B. Mawer and C. P. Sibley (1995). "Calbindin-D9K gene expression in rat chorioallantoic placenta is not regulated by 1,25-dihydroxyvitamin D3." Pediatr Res **37**(6): 720-725.

Glossmann, H. H. (2010). "Origin of 7-Dehydrocholesterol (Provitamin D) in the Skin." J Invest Dermatol **130**(8): 2139-2141.

Godfrey, K. M. and D. J. Barker (2001). "Fetal programming and adult health." Public Health Nutrition **4**(2b): 611-624.

Goldstein, D. A. (1990). Serum Calcium. Clinical Methods: The history, physical and laboratory examinations. H. K. Walker, W. D. Hall and J. W. Hurst. Boston, Butterworths: 677-679.

Grant, W. B. (2011). "Adequate vitamin D during pregnancy reduces the risk of premature birth by reducing placental colonization by bacterial vaginosis species." MBio **2**(2): e00022-00011.

Grover, S. R. and R. Morley (2001). "Vitamin D deficiency in veiled or dark-skinned pregnant women." Med J Aust **175**(5): 251-252.

Gude, N. M., C. T. Roberts, B. Kalionis and R. G. King (2004). "Growth and function of the normal human placenta." Thromb Res **114**(5-6): 397-407.

Guyton, A. and J. Hall (1996). Parathyroid Hormone, Calcitonin, Calcium and Phosphate Metabolism, Vitamin D, Bone and Teeth. Text book of Medical Physiology. Philadelphia, Pennsylvania, W.B. Saunders Company: 985 - 1001.

Halhali, A., L. Diaz, D. Barrera, E. Avila and F. Larrea (2014). "Placental calcitriol synthesis and IGF-I levels in normal and preeclamptic pregnancies." J Steroid Biochem Mol Biol **144 Pt A**: 44-49.

Halhali, A., L. Diaz, I. Sanchez, M. Garabedian, H. Bourges and F. Larrea (1999). "Effects of IGF-I on 1,25-dihydroxyvitamin D(3) synthesis by human placenta in culture." Mol Hum Reprod **5**(8): 771-776.

Halhali, A., A. R. Tovar, N. Torres, H. Bourges, M. Garabedian and F. Larrea (2000). "Preeclampsia is associated with low circulating levels of insulin-like growth factor I and 1,25-dihydroxyvitamin D in maternal and umbilical cord compartments." J Clin Endocrinol Metab **85**(5): 1828-1833.

Halhali, A., A. R. Villa, E. Madrazo, M. C. Soria, E. Mercado, L. Diaz, E. Avila, M. Garabedian and F. Larrea (2004). "Longitudinal changes in maternal serum 1,25-dihydroxyvitamin D and insulin like growth factor I levels in pregnant women who developed preeclampsia: comparison with normotensive pregnant women." J Steroid Biochem Mol Biol **89-90**(1-5): 553-556.

Halloran, B. P. and H. F. DeLuca (1980). "Effect of vitamin D deficiency on fertility and reproductive capacity in the female rat." J Nutr **110**(8): 1573-1580.

Hashemipour, S., F. Lalooha, S. Zahir Mirdamadi, A. Ziaee and T. Dabaghi Ghaleh (2013). "Effect of vitamin D administration in vitamin D-deficient pregnant women on maternal and neonatal serum calcium and vitamin D concentrations: a randomised clinical trial." Br J Nutr **110**(9): 1611-1616.

Hawfield, A. and B. I. Freedman (2009). "Pre-eclampsia: the pivotal role of the placenta in its pathophysiology and markers for early detection." Ther Adv Cardiovasc Dis **3**(1): 65-73.

Heaney, R. P., M. S. Dowell, C. A. Hale and A. Bendich (2003). "Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D." J Am Coll Nutr **22**(2): 142-146.

Hellems, J., G. Mortier, A. De Paepe, F. Speleman and J. Vandesompele (2007). "qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data." Genome Biol **8**(2): R19.

Hewison, M. (2012). "Vitamin D and immune function: autocrine, paracrine or endocrine?" Scand J Clin Lab Invest Suppl **243**: 92-102.

Hoffman, L., C. Nolan, J. D. Wilson, J. J. Oats and D. Simmons (1998). "Gestational diabetes mellitus--management guidelines. The Australasian Diabetes in Pregnancy Society." Med J Aust **169**(2): 93-97.

Hofmeyr, G. J., A. N. Atallah and L. Duley (2006). "Calcium supplementation during pregnancy for preventing hypertensive disorders and related problems." Cochrane Database Syst Rev(3): CD001059.

Hofmeyr, G. J., T. A. Lawrie, A. N. Atallah, L. Duley and M. R. Torloni (2014). "Calcium supplementation during pregnancy for preventing hypertensive disorders and related problems." Cochrane Database Syst Rev **6**: CD001059.

Holick, M. F. (2011). "Vitamin D: evolutionary, physiological and health perspectives." Curr Drug Targets **12**(1): 4-18.

Hollis, B. W. and W. B. Pittard, 3rd (1984). "Evaluation of the total fetomaternal vitamin D relationships at term: evidence for racial differences." J Clin Endocrinol Metab **59**(4): 652-657.

Hollis, B. W. and C. L. Wagner (2013). "Vitamin D and pregnancy: skeletal effects, nonskeletal effects, and birth outcomes." Calcif Tissue Int **92**(2): 128-139.

Holmes, V. A., M. S. Barnes, H. D. Alexander, P. McFaul and J. M. Wallace (2009). "Vitamin D deficiency and insufficiency in pregnant women: a longitudinal study." Br J Nutr **102**(6): 876-881.

Hostmark, A. T., S. E. Tomten and J. E. Berg (2005). "Serum albumin and blood pressure: a population-based, cross-sectional study." J Hypertens **23**(4): 725-730.

Huppertz, B. (2010). "IFPA Award in Placentology Lecture: Biology of the placental syncytiotrophoblast--myths and facts." Placenta **31** Suppl: S75-81.

Hustin, J. and J. P. Schaaps (1987). "Echographic [corrected] and anatomic studies of the maternotrophoblastic border during the first trimester of pregnancy." Am J Obstet Gynecol **157**(1): 162-168.

Ikezoe, T., S. Tanosaki, U. Krug, B. Liu, P. Cohen, H. Taguchi and H. P. Koeffler (2004). "Insulin-like growth factor binding protein-3 antagonizes the effects of retinoids in myeloid leukemia cells." Blood **104**(1): 237-242.

Irgens, H. U., L. Reisaeter, L. M. Irgens and R. T. Lie (2001). "Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study." BMJ **323**(7323): 1213-1217.

Jauniaux, E., N. Greenwold, J. Hempstock and G. J. Burton (2003). "Comparison of ultrasonographic and Doppler mapping of the intervillous circulation in normal and abnormal early pregnancies." Fertil Steril **79**(1): 100-106.

Jauniaux, E., A. L. Watson, J. Hempstock, Y. P. Bao, J. N. Skepper and G. J. Burton (2000). "Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure." Am J Pathol **157**(6): 2111-2122.

Johnson, L. E. and H. F. DeLuca (2001). "Vitamin D receptor null mutant mice fed high levels of calcium are fertile." J Nutr **131**(6): 1787-1791.

Jollie, W. P. (1990). "Development, morphology, and function of the yolk-sac placenta of laboratory rodents." Teratology **41**(4): 361-381.

Jones, G. (2008). "Pharmacokinetics of vitamin D toxicity." Am J Clin Nutr **88**(2): 582S-586S.

Jones, G. (2015). "Interpreting vitamin D assay results: proceed with caution." Clin J Am Soc Nephrol **10**(2): 331-334.

Jones, G., D. E. Prosser and M. Kaufmann (2014). "Cytochrome P450-mediated metabolism of vitamin D." J Lipid Res **55**(1): 13-31.

Keelan, J. A., M. Blumenstein, R. J. Helliwell, T. A. Sato, K. W. Marvin and M. D. Mitchell (2003). "Cytokines, prostaglandins and parturition--a review." Placenta **24 Suppl A**: S33-46.

Khong, T. Y., F. De Wolf, W. B. Robertson and I. Brosens (1986). "Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants." Br J Obstet Gynaecol **93**(10): 1049-1059.

Khong, T. Y., H. S. Liddell and W. B. Robertson (1987). "Defective haemochorial placentation as a cause of miscarriage: a preliminary study." Br J Obstet Gynaecol **94**(7): 649-655.

Kirby, B. J., Y. Ma, H. M. Martin, K. L. Buckle Favaro, A. C. Karaplis and C. S. Kovacs (2013). "Upregulation of calcitriol during pregnancy and skeletal recovery after lactation do not require parathyroid hormone." J Bone Miner Res **28**(9): 1987-2000.

Kovacs, C. S. (2008). "Vitamin D in pregnancy and lactation: maternal, fetal, and neonatal outcomes from human and animal studies." Am J Clin Nutr **88**(2): 520S-528S.

Kovacs, C. S. (2009). Chapter 3. Calcium Metabolism during Pregnancy and Lactation. Endocrine Disease and Pregnancy. B. R. Carr, Endotext.com. **2010**.

Kovacs, C. S. (2014). "Bone metabolism in the fetus and neonate." Pediatr Nephrol **29**(5): 793-803.

Kovacs, C. S. and H. M. Kronenberg (1997). "Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation." Endocr Rev **18**(6): 832-872.

Kovacs, C. S., M. L. Woodland, N. J. Fudge and J. K. Friel (2005). "The vitamin D receptor is not required for fetal mineral homeostasis or for the regulation of placental calcium transfer in mice." Am J Physiol Endocrinol Metab **289**(1): E133-144.

Kumar, N., J. Leverence, D. Bick and V. Sampath (2012). "Ontogeny of growth-regulating genes in the placenta." Placenta **33**(2): 94-99.

Kuro-o, M., Y. Matsumura, H. Aizawa, H. Kawaguchi, T. Suga, T. Utsugi, Y. Ohyama, M. Kurabayashi, T. Kaname, E. Kume, H. Iwasaki, A. Iida, T. Shiraki-Iida, S. Nishikawa, R. Nagai and Y. I. Nabeshima (1997). "Mutation of the mouse *klotho* gene leads to a syndrome resembling ageing." Nature **390**(6655): 45-51.

Lausman, A., R. Gagnon, M. Basso, H. Bos, J. Crane, G. Davies, M. Delisle, L. Hudon, S. Menticoglou and W. Mundle (2013). "Intrauterine growth restriction: screening, diagnosis, and management." Journal of obstetrics and gynaecology Canada: JOGC= Journal d'obstetrique et gynecologie du Canada: JOGC **35**(8): 741-757.

Lawson, D. E., D. R. Fraser, E. Kodicek, H. R. Morris and D. H. William (1971). "Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling kidney homeostasis." Nature **230**: 228-230.

Leffelaar, E. R., T. G. Vrijkotte and M. van Eijsden (2010). "Maternal early pregnancy vitamin D status in relation to fetal and neonatal growth: results of the multi-ethnic Amsterdam Born Children and their Development cohort." Br J Nutr **104**(1): 108-117.

Lerchbaum, E. and B. Obermayer-Pietsch (2012). "Vitamin D and fertility: a systematic review." Eur J Endocrinol **166**(5): 765-778.

Li, Y. C., J. Kong, M. Wei, Z. F. Chen, S. Q. Liu and L. P. Cao (2002). "1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system." J Clin Invest **110**(2): 229-238.

Li, Z., L. Hilder and E. Sullivan (2013). Australia's Mothers and Babies 2011. Canberra, Perinatal statistics series 28 Cat. no. PER 28.

Linkhart, T. A. and M. J. Keffer (1991). "Differential regulation of insulin-like growth factor-I (IGF-I) and IGF-II release from cultured neonatal mouse calvaria by parathyroid hormone, transforming growth factor-beta, and 1,25-dihydroxyvitamin D3." Endocrinology **128**(3): 1511-1518.

Lips, P. (2004). "Which circulating level of 25-hydroxyvitamin D is appropriate?" J Steroid Biochem Mol Biol **89-90**(1-5): 611-614.

Lips, P., N. M. van Schoor and R. T. de Jongh (2014). "Diet, sun, and lifestyle as determinants of vitamin D status." Ann N Y Acad Sci **1317**(1): 92-98.

Litonjua, A. A. (2012). "Vitamin D deficiency as a risk factor for childhood allergic disease and asthma." Curr Opin Allergy Clin Immunol **12**(2): 179-185.

Liu, J. P., J. Baker, A. S. Perkins, E. J. Robertson and A. Efstratiadis (1993). "Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r)." Cell **75**(1): 59-72.

Liu, N., A. T. Kaplan, J. Low, L. Nguyen, G. Y. Liu, O. Equils and M. Hewison (2009). "Vitamin D induces innate antibacterial responses in human trophoblasts via an intracrine pathway." Biol Reprod **80**(3): 398-406.

Liu, N. Q., A. T. Kaplan, V. Lagishetty, Y. B. Ouyang, Y. Ouyang, C. F. Simmons, O. Equils and M. Hewison (2011). "Vitamin D and the regulation of placental inflammation." J Immunol **186**(10): 5968-5974.

Liu, N. Q., Y. Ouyang, Y. Bulut, V. Lagishetty, S. Y. Chan, B. W. Hollis, C. Wagner, O. Equils and M. Hewison (2013). "Dietary vitamin D restriction in pregnant female mice is associated with maternal hypertension and altered placental and fetal development." Endocrinology **154**(7): 2270-2280.

Liu, W., M. Guo, S. Ezzat and S. L. Asa (2011). "Vitamin D inhibits CEACAM1 to promote insulin/IGF-I receptor signaling without compromising anti-proliferative action." Lab Invest **91**(1): 147-156.

Looker, A. C., C. M. Pfeiffer, D. A. Lacher, R. L. Schleicher, M. F. Picciano and E. A. Yetley (2008). "Serum 25-hydroxyvitamin D status of the US population: 1988-1994 compared with 2000-2004." Am J Clin Nutr **88**(6): 1519-1527.

Ma, R., Y. Gu, S. Zhao, J. Sun, L. J. Groome and Y. Wang (2012). "Expressions of vitamin D metabolic components VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR

in placentas from normal and preeclamptic pregnancies." Am J Physiol Endocrinol Metab **303**(7): E928-935.

Maghbooli, Z., A. Hossein-Nezhad, F. Karimi, A. R. Shafaei and B. Larijani (2008). "Correlation between vitamin D3 deficiency and insulin resistance in pregnancy." Diabetes Metab Res Rev **24**(1): 27-32.

Makgoba, M., S. M. Nelson, M. Savvidou, C. M. Messow, K. Nicolaidis and N. Sattar (2011). "First-trimester circulating 25-hydroxyvitamin D levels and development of gestational diabetes mellitus." Diabetes Care **34**(5): 1091-1093.

Malassine, A., J. L. Frendo and D. Evain-Brion (2003). "A comparison of placental development and endocrine functions between the human and mouse model." Hum Reprod Update **9**(6): 531-539.

Marya, R. K., S. Rathee and M. Manrow (1987). "Effect of calcium and vitamin D supplementation on toxemia of pregnancy." Gynecol Obstet Invest **24**(1): 38-42.

Masuyama, R., Y. Nakaya, S. Tanaka, H. Tsurukami, T. Nakamura, S. Watanabe, T. Yoshizawa, S. Kato and K. Suzuki (2001). "Dietary phosphorus restriction reverses the impaired bone mineralization in vitamin D receptor knockout mice." Endocrinology **142**(1): 494-497.

Maxwell, J. D., L. Ang, O. G. Brooke and I. R. Brown (1981). "Vitamin D supplements enhance weight gain and nutritional status in pregnant Asians." Br J Obstet Gynaecol **88**(10): 987-991.

Mellanby, E. (1919). "An experimental investigation of rickets." Lancet **1**: 407-412.

Moffett-King, A. (2002). "Natural killer cells and pregnancy." Nat Rev Immunol **2**(9): 656-663.

Moller, U. K., S. Streym, L. Mosekilde, L. Heickendorff, A. Flyvbjerg, J. Frystyk, L. T. Jensen and L. Rejnmark (2013). "Changes in calcitropic hormones, bone markers and insulin-like growth factor I (IGF-I) during pregnancy and postpartum: a controlled cohort study." Osteoporos Int **24**(4): 1307-1320.

Morgan, T. K., J. E. Tolosa, L. Mele, R. J. Wapner, C. Y. Spong, Y. Sorokin, D. J. Dudley, A. M. Peaceman, B. M. Mercer, J. M. Thorp, M. J. O'Sullivan, S. M. Ramin, D. J. Rouse, B. Sibai, H. Eunice Kennedy Shriver National Institute of Child and Human Development Maternal-Fetal Medicine Units (2013). "Placental villous hypermaturation is associated with idiopathic preterm birth." J Matern Fetal Neonatal Med **26**(7): 647-653.

Morley, R., J. B. Carlin, J. A. Pasco and J. D. Wark (2006). "Maternal 25-hydroxyvitamin D and parathyroid hormone concentrations and offspring birth size." J Clin Endocrinol Metab **91**(3): 906-912.

Morley, R., J. B. Carlin, J. A. Pasco, J. D. Wark and A. L. Ponsonby (2009). "Maternal 25-hydroxyvitamin D concentration and offspring birth size: effect modification by infant VDR genotype." Eur J Clin Nutr **63**(6): 802-804.

Morris, H. A. (2005). "Vitamin D: a hormone for all seasons--how much is enough?" Clin Biochem Rev **26**(1): 21-32.

Morris, H. A., G. W. Morrison, M. Burr, D. W. Thomas and B. E. Nordin (1984). "Vitamin D and femoral neck fractures in elderly South Australian women." Med J Aust **140**(9): 519-521.

Muntener, M. and Y. C. Hsu (1977). "Development of trophoblast and placenta of the mouse. A reinvestigation with regard to the in vitro culture of mouse trophoblast and placenta." Acta Anat (Basel) **98**(3): 241-252.

Murray, S. A., J. L. Morgan, C. Kane, Y. Sharma, C. S. Heffner, J. Lake and L. R. Donahue (2010). "Mouse gestation length is genetically determined." PLoS One **5**(8): e12418.

Nassar, N., G. H. Halligan, C. L. Roberts, J. M. Morris and A. W. Ashton (2011). "Systematic review of first-trimester vitamin D normative levels and outcomes of pregnancy." Am J Obstet Gynecol **205**(3): 208 e201-207.

Nathanielsz, P. W., L. Poston and P. D. Taylor (2007). "In utero exposure to maternal obesity and diabetes: animal models that identify and characterize implications for future health." Clin Perinatol **34**(4): 515-526, v.

Need, A. G., H. A. Morris, M. Horowitz and C. Nordin (1993). "Effects of skin thickness, age, body fat, and sunlight on serum 25-hydroxyvitamin D." Am J Clin Nutr **58**(6): 882-885.

Newbern, D. and M. Freemark (2011). "Placental hormones and the control of maternal metabolism and fetal growth." Curr Opin Endocrinol Diabetes Obes **18**(6): 409-416.

Nishimura, M. and S. Naito (2008). "Tissue-specific mRNA expression profiles of human solute carrier transporter superfamilies." Drug Metab Pharmacokinet **23**(1): 22-44.

Novakovic, B., J. C. Galati, A. Chen, R. Morley, J. M. Craig and R. Saffery (2012). "Maternal vitamin D predominates over genetic factors in determining neonatal circulating vitamin D concentrations." Am J Clin Nutr **96**(1): 188-195.

Novakovic, B., M. Sibson, H. K. Ng, U. Manuelpillai, V. Rakyant, T. Down, S. Beck, T. Fournier, D. Evain-Brion, E. Dimitriadis, J. M. Craig, R. Morley and R. Saffery (2009). "Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface." J Biol Chem **284**(22): 14838-14848.

Nowson, C. A. and C. Margerison (2002). "Vitamin D intake and vitamin D status of Australians." Med J Aust **177**(3): 149-152.

O'Brien, K. O., S. Li, C. Cao, T. Kent, B. V. Young, R. A. Queenan, E. K. Pressman and E. M. Cooper (2014). "Placental CYP27B1 and CYP24A1 expression in human placental tissue and their association with maternal and neonatal calcitropic hormones." J Clin Endocrinol Metab **99**(4): 1348-1356.

Ohata, Y., H. Arahori, N. Namba, T. Kitaoka, H. Hirai, K. Wada, M. Nakayama, T. Michigami, A. Imura, Y. Nabeshima, Y. Yamazaki and K. Ozono (2011). "Circulating levels of soluble alpha-Klotho are markedly elevated in human umbilical cord blood." J Clin Endocrinol Metab **96**(6): E943-947.

Pal, A., D. Roy, S. Adhikary, A. Roy, M. Dasgupta and A. K. Mandal (2012). "A prospective study for the prediction of preeclampsia with urinary calcium level." J Obstet Gynaecol India **62**(3): 312-316.

Panda, D. K., D. Miao, M. L. Tremblay, J. Sirois, R. Farookhi, G. N. Hendy and D. Goltzman (2001). "Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction." Proc Natl Acad Sci U S A **98**(13): 7498-7503.

Pariikh, G., M. Varadinova, P. Suwandhi, T. Araki, Z. Rosenwaks, L. Poretsky and D. Seto-Young (2010). "Vitamin D regulates steroidogenesis and insulin-like growth factor binding protein-1 (IGFBP-1) production in human ovarian cells." Horm Metab Res **42**(10): 754-757.

Pasco, J. A., M. J. Henry, G. C. Nicholson, K. M. Sanders and M. A. Kotowicz (2001). "Vitamin D status of women in the Geelong Osteoporosis Study: association with diet and casual exposure to sunlight." Med J Aust **175**(8): 401-405.

Paxton, G. A., G. R. Teale, C. A. Nowson, R. S. Mason, J. J. McGrath, M. J. Thompson, A. Siafarikas, C. P. Rodda and C. F. Munns (2013). "Vitamin D and

health in pregnancy, infants, children and adolescents in Australia and New Zealand: a position statement." Med J Aust **198**(3): 142-143.

Peleg, S., R. V. Abruzzese, C. W. Cooper and R. F. Gagel (1993). "Down-regulation of calcitonin gene transcription by vitamin D requires two widely separated enhancer sequences." Mol Endocrinol **7**(8): 999-1008.

Perez-Ferre, N., M. J. Torrejon, M. Fuentes, M. D. Fernandez, A. Ramos, E. Bordiu, L. del Valle, M. A. Rubio, A. R. Bedia, C. Montanez and A. L. Calle-Pascual (2012). "Association of low serum 25-hydroxyvitamin D levels in pregnancy with glucose homeostasis and obstetric and newborn outcomes." Endocr Pract **18**(5): 676-684.

Pijnenborg, R., L. Vercruysse and M. Hanssens (2006). "The uterine spiral arteries in human pregnancy: facts and controversies." Placenta **27**(9-10): 939-958.

Pijnenborg, R., L. Vercruysse and M. Hanssens (2008). "Fetal-maternal conflict, trophoblast invasion, preeclampsia, and the red queen." Hypertens Pregnancy **27**(2): 183-196.

Pike, J. W. and M. B. Meyer (2014). "Fundamentals of vitamin D hormone-regulated gene expression." J Steroid Biochem Mol Biol **144 Pt A**: 5-11.

Pike, J. W., M. B. Meyer and S. M. Lee (2011). The Vitamin D Receptor: Biochemical, Molecular, Biological, and Genomic Era Investigations. Vitamin D: 3rd Edition. D. Feldman. Waltham, USA, Academic Press. 1: 97-136.

Poel, Y. H., P. Hummel, P. Lips, F. Stam, T. van der Ploeg and S. Simsek (2012). "Vitamin D and gestational diabetes: A systematic review and meta-analysis." Eur J Intern Med **23**(5): 465-469.

Powe, C. E., E. W. Seely, S. Rana, I. Bhan, J. Ecker, S. A. Karumanchi and R. Thadhani (2010). "First trimester vitamin D, vitamin D binding protein, and subsequent preeclampsia." Hypertension **56**(4): 758-763.

Pringle, K. G., K. L. Kind, A. N. Sferruzzi-Perri, J. G. Thompson and C. T. Roberts (2010). "Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy." Hum Reprod Update **16**(4): 415-431.

Quimby, F. W. and R. H. Luong (2007). Clinical Chemistry of the Laboratory Mouse. The Mouse in Biomedical Research. J. G. Fox, S. W. Barthold, M. T. Davisson et al. MA, USA, Elsevier. **3**: 181.

RANZCOG (2014). Diagnosis of Gestational Diabetes Mellitus (GDM) and Diabetes Mellitus in Pregnancy. W. s. H. C. (RANZCOG), Women's Health Committee RANZCOG (Royal Australian and New Zealand College of Obstetricians): 7.

Reeves, P. G., F. H. Nielsen and G. C. Fahey, Jr. (1993). "AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet." J Nutr **123**(11): 1939-1951.

Roberts, C. T. (2010). "Review: Complicated interactions between genes and the environment in placentation, pregnancy outcome and long term health." Placenta.

Roberts, C. T., J. A. Owens and A. N. Sferruzzi-Perri (2008). "Distinct actions of insulin-like growth factors (IGFs) on placental development and fetal growth: lessons from mice and guinea pigs." Placenta **29 Suppl A**: S42-47.

Roberts, C. T., A. Sohlstrom, K. L. Kind, R. A. Earl, T. Y. Khong, J. S. Robinson, P. C. Owens and J. A. Owens (2001). "Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig." Placenta **22**(2-3): 177-185.



Robinson, C. J., M. C. Alanis, C. L. Wagner, B. W. Hollis and D. D. Johnson (2010). "Plasma 25-hydroxyvitamin D levels in early-onset severe preeclampsia." Am J Obstet Gynecol **203**(4): 366 e361-366.

Romero, R., S. K. Dey and S. J. Fisher (2014). "Preterm labor: one syndrome, many causes." Science **345**(6198): 760-765.

Romero, R., J. Espinoza, L. F. Goncalves, J. P. Kusanovic, L. Friel and S. Hassan (2007). "The role of inflammation and infection in preterm birth." Semin Reprod Med **25**(1): 21-39.

Ross, A. C., J. E. Manson, S. A. Abrams, J. F. Aloia, P. M. Brannon, S. K. Clinton, R. A. Durazo-Arvizu, J. C. Gallagher, R. L. Gallo, G. Jones, C. S. Kovacs, S. T. Mayne, C. J. Rosen and S. A. Shapses (2011). "The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know." J Clin Endocrinol Metab **96**(1): 53-58.

Ross, P. (2011). Osteoclasts. Vitamin D. D. Feldman, J. W. Pike and J. S. Adams. San Diego, Academic Press. **1**: 335-347.

Ross, R., J. Florer, K. Halbert and L. McIntyre (1989). "Characterization of 1,25-dihydroxyvitamin D<sub>3</sub> receptors and *in vivo* targeting of <sup>3</sup>H-1,25(OH)<sub>2</sub>D<sub>3</sub>." Placenta **10**: 553-567.

Roth, D. E., A. Al Mahmud, R. Raqib, E. Akhtar, N. Perumal, B. Pezzack and A. H. Baqui (2013). "Randomized placebo-controlled trial of high-dose prenatal third-trimester vitamin D3 supplementation in Bangladesh: the AViDD trial." Nutr J **12**(1): 47.

Rummens, K., S. J. van Cromphaut, G. Carmeliet, E. van Herck, R. van Bree, I. Stockmans, R. Bouillon and J. Verhaeghe (2003). "Pregnancy in mice lacking the vitamin D receptor: normal maternal skeletal response, but fetal hypomineralization rescued by maternal calcium supplementation." Pediatr Res **54**(4): 466-473.

Salle, B. L., E. E. Delvin, A. Lapillonne, N. J. Bishop and F. H. Glorieux (2000). "Perinatal metabolism of vitamin D." Am J Clin Nutr **71**(5 Suppl): 1317S-1324S.

Schneiderman, E. H. (2010). "Gestational diabetes: an overview of a growing health concern for women." J Infus Nurs **33**(1): 48-54.

Schodel, J., S. Oikonomopoulos, J. Ragoussis, C. W. Pugh, P. J. Ratcliffe and D. R. Mole (2011). "High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq." Blood **117**(23): e207-217.

Scholl, T. O. and X. Chen (2009). "Vitamin D intake during pregnancy: association with maternal characteristics and infant birth weight." Early Hum Dev **85**(4): 231-234.

Seki, K., K. Furuya, N. Makimura, C. Mitsui, J. Hirata and I. Nagata (1994). "Cord blood levels of calcium-regulating hormones and osteocalcin in premature infants." J Perinat Med **22**(3): 189-194.

Shand, A. W., N. Nassar, P. Von Dadelszen, S. M. Innis and T. J. Green (2010). "Maternal vitamin D status in pregnancy and adverse pregnancy outcomes in a group at high risk for pre-eclampsia." BJOG **117**(13): 1593-1598.

Shibata, M., A. Suzuki, T. Sekiya, S. Sekiguchi, S. Asano, Y. Udagawa and M. Itoh (2011). "High prevalence of hypovitaminosis D in pregnant Japanese women with threatened premature delivery." J Bone Miner Metab **29**(5): 615-620.

Shin, J. S., M. Y. Choi, M. S. Longtine and D. M. Nelson (2010). "Vitamin D effects on pregnancy and the placenta." Placenta **31**(12): 1027-1034.

Shuler, F. D., J. Hendrix, S. Hodroge and A. Short (2013). "Antibiotic-like actions of vitamin D." W V Med J **109**(1): 22-25.

Sibai, B. M. and A. Frangieh (1995). "Maternal adaption to pregnancy." Current Opinion in Obstetrics and Gynecology **7**: 420-426.

Soheilykhah, S., M. Mojibian, M. Rashidi, S. Rahimi-Saghand and F. Jafari (2010). "Maternal vitamin d status in gestational diabetes mellitus." Nutr Clin Pract **25**(5): 524-527.

Stegers, E. A., P. von Dadelszen, J. J. Duvekot and R. Pijnenborg (2010). "Pre-eclampsia." Lancet **376**(9741): 631-644.

Stephanou, A. and S. Handwerger (1995). "Identification of a composite steroid hormone response element on the human placental lactogen promoter." Mol Cell Endocrinol **112**(1): 123-129.

Stephanou, A., R. Ross and S. Handwerger (1994). "Regulation of human placental lactogen expression by 1,25-dihydroxyvitamin D3." Endocrinology **135**(6): 2651-2656.

Tabesh, M., A. Salehi-Abargouei, M. Tabesh and A. Esmailzadeh (2013). "Maternal vitamin D status and risk of pre-eclampsia: a systematic review and meta-analysis." J Clin Endocrinol Metab **98**(8): 3165-3173.

Tanaka, Y., B. Halloran, H. K. Schnoes and H. F. DeLuca (1979). "In vitro production of 1,25-dihydroxyvitamin D3 by rat placental tissue." Proc Natl Acad Sci U S A **76**(10): 5033-5035.

Tanimoto, K., K. Tsuchihara, A. Kanai, T. Arauchi, H. Esumi, Y. Suzuki and S. Sugano (2010). "Genome-wide identification and annotation of HIF-1 $\alpha$  binding sites in two cell lines using massively parallel sequencing." Hugo J **4**(1-4): 35-48.

Thorp, J., C. Camargo, P. McGee, M. Harper, M. Klebanoff, Y. Sorokin, M. Varner, R. Wapner, S. Caritis, J. Iams, M. Carpenter, A. Peaceman, B. Mercer, A. Sciscione, D. Rouse, S. Ramin and G. Anderson (2012). "Vitamin D status and recurrent preterm birth: a nested case-control study in high-risk women." BJOG **119**(13): 1617-1623.

Tranquilli, A. L., M. A. Brown, G. G. Zeeman, G. Dekker and B. M. Sibai (2013). "The definition of severe and early-onset preeclampsia. Statements from the International Society for the Study of Hypertension in Pregnancy (ISSHP)." Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health **3**(1): 44-47.

Tranquilli, A. L., G. Dekker, L. Magee, J. Roberts, B. M. Sibai, W. Steyn, G. G. Zeeman and M. A. Brown (2014). "The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP." Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health **4**(2): 97-104.

Ullah, M. I., C. A. Koch, S. Tamanna, S. Rouf and L. Shamsuddin (2013). "Vitamin D deficiency and the risk of preeclampsia and eclampsia in Bangladesh." Horm Metab Res **45**(9): 682-687.

van der Mei, I. A., A. L. Ponsonby, O. Engelsen, J. A. Pasco, J. J. McGrath, D. W. Eyles, L. Blizzard, T. Dwyer, R. Lucas and G. Jones (2007). "The high prevalence of vitamin D insufficiency across Australian populations is only partly explained by season and latitude." Environ Health Perspect **115**(8): 1132-1139.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." Genome Biol **3**(7): RESEARCH0034.

Wagner, C. L., R. McNeil, S. A. Hamilton, J. Winkler, C. Rodriguez Cook, G. Warner, B. Bivens, D. J. Davis, P. G. Smith, M. Murphy, J. R. Shary and B. W. Hollis (2013). "A randomized trial of vitamin D supplementation in 2 community

health center networks in South Carolina." Am J Obstet Gynecol **208**(2): 137 e131-113.

Wang, O., M. Nie, Y. Y. Hu, K. Zhang, W. Li, F. Ping, J. T. Liu, L. M. Chen and X. P. Xing (2012). "Association between Vitamin D Insufficiency and the Risk for Gestational Diabetes Mellitus in Pregnant Chinese Women." Biomed Environ Sci **25**(4): 399-406.

Wang, T. T., L. E. Tavera-Mendoza, D. Laperriere, E. Libby, N. B. MacLeod, Y. Nagai, V. Bourdeau, A. Konstorum, B. Lallemant, R. Zhang, S. Mader and J. H. White (2005). "Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes." Mol Endocrinol **19**(11): 2685-2695.

Watson, E. D. and J. C. Cross (2005). "Development of structures and transport functions in the mouse placenta." Physiology (Bethesda) **20**: 180-193.

Webb, A. R., L. Kline and M. F. Holick (1988). "Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin." J Clin Endocrinol Metab **67**(2): 373-378.

Wei, S. Q., F. Audibert, N. Hidiroglou, K. Sarafin, P. Julien, Y. Wu, Z. C. Luo and W. D. Fraser (2012). "Longitudinal vitamin D status in pregnancy and the risk of pre-eclampsia." BJOG **119**(7): 832-839.

Wei, S. Q., H. P. Qi, Z. C. Luo and W. D. Fraser (2013). "Maternal vitamin D status and adverse pregnancy outcomes: a systematic review and meta-analysis." J Matern Fetal Neonatal Med **26**(9): 889-899.

WHO (2013). Diagnostic Criteria and Classification of Hyperglycaemia First Detected in Pregnancy: 63.

Wieland, P., J. A. Fischer, U. Trechsel, H. R. Roth, K. Vetter, H. Schneider and A. Huch (1980). "Perinatal parathyroid hormone, vitamin D metabolites, and calcitonin in man." Am J Physiol **239**(5): E385-390.

Williams, D. (2011). "Long-Term Complications of Preeclampsia." Seminars in Nephrology **31**(1): 111-122.

Windaus, A., O. Linsert, A. Luttringhaus and W. G (1932). "Uber das krystallisierte Vitamin D2." Justis. Liebigs. Ann. Chem. **492**: 226-231.

Woodham, P. C., J. E. Brittain, A. M. Baker, D. L. Long, S. Haeri, C. A. Camargo, Jr., K. A. Boggess and A. M. Stuebe (2011). "Midgestational maternal serum 25-hydroxyvitamin D level and soluble fms-like tyrosine kinase 1/placental growth factor ratio as predictors of severe preeclampsia." Hypertension **58**(6): 1120-1125.

Wu, C., C. Orozco, J. Boyer, M. Leglise, J. Goodale, S. Batalov, C. L. Hodge, J. Haase, J. Janes, J. W. Huss, 3rd and A. I. Su (2009). "BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources." Genome Biol **10**(11): R130.

Wysolmerski, J. J. (2012). "Parathyroid hormone-related protein: an update." J Clin Endocrinol Metab **97**(9): 2947-2956.

Yamashita, T., M. Yoshioka and N. Itoh (2000). "Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain." Biochem Biophys Res Commun **277**(2): 494-498.

Yoshizawa, T., Y. Handa, Y. Uematsu, S. Takeda, K. Sekine, Y. Yoshihara, T. Kawakami, K. Arioka, H. Sato, Y. Uchiyama, S. Masushige, A. Fukamizu, T. Matsumoto and S. Kato (1997). "Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning." Nat Genet **16**(4): 391-396.

Zehnder, D., K. N. Evans, M. D. Kilby, J. N. Bulmer, B. A. Innes, P. M. Stewart and M. Hewison (2002). "The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua." Am J Pathol **161**(1): 105-114.

Zhang, C., C. Qiu, F. B. Hu, R. M. David, R. M. van Dam, A. Bralley and M. A. Williams (2008). "Maternal plasma 25-hydroxyvitamin D concentrations and the risk for gestational diabetes mellitus." PLoS ONE **3**(11): e3753.

Zhong, W., Q. T. Wang, T. Sun, F. Wang, J. Liu, R. Leach, A. Johnson, E. E. Puscheck and D. A. Rappolee (2006). "FGF ligand family mRNA expression profile for mouse preimplantation embryos, early gestation human placenta, and mouse trophoblast stem cells." Mol Reprod Dev **73**(5): 540-550.